Published in final edited form as:

Vet Immunol Immunopathol. 2015 November 15; 168(1-2): 118–130. doi:10.1016/j.vetimm.2015.09.002.

Characterization and expression of monoclonal antibody-defined molecules on resting and activated bovine $\alpha\beta$, $\gamma\delta$ T and NK cells

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Abstract

Monoclonal antibodies (mAbs) specific for leukocyte differentiation molecules (LDMs) were developed during the past few decades to expand reagents for research in ruminants, pigs, and horses. The specificity of some of the mAb-defined molecules was determined through participation in international workshops. Other molecules identified with mAbs during this time, and more recently with mAbs developed after the workshops, have remained partially characterized. Efforts are now underway to characterize the specificity of these mAbs. As reported here, flow cytometry (FC) was used to screen two sets of hybridomas to determine how many of the hybridomas produce mAbs that detect molecules with up-regulated expression on activated lymphocytes or NK cells. Thirty four hybridomas were identified. Comparison of the patterns of reactivity of the mAbs showed some of the mAbs formed clusters that recognize 5 different molecules. FC showed one cluster recognized CD25. Use of mass spectrometry showed 4 clusters recognized orthologues of CD26, CD50, gp96 and signaling lymphocytic activation molecule

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Conflict of interest

The authors of this manuscript do not have any commercial or other associations that might pose a conflict of interest for information presented in this manuscript.

Author's contribution

KTP identified the target molecules of NK29, CACT185A, and CACT200A, and participated in the study with *Map-relA* vaccinated animals and manuscript development. KSS identified the target molecule of NK93A. NAG and BJVW participated in development and characterization of the NK series of mAbs. YMG participated in establishing the IP protocol. YHP participated in development of the initial and later sets of CACT series of mAbs. WCD designed and coordinated the overall study, and participated in all mAb development and characterization, FA analysis, and manuscript development. All authors helped draft, read and approve the final draft of the manuscript.

family member 9 (SLAMF9). Verification and documentation that CD26, CD50, and SLAMF9 were only up-regulated on activated cells was obtained with PBMC from calves vaccinated with a *Mycobacterium avium paratuberculosis* mutant, *Map-relA*. CD26 and CD50 were up-regulated on NK cells, CD4 and CD8 T cells and $\gamma\delta$ T cells. SLAMF9 was only up-regulated on CD4, CD8, and $\gamma\delta$ T cells. gp96 was detected on granulocytes, monocytes and activated NK cells. Detection was attributable to the binding of gp96 to its receptor CD91.

Keywords

Monoclonal antibody; Bovine immunology; Johne's disease; *Mycobacterium paratuberculosis*

1. Introduction

During the past several decades various approaches have been taken to identify monoclonal antibodies (mAbs) to leukocyte differentiation molecules (LDMs, previously referred to as leukocyte differentiation antigens, LDA) for use in veterinary species. Initial approaches included development of sets of mAbs from mice immunized with leukocytes or activated lymphocytes from one or more species for characterization (Davis et al., 1987). International workshops were convened, similar to those convened for characterization of mAbs developed to human leukocyte differentiation antigens (HLDA, now referred to as human cell differentiation molecules, HCDM). Four workshops were convened for characterization of LDMs specific for ruminants (Davis, 1985; Howard et al., 1991; Howard and Naessens, 1993; Naessens and Hopkins, 1996; Teale et al., 1987), three for swine (Haverson et al., 2001; Lunney et al., 1994; Saalmuller et al., 1996), and two for horses (Kydd et al., 1994; Lunn et al., 1998). A statistical method and flow cytometry (FC) were used for initial clustering of mAbs with similar characteristics that appeared to recognize the same molecule. Further studies were then conducted to determine the specificity of the mAb-defined molecules. In some instances, cell lines transfected with a known molecule were used to document specificity. As part of the overall strategy, one additional workshop was convened as part of the eighth HDLA human workshop (Saalmuller et al., 2005). MABs specific or cross reactive with human LDM were screened to determine if they recognized epitopes conserved on orthologues in other species (Davis et al., 2007; Griebel et al., 2007; Saalmuller et al., 2005). Both strategies yielded many of the mAbs currently available for research. However, because of the limitations of methods available for characterization of mAbs during the early workshops, many of the potentially useful mAb-defined LDMs were not fully characterized. This is the case for many of the mAbs we developed. The objective of the present study was to extend studies on two sets of hybridomas developed from mice hyper-immunized with concanavalin A (ConA) activated bovine lymphocytes or cultures enriched for bovine NK cells to determine how many of the hybridomas produced mAbs that recognize molecules with up-regulated expression on activated $\alpha\beta$, $\gamma\delta$ T cells or NK cells. We were particularly interested in characterizing mAbs that recognized molecules with up-regulated expression on peripheral blood mononuclear cells (PBMC) from cattle experimentally and naturally infected with *Mycobacterium avium paratuberculosis* (*Map*) (Koo et al., 2004). FC and mass spectrometry (MS) were used to cluster and characterize the mAb-defined molecules.

2. Materials and methods

2.1. Animals

Three calves obtained from the WSU *Map* free dairy herd were used in the present study as a source of cells to determine the identity of the mAb-defined molecules and illustrate and document expression of the molecules only occurs on activated cells from animals with an immune response to a known pathogen. The calves were being maintained for studies on the immune response to a candidate live vaccine strain of *Map* with a deletion of *relA* gene (*Map-relA*). Two of the calves were vaccinated with the mutant one day after birth (Park et al., 2015). The third calf has been maintained as an unvaccinated control. Since previous studies had shown the mutant is immune eliminated, permission was obtained to maintain the calves in open unrestricted holding facilities at a lower per diem cost, according to standards outlined by Washington State University Institutional Animal Care and Use Committee (Park et al., 2011, 2014). At the time the calves were used as a source of cells in the present study, the calves had been maintained for 2 years. Multiple ex vivo studies, during this time, documented both of the vaccinated calves developed an immune response to live *Map* and *Map* antigens comparable to the immune response noted in previous studies (Allen et al., 2009; Koo et al., 2004; Park et al., 2011, 2014). The mAbs described in the present study were developed over a period of ~20 years, as part of an international effort to develop mAbs reagents for research, as referenced in the introduction and described below. BALB/c mice were used to develop the mAbs. All protocols and procedures were approved by the Washington State University Institutional Animal Care and Use Committee.

2.2. Development of mAbs to molecules expressed on resting and activated lymphocytes

Two strategies were used to develop mAbs to molecules expressed on resting unstimulated and activated NK cells and lymphocytes: (1) hyper-immunization with lymphocytes stimulated with ConA and (2) hyper-immunization with cultures of NK cells maintained with human IL-15 (huIL-15). Briefly, fresh PBMC were cultured for 6 days in culture medium containing 5 µg/ml of ConA. Mice were injected multiple times with $3-5 \times 10^6$ cells/mouse as previously described (Davis et al., 1987, 1996b). NK cells were prepared by continuous culture of PBMC in medium containing 1 ng/ml of huIL-15 (R&D Systems, Minneapolis, MN). FC analysis with anti-CD3 (Davis et al., 1993) showed the NK cell preparation used for immunization was 98% CD3 negative. Hybridomas were produced as previously described (Davis et al., 1984; Hamilton and Davis, 1995).

