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Phenotypic and functional analysis of bovine γδ lymphocytes

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The studies have provided the first comprehensive comparison of the factors regulating activation and proliferation of WC1$^+$ and WC1$^-$ γδ T cells. The investigation has shown that accessory molecules essential for activation and function of WC1$^+$ and WC1$^-$ γδ T cells and the sources and roles of cytokines in activation of γδ T cells through the T cell receptor (TCR). The study has also shown that the role of cytokines in activation and function of γδ T cells activated indirectly through cytokines secreted by ab T cells, accessory cells and antigen presenting cells (APC). Cytokines were differentially produced by subpopulations of γδ T cells under different conditions of activation. The investigation obtained in this study has revealed that factors account for activation and proliferation of γδ T cells in cultures designed to study MHC-restricted responses to antigens. Evidence obtained here has shown there is biological relevance to activation under these culture conditions that points to potential regulatory and effector functions of γδ T cells. The investigations have also provided the information needed to begin identifying and characterizing antigens recognized by the TCR repertoires of WC1$^+$ and WC1$^-$ γδ T cells. Finally, the investigations have provided the information needed to begin analysis of the mechanisms by which γδ T cells modulate MHC restricted immune responses to pathogens and derived vaccines.

Key words: WC1$^+$ γδ T cells, WC1$^-$ γδ T cells, T cell receptor, MHC restricted immune responses

Introduction

Investigations in both ruminants and pigs have shown the γδ T cell population differs in composition from that noted in other species. In ruminants and pigs, a subset of γδ T cells that expresses two unique high molecular weight molecules (WC1 and GD3.5 molecules in cattle and SWC6 and the orthologue of WC1 in pigs) have undergone expansion in the course of evolution. Little information is yet available on GD3.5 and SWC6 [4, 29, 32]. The WC1 molecule is a member of a newly defined scavenger receptor cysteine rich (SRCR) family of proteins that express CD3 and CD5 but differ in expression of other membrane molecules. WC1$^+$ cells are negative for CD2, CD4, CD6, and CD8. WC1$^-$ cells are positive for CD2 includes T cell molecules CD5 and CD6 [2, 45]. A subset of these cells co-expresses CD8. To date, no γδ CD4$^+$ T cells have been found. The WC1$^-$ population resembles populations of γδ T cells in humans and rodents. Although data remain limited, information obtained thus far indicate both populations of cells possess regulatory and effector activity and that both populations may modulate the response of αβ T (CD4 and CD8) cells to antigens [5, 8, 10, 42]. Our current working hypothesis is: Effector and regulatory activity of γδ T cell subpopulations are modulated by direct and indirect mechanisms either by 1) antigen recognition through the TCR and 2) activation through cytokines produced by antigen presenting cells (APC) and crossregulatory cytokines produced by both γδ and αβ T cells.

Materials and Methods

Determination of the requirements for stimulation and proliferation through the γδ TCR

Studies have shown γδ T cells are activated and proliferate following exposure to pathogenic organisms and parasites. Limited information is available on the specificity of the responses and the cellular and molecular events that lead to functional activation. Studies are needed to define what the γδ T cells recognize and also determine the sources of stimuli that lead to their activation and development of effector and regulatory activity.

i) Preparation of cells: Peripheral blood from young Holstein calves (3 to 12 months of age) were used as the

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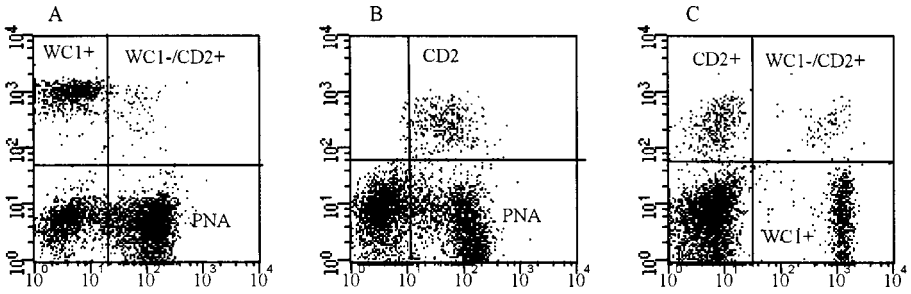