2.3. Screening of primary culture supernatants of hybridomas for mAbs reactive with resting and/or activated lymphocytes

The ConA activated (CACT) sets of mAbs were developed from 3 fusions made at different times from 1987 to 2003 as described (Davis et al., 1995, 1984). Flow cytometry was used with ConA stimulated PBMC to screen supernatants from the hybridomas generated from the first two fusions. Hybridomas producing mAbs of potential interest were cryopreserved for later analysis. A different strategy was used to screen the last fusion, in effort to simultaneously identify mAbs that recognize molecules only expressed on activated lymphocytes and mAbs that recognize molecules expressed on one or more subsets of leukocytes. As illustrated in Fig. 1, activated lymphocytes undergoing blastogenesis increase

in size and can be distinguished from unstimulated lymphocytes, using side vs forward light scatter (SSC vs FSC). When electronic gates and artificial color coding are used to distinguish the two populations (e.g. Fig. 1A, G1 orange/red and G2 blue) the two populations can be tracked simultaneously in the fluorescent channels (Fig. 1B). Additional gates can be used to isolate cell subsets for analysis of expression of other molecules (Fig. 1C) (Allen et al., 2009; Park et al., 2011). This gating and color coding strategy was used with a vital dye, hydroethidine (HE, dihydroethidium bromide, Life Technologies, Carlsbad, CA) to distinguish unstimulated preparations of PBMC from activated lymphocytes. HE loaded cells fluoresce in red and can be readily distinguished from unlabeled cells based on the signals seen in the FL-2 channel (Fig. 2A). To screen hybridoma supernatants from the third fusion, ConA activated PBMC were loaded with HE and then mixed with unstimulated PBMC. After mixing, the cells were labeled with supernatants from the primary hybridoma cultures and fluorescein conjugated polyclonal goat anti-IgG/IgM second step antibody. For analysis, G1 (orange) was placed on unstimulated PBMC. G2 was placed on the large cells comprised mainly of the HE loaded ConA stimulated cells. Some unstimulated PBMC were included in G2. Labeling of cells in one or both populations of cells could be detected in the FL-1 channel. MAbs reacting with molecules only expressed on activated cells could be distinguished from mAbs reacting with molecules expressed on unstimulated PBMC (Davis et al., 1995). Fig. 2B is representative of cells labeled with a mAb (CD45R0, memory cell marker) detecting a molecule expressed on resting and activated cells. As noted, unstimulated small memory cells (Fig. 1B, lower right quadrant, FL1) and all activated cells and some small lymphocytes (upper right quadrant, FL2) were present in the stimulated, HE loaded cell population. Fig. 1C is representative of cells labeled with a mAb (ACT 1) detecting a molecule only expressed on activated blast cells (upper right quadrant, FL2). As shown in this example, the unstimulated lymphocytes present in the HE loaded preparation of ConA stimulated cells did not express ACT1 and remained in the upper left FL-2 quadrant.

The NK series of hybridomas, producing mAbs reactive with molecules expressed on NK cells alone or on NK and other cell types, were identified by flow cytometry also, using freshly isolated PBMC mixed with cultures of IL-15 stimulated cells enriched for CD335⁺ NK cells. Anti-NK (AKS1, CD335) used during the development of this set of mAbs was a gift provided by Anne Storset (Norwegian School of Veterinary Science).

2.4. Flow cytometric analysis

Single and multi-color FC were used to identify and characterize mAbs that recognize molecules expressed on resting and/or activated lymphocytes (Allen et al., 2009; Davis et al., 1996a, 1995; Koo et al., 2004). Cultures of IL-15 activated NK cells and preparations of unstimulated and ConA stimulated PBMC were used to compare expression of mAb-defined molecules expressed on NK and/or $\alpha\beta$, and $\gamma\delta$ T cell subsets. For some studies, whole blood was lysed with NH₄Cl to obtain preparation of cells with granulocytes and mononuclear cells (Davis et al., 1987). The second step reagents used were isotype specific goat anti-mouse immunoglobulins conjugated with fluorescein (FL), phycoerythrin (PE), Cy5, or PE-Cy5.5 (Invitrogen, Life Technologies, Carlsbad, CA). When the mAbs were the same isotype (IgG1), Zenon™ labeling kits were used (Molecular Probes, Life

Technologies, Carlsbad, CA). Zenon-Fab fragments of IgG1 specific goat anti-mouse antibody conjugated with different fluorochromes (FL, PE or Cy5, or PE-Cy5) were used according to the manufacturer's instructions. All cell preparations were processed as described (Davis et al., 1995) and fixed in 2% PBS-buffered formaldehyde and kept at 4 °C until analyzed.

Data were collected on a Becton Dickinson FACS Calibur flow cytometer equipped with a MAC computer and Cell Quest software (BD Immunocytometry Systems, San Jose, CA). FCS Express software (DeNovo Software, Glendale, CA) was used to analyze the data.

2.5. Clustering of mAbs with apparent specificity for the same molecule

Single color FC was used in preliminary studies to compare the patterns of reactivity of mAbs with molecules expressed on subsets or all leukocytes. MAb exhibiting similar or identical patterns of labeling were grouped to form clusters as described (Davis et al., 1995). Each cluster was given a temporary name to be used until the cluster was characterized. A numerical designation was given to mAbs reacting with molecules up-regulated on lymphocytes starting with ACT1. Where similarity of expression was strong, mAbs were given the same numerical designation forming a cluster. Where there was uncertainty about similarity or there was a clear difference, a separate numerical designation was used (Koo et al., 2004). A formal numerical workshop designation was established during the first international workshop to use until a cluster of mAbs was characterized (Howard et al., 1991). This workshop designation was adapted and used with the mAbs submitted to the workshops except for mAbs that recognized molecules with upregulated expression on activated lymphocytes (Davis et al., 1996b).

2.6. Characterization of mAb-defined molecules

ConA stimulated PBMC and cultured NK cells were prepared as described above, and used as indicated below. Two different approaches were performed to characterize the target antigen of each mAb cluster.

The first approach was done with two-dimensional gel electrophoresis (2-DE) followed by western blotting analysis (WB) to characterize one of the molecules identified with one cluster of mAbs (ACT39) that recognized a molecule with apparent up-regulation in expression on activated NK cells. In brief, using previously described methods (Seo et al., 2009), whole cell lysates from cultures of NK cells were prepared using lysis buffer [7 M urea (Bio-Rad Laboratories, Hercules, CA), 2 M thiourea (Bio-Rad Laboratories), 2% ASB-14 (Sigma, St. Louis, MO), 0.5% Triton X-100 (Sigma), 2 mM tributylphosphine (Bio-Rad), and 0.1% bromophenol blue], followed by proteinase inhibitor cocktail for 5 min on ice and nuclease treatment (GE Healthcare) for 15 min at room temperature. Lysates were clarified by centrifugation ($45,000 \times g$ for 30 min). The lysates were analyzed by 2-DE. Two IPG strips (Immobiline™ Drystrip gels, pH 3–11 non-linear, GE Healthcare) were rehydrated with the lysates supplemented with 2% IPG-buffer (GE Healthcare) for 24 h. Isoelectric focusing (1st dimension) was performed using a Multiphor II unit (GE Healthcare) in three running phases: phase 1, 300 V/0.01 h; phase 2, 3500 V/1.50 h; and phase 3, 3500 V/4.50 h. SDS-PAGE (2nd dimension) was conducted in 12.5% acrylamide

gels. One gel was stained with Coomassie blue G-250 (Bio-Rad, Hercules, CA) and used for MS (see below). The other was used for WB. The spot, in the Coomassie blue-stained 2-DE gel identical to the spot in the WB, was excised and digested with trypsin for analysis (Shevchenko et al., 2002). The resulting peptides were analyzed by matrix-assisted laser desorption ionization (MALDI) and nano-electrospray LC-MS/MS using a Nanoacquity Ultra Performance Liquid Chromatograph (Waters, Milford, MA). Peptide sequencing data were generated using a Quadrupole-TOF Premier mass spectrometer equipped with a Nano-ESI source (Waters, Milford, MA). Spectra were analyzed by the MASCOT search engine using the Swiss protein database (Perkins et al., 1999). Probability-based protein identification was performed by searching the sequence databases using MS data (Perkins et al., 1999).

In the second approach, the other three mAb-defined molecules were identified following immunoaffinity purification (IP) using the Pierce Direct IP Kit (Pierce, IL). The kit uses an amine-reactive support (AminoLink Plus Coupling Resin) which binds directly to purified mAbs. The immobilized mAbs remain bound to the resin, which allows elution of the target antigen free from Ab contamination. Since the resin non-specifically binds any primary amine-containing molecules, the mAb was first purified from ascites to remove non-relevant proteins, such as albumin and transferrin present in ascites, using Melon Gel Monoclonal IgG Purification Kit (Pierce, IL) following the company's protocol. The purity of purified mAb solution was evaluated by SDS-PAGE analysis. The purified mAb was coupled to AminoLink Plus Coupling Resin following the manufacturer's instruction, and used for IP of the target antigen. The lysates were prepared from ConA stimulated PBMC in IP lysis buffer (included in the kit) with protease inhibitor cocktail (Halt Protease Inhibitor Cocktail, Pierce, IL) as recommended in the kit protocol. The lysates were pre-cleared by incubation with the control agarose resin provided in the kit for 1 h at 4 °C followed by incubation with the resin coupled with an isotype control mAb (Colis69: IgG1) at 4 °C overnight to reduce non-specific binding. The pre-cleared lysates were used for IP of mAb defined molecules by incubation with the resin coupled with a mAb specific for the target molecule. The rest of steps were conducted following the manufacturer's recommendation (Pierce Direct IP Kit, Pierce, IL). All procedures were repeated 3–5 times using each new lysate to obtain enough of the molecule for analysis. All eluates obtained after each repeat were pooled and concentrated approximately 10 times (based on volume) using Ultrafree MC Centrifuge Filter (Millipore, MA). The resultant eluates were subjected to SDS-PAGE analysis. Bands were visualized by silver staining (SilverQuest™ Staining Kit, Invitrogen, CA). Comparison of the band patterns revealed which band was specific for the mAb defined molecule as previously shown (Lee et al., 2001). Where possible, the specific band was further confirmed by WB with the mAb used for the IP following reducing and non-reducing SDS-PAGE conditions. The identified bands were excised and used for LC-MS/MS analysis. The acquired MS/MS fragment ion lists were compared to the bovine protein databases (ftp://ftp.ensembl.org/pub/release-78/fasta/bos_taurus/pep/) using the MASCOT search engine as previously described (Noh et al., 2008).

2.7. Expression of mAb-defined molecules on PBMC from cattle vaccinated with Map-relA

The mAbs selected for analysis and the mAbs used to facilitate characterization are shown in Tables 1 and 2. As mentioned, the unvaccinated calf and the two calves vaccinated with the *Map-relA* mutant were used as a source of cells to document the mAb-defined molecules were only up-regulated in expression on cells from the two immunized calves. Combinations of 3 or 4 mAbs, shown in Table 3, were used to determine the level of expression of the mAb-defined molecules on NK and T cell subsets. PBMC were isolated as previously described and cultured in 6 well culture plates (10^7 cells/well, alone or with live *Map* as previously described, Park et al., 2011). Cells were stimulated with live *Map* at a MOI of 10 (Park et al., 2011). Aliquots of cells were labeled at the initiation culture day 0 and 6 days post stimulation, as previously described (Koo et al., 2004; Park et al., 2011). Selective electronic gating was used to collect and analyze the data (Fig. 1). As illustrated in the example of selective gating of CD4 cells in Fig. 1B and C, selective gates were also placed on NK, $\gamma\delta$ T cells, or CD8 cells to determine the pattern of expression of the mAb-defined molecules on the respective gated cell subsets.

3. Results

3.1. Development and characterization of mAbs that recognize molecules expressed on resting and activated lymphocytes

The fusions made with spleens from mice hyper-immunized with ConA activated lymphocytes yielded 317 hybridomas secreting mAbs specific for molecules expressed on resting and/or activated lymphocytes. Thirty four mAbs were identified that reacted with molecules with up-regulated expression on activated lymphocytes (Table 1). Some of the mAbs developed from the first fusion were given a temporary name designation of ACT1–ACT5. These were submitted for evaluation in the third ruminant workshop (Davis et al., 1996a). Two of the mAbs, CACT7A and CACT101A, formed a cluster, referred to as ACT1. They were not completely characterized and were not given an official workshop cluster designation (Davis et al., 1996a). Further studies reported here revealed five additional mAbs, developed at a later date, had identical specificities with mAbs in the ACT1 cluster (Table 1). The criterion used to include the mAbs in this cluster was that, cross comparison of the mAbs in two color analysis, showed the mAbs formed a diagonal labeling pattern or blocked labeling with one of the mAbs, indicating the mAbs recognized epitopes on the same molecule. A mAb designated as ACT3 clustered with mAbs submitted to the second ruminant workshop that formed workshop cluster WC10 (Naessens and Hopkins, 1996). Further studies showed the mAb ACT3 recognized CD26 (Lee et al., 2001). As mentioned below, this mAb was used in the validation of the specificity of a cluster of mAbs (ACT40) developed at a later date. Studies with mAbs in cluster ACT40 did not suggest they might recognize CD26. Three mAbs with identical specificity, designated as cluster ACT5, reacted with CD25 (Table 1) (Naessens et al., 1992). As reported below, these mAbs were used in cross comparison studies to verify the specificity of 9 of the mAbs, developed from the later fusions, that formed one of the clusters reported in the present study. Cross comparison of labeling of pairs of a group of five mAbs designated as ACT28, ACT29, ACT30, ACT32, and ACT34, initially thought to recognize different molecules, showed they yielded diagonal patterns of labeling or blocking by one of the pair of mAbs. These mAbs formed a cluster

that recognized a molecule constitutively expressed on granulocytes, with low expression on unstimulated lymphocytes and increased expression on activated lymphocytes (Koo et al., 2004).