Fig. 1. Representative profiles of peripheral blood mononuclear cells and granulocytes labeled for three-color analysis. The cells were labeled with PNA conjugated with Fluorescein, anti-δ chain and PE-conjugated goat anti-IgG2b, and anti-CD2 and TRI-color-conjugated goat anti-IgG1. Panel A is a comparison of labeling with anti-δ chain mAb (that reacts with $WC1^+$ and $WC1^-$ γδ T cells) and PNAF (FL-2, Y axis, FL-1, X axis). Panel B is a comparison of labeling with anti-CD2 and PNA (FL-3, Y axis, FL-1, X axis). Panel C is a comparison of labeling with anti-CD2 and anti-δ chain mAb (FL-3, Y axis; FL-2, X axis). As shown in panel A, $WC1^+$ γδ T cells are negative for PNA (upper left quadrant) and that $WC1^-$ γδ T cells are positive for PNA (upper right quadrant). As shown in panel B, CD2 positive cells are positive for PNA (upper right quadrant). As shown in panel C, $CD2^+$, $CD2^+/WC1^-$, and $WC1^+$ populations can be distinguished as distinct populations which can be selectively sorted for isolation of mRNA. Proof that the PNA positive γδ T cells were the $WC1^-/CD2^+$ cells was obtained with the PAINT-A-GATE-PRO software program that permits a direct comparison of cell populations for presence of 1, 2, or 3 labels.

primary source of $WC1^+$ cells and spleens as a source of $WC1^-$ cells. Spleens were obtained from cattle processed through the Washington State University(WSU) slaughter-house. All cell separation procedures were performed at 4°C to prevent activation. Peripheral blood mononuclear cells(PBMC) were obtained from peripheral blood by density gradient separation on Accupaque (Accurate Chemical, USA). PBMC depleted of monocytes and B cells (MD-PBMC) were obtained by passing PBMC through acid-washed nylon wool columns [21]. Purified $WC1^+$ cells were obtained from MD-PBMC using peanut agglutinin (PNA). The cells were incubated on petri plates coated with PNA to remove αβ and $WC1^-$ γδ T cells and any remaining monocytes and nonadherent dendritic cells. The choice of PNA to remove αβ T cells and monocytes was based on an early observation that $WC1^+$ γδ T and B cells do not express the receptor for peanut agglutinin (Fig. 1) [16]**.** To purify $WC1^-$ population, cells were incubated on petri plates coated with anti-WC1, anti-IgM, anti-B, anti-CD4, and anti-monocyte/macrophage mAbs to remove αβ and $WC1^+$ γδ T cells, B cells, and monytes/macrophages. mAbs specific for CD2, WC1, and TCR1δ chain (Tables 1 and 2) were used in two color staining to sort cells for isolation of cytokine mRNA using a Becton Dickinson FACSort equipped with a cell concentrator.

ii) Purification of anti-TCR and other anti-accessory molecule mAbs and preparation of mAb-coated plates: Purified mAbs used in culture for direct and costimulation studies of the TCR were prepared from mouse ascites. The mAbs were purified using a salt-promoted adsorption chromatography thiophilic matrix (Affi-T, Kem-En-Tec, Copenhagen, Denmark) using previously described methods [3, 30]. Ninety six well plates were coated with different concentrations of purified mAb diluted in sterile PBS in final volume of 100 ml and kept at 4°C overnight. For costimulation assays where more than one mAb was used, stock concentrations were adjusted accordingly to maintain the appropriate concentration. To coat the six well plates for bulk cultures, 1 ml of mAbs at different concentrations were used.