3.2. Development of mAbs that recognize molecules expressed on IL-15 activated NK cells

FC screening of primary culture supernatants from hybridomas obtained from mice immunized with activated NK cells yielded 158 hybridomas producing antibodies that recognized molecules expressed on unstimulated and/or activated NK cells. Eight of the hybridomas that appeared to recognize molecules exclusively expressed on activated NK cells were selected and cloned for further analysis. Further studies revealed the mAbs formed two clusters (ACT39, ACT40) (Table 1). Analysis of expression of mAbs in cluster ACT39 on whole blood preparations containing granulocytes and mononuclear cells revealed the mAbs in cluster ACT39 recognized a molecule with apparent high expression on granulocytes and low expression on monocytes (Fig. 3B). Expression was not detected on lymphocytes (Fig. 3B). Comparison of expression in a mixed population of PBMC comprised mainly of CD3 positive lymphocytes and CD335 positive NK cells (Fig. 3D) showed the molecule detected was also expressed on activated NK cells (Fig. 3E). MAb in the ACT40 cluster recognized a small population of lymphocytes on unstimulated lymphocytes in PBMC (Fig. 3C). Further analysis of the lymphocyte population in subsequent studies revealed the molecule was expressed on CD4 and CD8 T cells, WC1⁺ $\gamma\delta$ T cells, and B cells but not on resting NK cells (data not shown). Analysis of expression in the mixed cell population revealed it was highly expressed on activated NK cells (Fig. 3F).

Comparison of labeling of NK64A with NK93A and NK29A with NK47A showed each combination yielded a diagonal labeling pattern. Further comparison of labeling with the other mAbs in cluster 40 demonstrated that pairs of mAbs yielded a diagonal pattern of labeling or blocking of labeling by one of the pairs, indicating they recognized the same molecule (data not shown).

3.3. Characterization of mAb defined molecules

Attempts to use 2-DE and WB to identify mAb-defined molecules by MS proved difficult because most of the mAbs under study recognized epitopes that were disrupted in the presence of SDS. The one success was with NK93A in the ACT39 cluster. The spot submitted for MS revealed the cluster recognized the bovine orthologue of gp96 (Fig. 4). An alternative strategy for characterizing the rest of the mAb-defined molecules was more successful. This involved use of immunoaffinity resins conjugated with a nonspecific isotype control mAb and the specific mAb. To prepare the immunoaffinity resins, the mAb was first purified from ascites (Fig. 5A). Once the immunoaffinity resins were prepared with the purified mAbs, cell lysates were pre-cleared by incubating with the control agarose resin, and subsequently with the resin coupled with the isotype control mAb, to reduce non-specific binding. The resultant lysate was used for the IP of the target antigen with resins coupled with specific mAbs. These steps were repeated multiple times to obtain sufficient product for MS as explained in Section 2. Following concentration, the eluates were subjected to SDS-PAGE followed by silver staining, and then compared. The bands only detected in the eluates from the mAb specific IP were extracted and submitted to the

Molecular Biology and Genomics Core at Washington State University for MS as described (Lee et al., 2001; Noh et al., 2008). The first IP was done with CACT185A (ACT30) since the mAb recognized an epitope prepared with SDS-PAGE under non-reducing conditions. This feature helped identify the target antigen and establish the IP protocol. MS of the IP band revealed the molecule recognized by CACT185A is CD50 (Fig. 5B and C). Since the epitopes detected with the other mAbs in this cluster (ACT28, ACT29, ACT30, ACT32, and ACT34) were destroyed during SDS-PAGE, their specificity was validated by FC. Cross comparing labeling demonstrated pairs of mAbs yielded a diagonal pattern of labeling or blocking of labeling by one of the pair of mAbs (data not shown). The IP of NK29A (ACT40 cluster) was conducted using the same lysates used for the IP with CACT185A. The resultant protein sample was compared with the eluates from the CACT185A IP (served as an isotype control). The MS results showed the mAbs in the ACT40 cluster recognize CD26 (Fig. 6A). FC comparison with ACT3 (CD26), characterized in the second ruminant workshop, provided additional proof that the mAbs in the ACT40 cluster recognized CD26. New cell lysates were used to obtain the IP for CACT200A (ACT1). The band pattern was compared with the IP of the isotype control (ColiS69, IgG1). Analysis of SDS-PAGE gel revealed 4 specific bands were present in the mAb specific IP compared to the isotype control (Fig. 6B). All four specific bands (b, c, e, and f, Fig. 6B) and one non-specific band (d, Fig. 6B), as a control, were submitted for MS analysis. Analysis revealed all the specific bands contained peptide fragments of the same molecule, the signaling lymphocytic activation molecule family member 9 (SLAMF9). The bands, b, c, and e, only matched a sequence in bovine SLAMF9. The f band contained peptide fragments of two bovine proteins, SLAMF9 and casein alpha S1. In contrast, the nonspecific band (band d) only matched a sequence in a different bovine protein, a serine/arginine-rich splicing factor 7 (NCBI reference sequence No. NP_001029449.1). The molecular weight (MW) of band d matched the MW of the protein (26.9 kDa). The estimated MW of SLAMF9 (NCBI reference sequence No. NP_001289591.1) is 32.4 kDa. One of the specific bands (band e) is approximately the same size. Bands b and c might be splice variants or fragmentation of SLAMF9 during protein extraction. The estimated MW of casein alpha S1 (NCBI reference sequence No. NP_851372.1), detected in band f, is 24.5 kDa. Taken together, the results indicate mAbs in ACT1 recognize bovine SLAMF9.

3.4. Expression of gp96, CD25, CD26, CD50, and SLAMF9 on resting and Map stimulated PBMC from cattle vaccinated with Map-relA

As mentioned in Section 2, selective gating was used to distinguish resting from activated mononuclear cells (Fig. 1) and determine the percent of the respective cell subsets in a cell preparation (Fig. 7), and to determine the relative proportion of resting and activated cells in each subset (Fig. 8).

3.4.1. Expression on NK cells—Previous studies showed NK (CD335⁺) cells proliferated in cultures with and without antigenic stimulation with no apparent difference in the phenotype of proliferating cells from control uninfected calves and calves experimentally infected with *Map* (Koo et al., 2004; Park et al., 2014). gp96 was detected on activated NK cells from the unvaccinated and vaccinated calves (data not shown). Further analysis showed NK cells from the control and vaccinated calves co-expressed CD8, CD25, and CD50. They

did not express SLAMF9 (data not shown). The majority of NK cells from the control calf cultured with *Map* did not express CD26 (Fig. 9). In contrast, CD26 was highly expressed on CD8⁺ NK cells from the vaccinated calves (Fig. 10). Of interest and importance to the analysis of expression of CD25, CD26, CD50, and SLAMF9 on $\alpha\beta$ CD8 T cells and CD8⁺ NK cells, the level of expression of CD8 on NK cells was clearly distinct, allowing for selective gating to be used to demonstrate the presence of CD8 on NK cells and also to exclude this population when examining expression of the latter molecules on $\alpha\beta$ CD8 T cells (compare Figs. 9 and 10). Further studies showed SLAMF9 was not expressed on resting or activated NK cells (not shown). An additional observation showed 2–5% of NK cells present in the cultures expressed the $\gamma\delta$ TCR. They were large and present in the G2 gate, showing they were activated. They did not appear to increase in frequency during culture.

3.4.2. Expression on $\gamma\delta$ T cells—Analysis of $\gamma\delta$ T cells, using selective gating, showed they were comprised of CD8⁺ and CD8[−] $\gamma\delta$ T cells (Figs. 11 and 12). A small proportion of cells from both the control and vaccinated calves were activated following culture in the presence of *Map* (Figs. 11 and 12). Additional studies showed expression of CD25, CD50, and SLAMF9 were up-regulated on both populations of activated CD8⁺ and CD8[−] $\gamma\delta$ T cells (data not shown). Previous studies had shown CD26 was only up-regulated on WC1[−] $\gamma\delta$ T cells.

3.4.3. Expression on CD4 and CD8 T cells—Analysis of expression of CD25, CD26, CD50, and SLAMF9 on PBMC from the control un-vaccinated calf, before and after stimulation with *Map*, confirmed there was little or no increase in expression on CD4 and CD8 T cells. There was clear up-regulation of expression of these molecules in cultures of cells from both vaccinated calves stimulated with *Map*, as illustrated with representative cells from one of the vaccinated calves and the control calf (Figs. 13 and 14). As noted previously, the proliferative response of CD4 T cells to *Map* was more vigorous than the CD8 T cell response (Figs. 8, 13 and 14) (Allen et al., 2009; Koo et al., 2004; Park et al., 2011). Consistent with previous studies, all activated cells expressed CD25 and CD45R0. CD45R0 was not expressed on naïve cells. CD25 was expressed on some resting non-replicating cells.

4. Discussion

4.1. mAb characterization

The improved methods for characterization of mAb-defined molecules have provided an opportunity to accelerate efforts to determine the specificity of a backlog of mAbs that recognize molecules involved in differentiation and function of lymphocyte subsets in cattle. As reported here, we were able to determine the specificity of mAbs shown to identify molecules with up-regulated expression on NK and T cell subsets from cattle stimulated with ConA or huIL-15. We were also able to verify their pattern of expression on PBMC from cattle with an immune response to *Map*, using calves vaccinated with a candidate *Map-relA* deletion mutant as a source of cells. Analysis of the 2 clusters of mAbs that recognize molecules expressed on activated NK cells revealed one cluster (ACT39) recognized gp96

(Table 1 and Fig. 3), a chaperone molecule that is a master regulator involved in antigen transport (Binder et al., 2001; Binder and Srivastava, 2004; Basu et al., 2001). Its extracellular form interacts with its receptor, CD91, expressed on antigen presenting cells and other cell types (Dai et al., 2003; Yang et al., 2007; Zheng et al., 2001). Its relevance to NK function is currently unknown. However, it has been recently detected on human NK cells and reported in an abstract (Sedlacek and Binder, 2014). As reported in the abstract, studies are underway to demonstrate gp96 is bound to its receptor CD91 on NK cells. The data presented here are the first to report detection of gp96 on bovine activated NK cells (Fig. 3). Its presence and apparent appearance as an activation molecule can now be explained. The lack of detection on resting NK cells indicates CD91, the gp96 receptor, is the molecule actually up-regulated following NK activation. Gp96 has also been detected on human neutrophils (Radsak et al., 2003) and now bovine neutrophils. The finding that detection of gp96 on neutrophils and low expression on other cell types, in the present study, appeared variable suggesting expression of CD91 may vary on freshly isolated cells. The development of the mAbs to gp96 affords new opportunities to expand studies on the role of gp96 in NK and neutrophil function through interaction with its receptor CD91. One intriguing area to explore is elucidation of the role of NK cells in cross presentation of antigens to antigen presenting cells (APC). Activated NK cells could deliver antigens to APC chaperoned by gp96 bound to CD91 (Pawaria and Binder, 2011). This is an area under intense investigation where an investigation using cattle as a model could make a major contribution. The second cluster (ACT40) recognized CD26 (a dipeptidyl peptidase IV, adenosine deaminase binding protein) (Table 1 and Fig. 6A), a multifunctional type II cell surface glycoprotein that is widely expressed on T and B cells and activated NK cells and also on epithelial, endothelial and acinar cells of a variety of tissues (Buhling et al., 1994, 1997; Gorrell et al., 2001). A soluble functionally active form occurs in serum. It is a marker for activated memory and migratory T cells. Studies have shown it has co-stimulatory activity similar to CD28 and CD49d (Waldrop et al., 1998). It augments T-cell responses to antigens, initiates signal transduction, increases cytokine secretion and proliferation, upregulates activation markers such as CD25, CD71 and CD69, induces differentiation into effector cells, and enhances provision of help to B cells and cytotoxic T lymphocytes (CTL) (Gorrell et al., 2001). Consistent with previous reports, the level of expression is low on resting lymphocytes and highly up-regulated on activated cells. The finding that CD26 is only expressed on activated NK cells was unexpected and it suggested the ACT40 cluster of mAbs recognized something else. Consequently we overlooked cross comparing the mAbs with ACT3, previously demonstrated to recognize CD26. The availability of the mAbs now offers opportunities to expand research of NK cells, CD4, CD8, and $\gamma\delta$ T cells in cattle. Of special interest, the studies presented here show expression of CD26 is associated with activated NK cells that express CD8. It is the primary subset of NK cells detected in cultures of PBMC from cattle vaccinated with the *Map-relA* mutant. Although further studies are needed, it appears CD26 may provide a way to discriminate a specific NK memory response to antigens from non-specific proliferative responses that we have observed in cultures of PBMC from calves unexposed to known pathogens like *Map*. It was not expressed on activated NK cells from the control calf not vaccinated with *Map-relA*. However, it was expressed on NK cells from the calves vaccinated with *Map-relA*.

Analysis of expression on $\gamma\delta$ T cells has shown CD26 is expressed on activated WC1⁺ $\gamma\delta$ T cells (new observation and see below for further discussion).

Analysis of the mAbs that formed the ACT1 cluster revealed they recognize the most recently identified member of the signaling lymphocyte activation molecules, SLAMF9 (Table 1 and Fig. 6B). As reviewed by Calpe et al. (2008) SLAM-family receptors and their adapters SAP and EAT-2 function in early phases of hematopoiesis and in lineage commitment. The SLAM-family receptors serve as adhesion molecules on the surface of a variety of mature hematopoietic cells. In addition, they are involved in regulating innate and adaptive immune responses. SLAM-family receptor–ligand pairs not only regulate proliferation, cytotoxicity, and cytokine production of T lymphocytes, but also modulate lytic activity, cytokine production, and MHC-independent cell inhibition of NK cells, B cell activation and memory generation, and regulation of neutrophil and macrophage killing and platelet aggregation. SLAMF9 is the most recently identified member of the family to be described. It has a short cytoplasmic tail, lacking tyrosine-based motifs or other known signaling motifs. Thus far, no signal transduction mode has been identified (Calpe et al., 2008). The ligand for this molecule is unknown. SLAMF9 mRNA has been found in human monocytes, T cells, B cells, and DCs suggesting the SLAMF9 may be expressed by multiple cell types (Fennelly et al., 2001; Zhang et al., 2001). Bovine SLAMF9 has only been detected on activated CD4 and CD8 cells (this report). It is not expressed on resting or activated NK cells. As illustrated, the expression level on CD4 and CD8 cells only increased in PBMC from the vaccinated calves following stimulation with *Map*, but not in PBMC from the control calf (Figs. 13 and 14).