iii) Analysis of stimulated cells for activation and proliferation: Cells in culture stimulated with anti-TCR and other anti-accessory molecule mAbs were analyzed for states of activation by: a) flow cytometry (FC) to determine the levels of expression of membrane molecules upregulated or only expressed on activated cells and b) direct proliferation in culture, and c) quantification of cytokine mRNA. Proliferation was measured by a non-radioactive assay incorporating Alamar blue (Serotec Inc. Raleigh, NC). The reduction of Alamar blue in lymphoproliferative assays had been shown to closely match results obtained with tritiated thymidine incorporation [1, 30]. Alamar blue was added at 10% assay volume for the last 24-48 hrs of culture and plates were read by spectrophotometry according to the instructions of the manufacturer, at two wavelengths suitable for measuring the oxidized and reduced forms of Alamar blue. The percent reduced Alamar blue was determined and used as an indicator of the level of proliferation.

For the analysis of activation and proliferation, cells were cultured in 96-well culture plates (5×10^5 cells/well) in triplicate with each treatment. Bulk cultures were prepared to obtain enough cells for FC and for cytokine mRNA isolation as detailed below. For bulk cultures, cells were cultured in 6 well plates (10^7 cells/well) coated with 1 ml of antibody at different concentrations. Cells were collected at selected time points and processed for FC and preparation of mRNA. For sorting, the cells were labeled

Table 1. List of mAbs used in this study.

mAb	Ig isotype	Specificity	mAb	Ig isotype	Specificity
H58A	IgG2a	MHC CL I	CACT38A	IgG1	WC1-N3 CL
H42A	IgG2a	MHC CL II	CACT47A	IgM	WC1-N3 CL
TH14B	IgG2a	MHC CL II	BAQ53A	IgM	WC1-N3 CL
TH81A	IgG2a	MHC CL II	BAQ72A	IgM	WC1-N3 CL
BAQ95A	IgG1	CD2	BAQ76A	IgG1	WC1-N3 CL
MUC2A	IgG2a	CD2	BAQ99A	IgG1	WC1-N3 CL
MM1A	IgG1	CD3	BAQ108A	IgG1	WC1-N3 CL
GC50A	IgM	CD4	BAS1A	IgG1	WC1-N3 CL
IL-A11A	IgG2a	CD4	BAQ89A	IgG1	WC1-N4 CL
CACT138A	IgG1	CD4	BAQ159A	IgG1	WC1-N4 CL
B29A	IgG2a	CD5	CACTB7A	IgG1	WC1-N4 CL
CACT105A	IgG1	CD5	BAS2A	IgG1	WC1-N-SUBPOP
BAG8A	IgG3	CD5	BAS6A	IgM	WC1-N-SUBPOP
BAQ82A	IgM	CD6	BAG2B	IgG1	WC1-N-SUBPOP
BAQ83A	IgG2b	CD6	BAG20A	IgM	WC1-N-SUBPOP
BAQ91A	IgG1	CD6	BAG25A	IgM	WC1-N-SUBPOP
CACT141A	IgG2b	CD6	PIG45A	IgG2b	sIgM
7C2B	IgG2a	CD8	BIG73A	IgG1	sIgM
CACT80C	IgG1	CD8α	BIG715A	IgG1	IgG1
BAT82A	IgG1	CD8β	BIG623A	IgG3	IgG2
CACT61A	IgM	TCR1-N12	BIG501E	IgG1	λ light chain
CACT148A	IgM	TCR1-N21	BIG43A	IgG1	κ light chain
GB21A	IgG2b	TCR1-N24	BAQ44A	IgM	B B-B2 antigen
CACTB6A	IgM	TCR1-N6	BAQ155A	IgG1	B B-B4 antigen
CACTB14A	IgG1	TCR1-N6 CL	CH27A	IgM	B B-B5 antigen
CACTB81A	IgG1	TCR1-N7	GC65A	IgM	B B-B6 antigen
86D	IgG1	TCR1-N7 CL	GB25A	IgG1	CD21
CACT22B	IgM	TCR1-N7 CL	CAM36A	IgG1	CD14
B7A1	IgM	WC1-N-BROAD	MM29A	IgM	Monocytes/macrophages
BAQ4A	IgG1	WC1-N-BROAD	BAQ151A	IgG1	Monocytes/macrophages
BAQ84A	IgG1	WC1-N-BROAD	BAT75A	IgG1	CD11a-LIKE
BAQ90A	IgG3	WC1-N-BROAD	MM10A	IgG2b	CD11b
BAQ109A	IgG3	WC1-N-BROAD	MM12A	IgG1	CD11b
BAQ113A	IgG1	WC1-N-BROAD	BAQ153A	IgM	CD11c
BAQ128A	IgG1	WC1-N-BROAD	BAQ30A	IgG1	CD18
CACTB19A	IgG1	WC1-N-BROAD	BAT31A	IgG1	CD44
BAS6A	IgM	WC1-N-BROAD	BAG40A	IgG3	CD44
GB24A	IgG1	WC1-N-BROAD	CACTB51A	IgG2a	CD45
GB54A	IgG2a	WC1-N-BROAD	GS5A	IgG1	CD45R
GB45A	IgG1	WC1-N-BROAD	GC6A	IgM	CD45R
CGB24A	IgG1	WC1-N-BROAD	GC42A	IgG1	CD45R0
CACT60A	IgM	WC1-N-BROAD	GC44A	IgG3	CD45R0
CACT73A	IgG1	WC1-N-BROAD	BAQ92A	IgG1	CD62L
CACT45A	IgG1	WC1-N-BROAD	CACT7A	IgM	ACT1
CACTB28A	IgG1	WC1-N-BROAD	CACT26A	IgG1	ACT2
CACTB31A	IgG2b	WC1-N-BROAD	CACT77A	IgM	ACT2 CL
CACTB37A	IgG1	WC1-N-BROAD	CACT100A	IgG1	ACT4
CACTB39A	IgG1	WC1-N-BROAD	CACT108A	IgG2a	CD25
CACTB42A	IgG1	WC1-N-BROAD	CACT114A	IgG2b	ACT3
CACTB1A	IgG1	WC1-N3 CL	CACT116A	IgG1	CD25
CACTB15A	IgG1	WC1-N3 CL	GB110A	IgM	ACT16
CACTB18A	IgG1	WC1-N3 CL	GB127A	IgM	ACT17
CACTB32A	IgG1	WC1-N3	LCTB28A	IgG2a	ACT13
CACTB33A	IgG1	WC1-N3 CL	LCTB50A	IgG2a	ACT14

CL = cluster, Broad = antigen expressed on most $WC1^+$ cells, Subpop = small unclustered subpopulation

with anti-δ and anti-CD2 mAbs (Fig. 1, profile C). To assess the state of activation, aliquots of cells were triple labeled with combinations of mAbs specific for CD4, CD8, and CD25 or MHC class II; CD2, TCRδ, and CD25 or MHC class II. Other mAbs to be used for analysis of the state of activation were: anti-CD25, -ACT1, -ACT2, -ACT3, -ACT4, -ACT13, -ACT14, -ACT16, and -ACT17 (Table 1) [17]. The sorting combination of mAbs divided the cells into $WC1^+$ and $WC1^-$ γδ T cells and αβ T cells (Fig. 1, profile C). Each of the populations was sorted and analyzed for the presence of cytokine mRNAs. The triple labels divided the major populations of cells and showed the state of activation. The purity of the isolated populations of cells was checked by FC for each sample.

Table 2. Properties of monoclonal antibodies specific for the gd TCR

mAb		Isotype	Group
GB21A	(TCR1-N24)	IgG2b	1
CACT18A	(TCR1-N19)	IgM	1
CACT61A	(TCR1-N12)	IgM	1
CACT71A	(TCR1-N20)	IgM	1
CACT148A	(TCR1-N21)	IgM	1
CACTB6A	(TCR1-N6)	IgM	2
CACTB10A	(TCR1-N6cl)	IgM	2
CACTB14A	(TCR1-N6cl)	IgG1	2
CACTB16A	(TCR1-N6cl)	IgG1	2
CACTB17A	(TCR1-N6cl)	IgG1	2
CACTB41A	(TCR1-N6cl)	IgG1	2
CACT19C	(TCR1-N6cl)	IgM	2
GB22A	(TCR1-N6cl)	IgG1	2
CACT16A	(TCR1-N7cl)	IgM	3
CACT17A	(TCR1-N7cl)	IgG1	3
CACT22B	(TCR1-N7cl)	IgM	3
CACTB12A	(TCR1-N7cl)	IgG1	3
CACTB44A	(TCR1-N7cl)	IgG1	3
CACTB81A	(TCR1-N7)	IgG1	3
86D	(TCR1-N7cl)	IgG1	3

Group 1 mAbs react with the δ chain. Group 2 mAbs react with a set of determinants expressed on a family of the γδ TCR molecule expressed on $WC1^+$ γδ T cells. Group 3 mAbs react with a set of determinants expressed on a group 2 negative family of the γδ TCR molecule expressed on $WC1^+$ γδ T cells. A fourth family of γδ TCR molecules coexpress the group 2 and group 3 clusters of determinants. It is not yet clear whether the determinants are expressed on V_γ or C_γ segments.

iv) Preparation of RNA for RT-PCR: RNA was isolated from 5 to 2 × 10^5 cells using Qiagen RNeasy total RNA kits with QIAshredders to prepare cell lysates for extraction. The mRNA in the RNA was reverse-transcribed and the cDNA subjected to PCR with primers for the respective cytokines. PCR products was analyzed by agarose gel electrophoresis followed by staining with ethidium bromide. The