Analysis of the cluster of mAbs comprised of ACT28, ACT29, ACT30, ACT32, and ACT34 revealed they recognize CD50 (Table 1 and Fig. 6), an intercellular adhesion molecule expressed on leukocytes of all lineages, epidermal Langerhans cells and endothelial cells. It is generally absent from cells and tissues of non-hematopoietic origin. CD50 is released from activated lymphocytes and neutrophils, probably by proteolytic cleavage. Soluble forms of CD50 are detectable in the blood (Montoya et al., 2002; Pino-Otin et al., 1995). It is the primary receptor for CD209 expressed on DC (Geijtenbeek et al., 2002; Geijtenbeek and Gringhuis, 2009). The level of expression is low on freshly isolated PBMC in cattle with increased expression following stimulation with polyclonal activators and *Map* antigens in animals naturally and experimentally infected with *Map* (Figs. 13 and 14) (Koo et al., 2004; Park et al., 2011).

4.2. Expression of CD25, CD26, CD50, SLAMF9 on activated NK cells

Further analysis of expression of the activation molecules has provided a more complete phenotypic profile of PBMC from cattle experimentally infected with wild type *Map* or a *Map-re1A* deletion mutant under study as a potential live vaccine (Koo et al., 2004; Park et al., 2011, 2014). PBMC are comprised of NK cells, WC1 positive and negative subsets of $\gamma\delta$ T cells, CD4 and CD8 $\alpha\beta$ T cells. CD8 is expressed on a subset of CD2⁺, WC1⁺ $\gamma\delta$ T cells, and as shown in the present study, it is highly expressed on activated CD26 positive NK cells in response to *Map* stimulation in vaccinated animals (Fig. 10). Previous studies by Storset et al. (2004) have shown a small population of CD8 positive NK cells is present in

freshly isolated PBMC, consistent with our observations. They reported the population was comprised of $\alpha\alpha$ and $\alpha\beta$ CD8 subsets. Further studies are needed to characterize this unique subset of NK cells and determine if proliferation is attributable to expansion of either subset of CD8 cells as described by Storset et al. (2004) and whether it represents a memory response. Analysis has shown this subset is positive for CD45R0. We show it is also positive for CD26. This distinguishes this population of NK cells from NK cells from uninfected cattle that proliferate in cultures stimulated with antigen. Further studies are needed to compare the phenotype of NK cells that proliferate spontaneously, especially in calves, without antigenic stimulation.

Of importance to further investigations of CD8 $\alpha\beta$ T cell responses to antigenic stimulation, the present study shows that CD8⁺ NK cells may comprise a significant proportion of CD8⁺ T cells in cultures with and without antigen stimulation. FC results from earlier studies suggested this might account for the high proportion of CD8 positive cells in some cultures, especially from young calves (Koo et al., 2004). The CD8⁺ NK CD335⁺ population could be clearly visualized, using FSC vs fluorescence, as a dense population in dot plot configuration in 6 day cultures of PBMC with and without presence of *Map* (Figs. 9 and 10). Use of an additional electronic gate showed this population accounted for a proportion of activated CD8 cells in the cultures. The gate could be used to isolate the population for analysis and also to exclude the population to study the CD8 $\alpha\beta$ T cell response to antigenic stimulation.

An additional observation made during the course of the studies is detection of a small subset of WC1⁻ CD8⁺ NK cells that co-expresses the $\gamma\delta$ TCR. In our initial studies reporting on this population, it appeared it was only detectable in cultures of PBMC and spleen cells following stimulation with IL-15 (Johnson et al., 2008). Expression of CD8 was not examined in this study. The proliferative response of PBMC cultured in the presence of IL-15 was low whereas the response of spleen derived cells was robust approaching 50% of NK cells following 2 weeks of culture. Addition of IL-12 and IL-18 to cultures, post stimulation with IL-15, increased the number of CD335⁺ $\gamma\delta$ T cells in cultures of PBMC and spleen. Analysis showed the CD335⁺ $\gamma\delta$ T cells produced IFN- γ in response to exogenous IL-12 and IL-18. Further studies are needed to determine the role of this unique subset of CD335⁺ $\gamma\delta$ T cells in immune responses.

4.3. Expression of CD25, CD26, CD50, SLAMF9 on $\gamma\delta$ T cells

Activation and proliferation of $\gamma\delta$ T cells has been observed in previous studies (Koo et al., 2004; Park et al., 2014) but it has not been clear whether it was an antigen specific response. A small proportion of CD8⁺ and CD8⁻ $\gamma\delta$ T cells in cultures from the control calf and vaccinated calves proliferated following stimulation with *Map* (Figs. 11 and 12). The CD8⁺ population is a subset of the CD2⁺ WC1⁻ population of bovine $\gamma\delta$ T cells (Ahn et al., 2002; Davis et al., 1996a). Subsets of $\gamma\delta$ T cells proliferated in cultures of PBMC in the presence of *Map*, as noted in previous studies, providing data that now suggest proliferation is not attributable to an antigen specific response (Figs. 11 and 12) (Koo et al., 2004; Park et al., 2014). Ongoing studies have shown that expression of CD25, CD50, and SLAMF9 are upregulated on WC1⁺ and WC1⁻ $\gamma\delta$ T cells (unpublished observation this report). Analysis

of expression of CD26 showed that it is only expressed on WC1⁺ $\gamma\delta$ T cells (this report). This observation provides further evidence that the mechanisms regulating the function of WC1⁺ and WC1⁺ $\gamma\delta$ T cells differ.

4.4. Expression of CD25, CD26, CD50, SLAMF9 on $\alpha\beta$ CD4 and CD8 T cells

As noted previously (Koo et al., 2004; Park et al., 2014), the predominant population proliferating in response to *Map* was CD4 T cells. CD8 T cells proliferated in response to *Map* also but to a lesser extent than CD4 T cells (Figs. 7 and 8). Co-labeling with CD45R0 and the respective molecules up-regulated following stimulation with *Map* showed increased expression only occurred on CD25/CD45R0 positive memory T cells. The activated cells co-expressed all the molecules characterized in the present study (Figs. 13 and 14). Cross-comparisons of expression are needed to determine if subsets of CD4 cells can be identified that might have different functional activity.