primers available for use in the initial studies are listed in Figure 2. The choice of which primers to be used was depend on the particular study. In addition, we have obtained plasmids containing ovine genes for IL-1b, IL-2, TNF-β, IL-4, IL-8, IL-13, IL-15, MCP1, GM-CSF, and IFN-γ from Drs. Paul Wood and Heng-Fong Seow in Australia [23]. Dr. Seow verified that these probes hybridized with bovine mRNA. We also had a probe for CD25 (IL-2Rα) from Dr. Nancy Magnuson, Washington State University, USA. We probed for cytokine mRNAs in isolated subpopulations of αβ and γδ T cells using RT-PCR. The cytokines of interest for these studies were IL-1b, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IL-13, IL-15, TNF-α, TGF-β, GM-CSF, and IFN-γ. Primers for GAP mRNA were used as positive control. A software program provided by Alpha Innotech was used to quantitate the levels of expression of mRNA for the different cytokines. A standard curve was generated in each assay with known concentrations of cDNA. Con A stimulated cells were used as a positive control to compare differences in the levels of expression of cytokine mRNAs elicited following different treatments with antibody and/or antigen.

IL-1β	5' AAT GAA CCG AGA AGT GG 3'
	5' TTC TTC GAT TTG AGA AG 3'
IL-2	5' CAG TTG CTT TTG GAG AA 3'
	5' TGT CAA AGC ATC TAC TC 3'
IL-4	5' CTG GTC TGC TTA CTG GT 3'
	5' TGT GAA TGA AGC CAA GA 3'
IL-5	5' ATG AGA ATG CAT CTG CAT TTG AC 3'
	5' ACT TTC CAT TGT CCA CTC TGT G 3'
IL-6	5' CTA TGA ACT CCC GCT TCA CAA 3'
	5' TGC CCA GTG GAC AGG TTT CT 3'
IL-7	5' CCC CTG ATC CTT GTT CTG 3'
	5' GCC CTG TGA AAC TGT TGA T 3'
IL-8	5' TTG GCA GCC TTC CTG ATT TCT GCA 3'
	5' AAC CCT GCA CCC AGT TTT CCT T 3'
IL-10	5' ATG CAT AGC TCA GCA CTA CTC TGT TGC CTG 3'
	5' TCA CTT TTG CAT CTT CGT TGT CAT GTA GGT T 3'
IL-12	5' GAG GCG AGG CTC TGA GTC 3'
	5' CAG GTT CTT GGG TGG GTC 3'
IL-13	5' ATG GCA GCA TGG TAT GG 3'
	5' TCC CTC GCG AAA AAG TT 3'
IL-15	5' GAT TTA CCG TGG CTT TGA GTA ATG 3'
	5' TCA AGA AGT GTT GAT GAA CAT TTG GAC 3'
IFN-γ	5' CCT CAA AGA TAA CCA GGT C 3'
	5' CGC TTT CTG AGG TTA GAT T 3'
TNFα	5' CGG TAG CCC ACG TTG TA 3'
	5' TGG CCT CAG CCC ACT CT 3'
GM-CSF	5' CAT GGG CTC CTT GAC C 3'
	5' TTG TTT GGA TCT TTG TGG 3'
TGFβ	5' CAG AAA TAT AGC AAC AAT TCC 3'
	5' GGC TTG CGG CCC ACG TAG TAC AC 3'
iNOS	5' TAG AGG AAC ATC TGG CCA GG 3'
	5' TGG CAG GGT CCC CTC TGA TG 3'