In summary, the results from the present study show further effort to determine the specificity of mAbs developed during and after the workshops is warranted. The molecules identified here expand opportunities to advance research on the mechanisms regulating immune responses, using cattle as the model species. CD26 and gp96 are under intense investigation for their roles in immune regulation and antigen delivery. SLAM9 has only been characterized recently as a member of a family of molecules centrally involved in immune responses. MAb to these molecules are now available for use. Additional mAbs left over from the workshops may recognize other molecules useful in advancing research in immunology, using cattle as a model species.

Acknowledgments

The studies were supported by the Washington State University Monoclonal Antibody Center. All the mAbs are available through the center's website <http://vmp.vetmed.wsu.edu/resources/monoclonal-antibody-center>.

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Fig. 1.

Representative profiles showing the gating and color coding strategy used to simultaneously obtain data on molecules expressed on resting and/or activated lymphocytes undergoing blastogenesis. As illustrated in (A), electronic gates were placed on cells imaged in SSC vs FSC light scatter using G1 to define small resting unstimulated cells (artificially colored orange/red) and G2 to define large cells (colored blue). As illustrated in (B) with a mAb specific for CD4, CD4⁺ unstimulated cells (lower right quadrant) could be distinguished from activated cells (upper right quadrant) (FSC vs CD4). As illustrated in panel C, use of an additional gate on provided a way to isolate a subset of cells, in this case CD4, for further analysis of molecules expressed on unstimulated and/or activated cells. The profiles shown here were prepared from a culture of PBMC from one of the vaccinated animals stimulated with *Map* for 6 days. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 2.

Representative profiles from a mixture of unstimulated PBMC mixed with ConA stimulated PBMC loaded with hydroethidine. As described in Fig. 1, electronic gates and artificial color coding were used to define small unstimulated lymphocytes (G1, orange/red) and large lymphocytes undergoing blastogenesis (G2, blue). An example of a mixture of unstimulated cells with ConA stimulated HE loaded cells is shown in (A). The HE loaded cells (ConA stimulated, upper left quadrant) are clearly separated from the HE unloaded cells (unstimulated cells). As noted in the upper left quadrant (FL-2), some inactivated cells were present in the ConA stimulated culture of PBMC (orange/red profiles present in the quadrant). An example of cells labeled with a mAb specific for a molecule (CD45R0, memory cell marker) expressed on unstimulated and stimulated cells (upper right and lower left quadrants) is shown in (B). An example cell labeled with a mAb (ACT1) specific for a molecule only expressed on stimulated cells (upper right quadrant) is shown in (C). HE loaded unstimulated cells present in the ConA culture of cells did not express ACT1 (orange/red cells, upper left quadrant). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 3.

Representative profiles of NK64A (ACT39) and NK47A (ACT40) expression in leukocyte subsets. (A–C) The leukocyte preparation was comprised of granulocytes (GR, red), monocytes (M, blue) and lymphocytes (L, orange/red). (D–F) Activated NK cells maintained with huIL-15 were mixed with fresh PBMC. The mixture was visualized in SSC vs FSC to place a gate on PBMC (G1, orange/red) and activated NK cells (G2, blue). Two color labeling with anti-CD3 (detecting T lymphocytes) and anti-CD335 (detecting NK cells) clearly showed the mAbs labeled mutually exclusive populations. (D). NK64A (E) and NK47A (F) only labeled NK cells (blue cells). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 4. Identification of the target molecule of NK93A by 2-DE and WB. Two identical 2-DE gels were prepared using NK cell lysates. One gel was visualized by Coomassie blue staining (A), the other was used for WB with NK93A mAb. WB signal was detected only on a single spot (indicated by an arrow in B) and the identical spot in 2-DE gel (indicated by an arrow in A) was excised for mass spectrophotometry.

Fig. 5.

Identification of the peptide recognized by CACT185A by IP. (A) Purification of the mAb from ascites. The figure shows SDS-PAGE analysis of ascites (left lane) and purified mAb of CACT185A (right panel). (B and C) Identification of the target antigen of CACT185A. The eluates of IP were analyzed by SDS-PAGE (non-reducing conditions) followed by silver staining (B) and WB (C). The target band (a) was subjected to mass spectrometry. M, protein marker; lane 1, whole lysate; lane 2, washed portion; lane 3, IP eluate.

Fig. 6.

Identification of the epitopes of NK29A and CACT200A by IP. (A) SDS-PAGE of NK29A IP eluates. The target band is indicated by (a). M, protein marker; lane 1, IP eluate of CACT185A (isotype control); lane 2, IP eluate of NK29A. (B) SDS-PAGE of CACT200A IP eluate. Four specific bands (b, c, e, and f) and one nonspecific band (d) were subjected to mass spectrometry. M, protein marker; lane 1, isotype control (IP of ColiS69); lane 2, IP eluate of CACT200A.

Fig. 7.

Frequency of cell subsets in PBMC from the control calf (DP4) and one of the calves (DP1) vaccinated with *Map-relA*. Data were collected at day 0 and after 6 days post stimulation and culture with live *Map*. The data show there was little difference in the frequency of subpopulations of cell subsets before and after stimulation. Selective gating was needed to show there was a shift in the relative proportion of activated cells. See Fig. 8.

Fig. 8.

Proportion of activated PBMC from the control calf (DP4) and one of the calves (DP1) vaccinated with *Map-relA*. Data were collected at day 0 and after 6 days of stimulation with live *Map*. The percent activation of each cell subset was obtained after selective gating for the cell subset. In each cluster, left bar graph indicates the value at day 0, and right bar, at day 6.

Fig. 9.

Co-expression of CD8 and CD26 with CD335 on activated NK cells in the control calf (DP4). Cells were labeled with anti-CD335, -CD8, or -CD335 and -CD26. Cells were visualized in SSC vs FSC to place gates on small (G1, orange/red) and large cells (G2, blue). (A) Percent of NK cells in PBMC at day 0. Few NK cells were activated at initiation of culture. (B–H) Cells cultured for 6 days with *Map*. (B) Percent of activated NK cells in the culture ungated. (C) Percent of activated NK cells gated. (D) Percent of gated NK cells expressing CD26. Few NK cells expressed CD26. (E) Data show the total population of CD8 cells present in the culture. (F) The NK cells were gated to show most of the NK cells are CD8⁺. (G) G2 was used to isolate activated cells for analysis. The data show the proportion of CD8⁺ CD335⁺ cells (in the circle used for gating and analysis) above diagonal bar and proportion of CD8⁺ CD335⁻ cells below the diagonal bar. (H) The data show cells gated in the circle are predominantly CD8⁺ CD335⁺. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 10.

Co-expression of CD8 and CD26 with CD335 on activated NK cells in a calf vaccinated with *Map-re1A* (DP3). Cells were labeled as described in Fig. 9. (A) NK cells at day 0. (B–H) Cells cultured for 6 days with *Map*. (B) Percent of activated NK cells ungated. (C) Percent of NK cells gated. (D) Percent of gated NK cells expressing CD26. Most of the NK cells express CD26. (E) Data show the total population of CD8 cells in the culture. (G) G2 was used to isolate activated cells for analysis. The data show the proportion of CD8⁺ CD335⁺ cells above diagonal bar and the proportion of CD8⁺ CD335⁻ cells below the bar. (H) The data show cells gated in the circle are predominantly CD8⁺ CD335⁺. The data show activated NK cells from the vaccinated calf express CD8 and CD26.