Fig. 2. The sequences of the primers used in the study

Results

Identification and characterization of N-cells (γδ T cells)

Antibodies, reactive with an unique population of nonT/nonB cells, were identified and termed N-cells [12, 15, 16, 18, 19]. Two color FC revealed these cells did not express CD2, CD4, CD6, CD8, or CD45R. The studies also revealed that these cells did not react with peanut agglutinin, a lectin specific for T cells, granulocytes and monocytes [16]. Subsequent studies revealed mAbs reactive with N-cells formed two clusters, one that recognized a

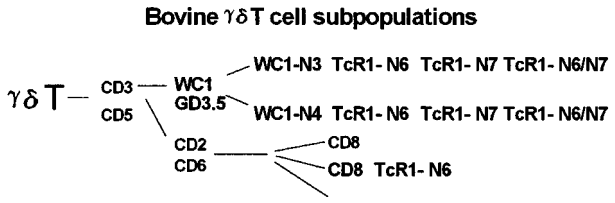

Fig. 3. Schematic diagram showing the subsets of γδ T cells defined with mAbs. The GD3.5 Ag is expressed only on WC1 positive γδ T cells.

high molecular weight molecule (now designated WC1) and a second that recognized a heterodimer comprised of peptides with M_r of approximately 37 and 47 kD (initially designated WC2) [36]. mAbs in the WC2 cluster were later shown to recognize determinants differentially expressed on family subsets of the γδ TCR [15, 27]. Similar studies in sheep [26, 35], goats [14, 44] and other ruminants revealed orthologues of WC1 were present in all species examined [37] and that many of the anti-WC1 mAbs recognized highly conserved determinants expressed on WC1 or the γδ TCR in many species of ruminants [11, 14, 15, 43].

One population was shown to express CD3, CD5, and WC1. Analysis of this population revealed it was comprised of at least two subsets that express mutually exclusive forms of WC1 identified with mAbs that reacted with a set of determinants associated with prototype determinants WC1-N3 or WC1-N4 [15, 34]. The second population was shown to express CD2, CD3, CD5, and CD6. A subset of this population was shown to express CD8 (Fig. 3) [13, 33, 47].