Fig. 11.

Expression of CD8 on resting and activated $\gamma\delta$ T cells from the control calf, DP4. Cells were labeled with anti-CD8 and anti-TCR δ chain. Cells were visualized in SSC vs FSC to place and color code gates on small/resting, G1 (orange/red), and large/activated, G2 (blue) lymphocytes. (A–D) $\gamma\delta$ T cells in PBMC at day 0. (A) Percent of $\gamma\delta$ T cells with no gate and (B) percent of small and large $\gamma\delta$ T cells with a gate on $\gamma\delta$ T cells. (C) Percent of small and large $\gamma\delta$ T cells expressing CD8 with a gate on $\gamma\delta$ T cells. (D) Percent of large $\gamma\delta$ T cells in G2 expressing CD8 with a gate on $\gamma\delta$ T cells. (E–H) Same gating strategy on $\gamma\delta$ T cells after 6 days of culture with *Map*. (E) Percent of $\gamma\delta$ T cells no gate. (F) Percent of small and large $\gamma\delta$ T cells with a gate on $\gamma\delta$ T cells. (G) Percent of small and large $\gamma\delta$ T cells expressing CD8. (H) Percent of large $\gamma\delta$ T cells in G2 expressing CD8 with a gate on $\gamma\delta$ T cells. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 12.

Expression of CD8 on resting and activated $\gamma\delta$ T cells from the vaccinated calf, DP3. Cells were labeled with anti-CD8 and anti-TCR δ chain. Cells were visualized in SSC vs FSC to place and color code gates on small/resting, G1 (orange/red), and large/activated, G2 (blue) lymphocytes. (A–D) $\gamma\delta$ T cells in PBMC at day 0. (A) Percent of $\gamma\delta$ T cells with no gate and (B) percent of small and large $\gamma\delta$ T cells with a gate on $\gamma\delta$ T cells. (C) Percent of small and large $\gamma\delta$ T cells expressing CD8 with a gate on $\gamma\delta$ T cells. (D) Percent of large $\gamma\delta$ T cells in G2 expressing CD8 with a gate on $\gamma\delta$ T cells. (E–H) Same gating strategy on $\gamma\delta$ T cells after 6 days of culture with *Map*. (E) Percent of $\gamma\delta$ T cells no gate. (F) Percent of small and large $\gamma\delta$ T cells with a gate on $\gamma\delta$ T cells. (G) Percent of small and large $\gamma\delta$ T cells expressing CD8. (H) Percent of large $\gamma\delta$ T cells in G2 expressing CD8 with a gate on $\gamma\delta$ T cells. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 13.

Comparison of the frequency of activation molecules on CD4 T cells in cultures of PBMC from the control (DP4) and a calf vaccinated with *Map-re1A* (DP1). The data represent the frequency of the indicated molecules on CD4 T cells in PBMC before and after stimulation with live *Map* (CACT116A – CD25, CACT185A – CD50, CACT200A – SLAMF9, NK29A – CD26). The data of CACT185 expression at day 0 are not available.

Fig. 14.

Comparison of the frequency of activation molecules on CD8 T cells in cultures of PBMC from the control (DP4) and a calf vaccinated with *Map-re1A* (DP1). The data represent the frequency of the indicated molecules on CD8 T cells in PBMC before and after stimulation with live *Map* (CACT116A – CD25, CACT185A – CD50, CACT200A – SLAMF9, NK29A – CD26). The data of CACT185 expression at day 0 are not available.

Table 1

MAbs characterized in the present study.

mAb	Isotype	Specificity	Expression
CACT7A	IgM	ACT1 (SLAMF9)	Activated $\alpha\beta$, $\gamma\delta$ T cells
CACT101A	IgM	ACT1 (SLAMF9)	
CACT177A	IgG1	ACT1 (SLAMF9)	
CACT200A	IgG1	ACT1 (SLAMF9)	
CACT206A	IgG2a	ACT1 (SLAMF9)	
CACT219A	IgM	ACT1 (SLAMF9)	
CACT276A	IgG2a	ACT1 (SLAMF9)	
CACT298A	IgG2a	ACT1 (SLAMF9)	
CACT114A	IgG2b	ACT3 (CD26)	Expressed on WC1 ⁻ $\gamma\delta$ T cells, CD4, CD8, B cells, activated CD335 ⁺ NK cells
CACT108A	IgG2a	ACT5 (CD25)	Activated $\alpha\beta$, $\gamma\delta$ T cells, NK cells
CACT109A	IgG1	ACT5 (CD25)	
CACT116A	IgG1	ACT5 (CD25)	
CACT151	IgG2a	CD25	
CACT159	IgG2a	CD25	
CACT164A	IgM	CD25	
CACT167	IgM	CD25	
CACT179	IgG1	CD25	
CACT195A	IgM	CD25	
CACT260A	IgM	CD25	Granulocytes, up-regulated on lymphocytes and NK cells
CACT282A	IgM	CD25	
CACT286	IgG1	CD25	
CACT216A	IgM	ACT28 (CD50)	
CACT225A	IgM	ACT29 (CD50)	
CACT185A	IgG1	ACT30 (CD50)	
CACT191A	IgM	ACT32 (CD50)	
CACT180A	IgG1	ACT34 (CD50)	
NK64A	IgG1	ACT39 (gp96)	Granulocytes, activated NK cells
NK93A	IgG1	ACT39 (gp96)	
NK29A	IgG1	ACT40 (CD26)	
NK42A	IgG1	ACT40 (CD26)	
NK47A	IgG2a	ACT40 (CD26)	
NK86A	IgG1	ACT40 (CD26)	
NK134A	IgG2a	ACT40 (CD26)	
NK137A	IgG1	ACT40 (CD26)	

Table 2MAbs used in flow cytometric analysis of the immune response to *Map*.

mAb	Isotype	Specificity
AKS1	IgG1	CD335
MUC2A	IgG2a	CD2
IL-A11A	IgG2a	CD4
7C2B	IgG2a	CD8
GB21A	IgG2b	$\gamma\delta$ TCR δ chain specific
LCTB2A	IgG3	CD25
CACT116A	IgG1	CD25
NK134A	IgG2a	CD26
CACT185A	IgG1	CD50
CACT200A	IgG1	SLAMF9
IL-A116A	IgG3	CD45R0

Table 3

MAB combinations used in flow cytometric analysis of the immune response to live *Map* stimulation.

Cocktail no.	Combination of mAbs
1	AKS1, NK134A, GB21A, LCTB2A
2	AKS1, 7C2B, GB21A, LCTB2A
3	CACT116A, IL A11A, IL-A116A
4	CACT185A, IL A11A, IL-A116A
5	CACT200A, IL A11A, IL-A116A
6	CACT116A, 7C2B, IL-A116A
7	CACT185A, 7C2B, IL-A116A
8	CACT200A, 7C2B, IL-A116A