As illustrated in Figure 3, comparison of the patterns of expression revealed the $WC1^+$ population could be subdivided into six subsets based on expression of WC1-N3 and WC1-N4 isoforms and expression of families of the γδ TCR that express determinants TCR1-N6, TCR1-N7, or TCR1-N6 and -N7. Only a subset of $WC1^-$ γδ T cells expressed a form of TCR1 positive for the TCR1-N6 determinant. Grouping and analysis of the mAbs which reacted with γδ T cell receptor have shown one set of mAbs reacted with a cluster of determinants expressed on TCR1 in $WC1^+$ and $WC1^-$ cells and the second with clusters of determinants expressed predominantly on TCR1-N6 related or only on TCR1-N7 related forms of TCR1 [13]. Examination of Cγ gene usage with 10γ clones has shown $WC1^+$ cells appeared to use only one of five possible Cγ genes, Cγ5. In these studies, Jγ segment usage in rearranged g genes appeared to be restricted to Jγ5 and Jγ2. In contrast, γ chain usage by $WC1^-$ cells appeared to be restricted to Cγ3 linked to Jγ3 and Cγ2 linked to Jγ1. Vγ gene usage also appeared to differ in $WC1^+$ and $WC1^-$ cells. Usage was restricted to Vγ3 and Vγ7.1 and Vγ7.2 in clones derived from $WC1^+$ cells. In WC1- clones, usage was restricted to Vγ2.4, Vγ2.3, and Vγ5.2. In contrast, Vδ gene usage appeared similar for both $WC1^+$ and $WC1^-$ cells. Vδ1 and Vδ3 genes were identified in association with the single Cδ gene derived from both populations of cells(Table 2).

Determination of the antigenic phenotype and frequency of subsets of $WC1^+$ and $WC1^-$ γδ T cells in peripheral blood and lymphoid tissues:

i) Flow cytometric analysis: Analysis of the tissue distribution of the two populations of γδ T cells by FC revealed the $WC1^+$ population was present in high concentration in peripheral blood (30-60% in young animals) and low in secondary lymphoid organs (5-10%) and that the $WC1^-$ population was low in peripheral blood (3-5%) and high in spleen, mammary gland, and mucosal epithelium of the intestine (20-60%). Approximately, fifty percent of the $WC1^-$ cells in these tissues expressed CD8. A $CD4^+$ population had not been identified in studies conducted thus far. Approximately 17% were TCR1-$N6^+$, 20% TCR1-$N7^+$, and 13% TCR1-$N6/N7^+$. Approximately 50% of the d^+ cells were negative for these mAbs defined determinants. $WC1^+$ cells comprise ~15% of the δ^+ cells.

ii) Immunohistochemistry: Analysis of the distribution of the $WC1^+$ and $WC1^-$ cells by immunohistochemistry showed the patterns of distribution of the two populations differ in some tissues. In the lymph node (LN), both populations of cells were localized in the subcapsular cortical and medullary sinuses. A few cells had been observed sparsely distributed in the T dependent paracortical areas. This pattern of distribution was similar to the pattern of distribution of macrophages and dendritic cells in LN. In the spleen, distribution differed. $WC1^-$ cells were abundant in the red pulp. $WC1^+$ cells were predominantly present in the periarteriolar region and marginal zones(Fig. 4). In the thymus, $WC1^+$ cells were widely distributed and few in number in the cortex. They were present in higher concentration in the medulla localized in clusters close to

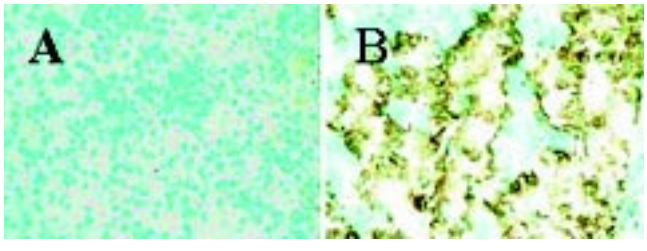

Fig. 4. Representative profiles of lymph node stained with fusion proteins WC1.1-3 (A) and WC1.9-11 (B). Macrophages, dendritic cells, and cells lining the medullary sinuses express BGAM. Tissue reacted with second step reagent alone or WC1.1-3 and second step reagent were negative.

Hassall's corpuscles [33].

Analysis of functional activity of γδ T cells

The ultimate objective has been to detail effector activity mediated directly through antigen specific interaction with the γδ TCR and indirectly through activation by cytokines produced by αβ and γδ T cells and accessory cells (monocytes, macrophages, epithelial cells).

Polyclonal activation with lectins

ACT1, a 30-37 kD molecule, was expressed on both γδ T and αβ T cells. ACT2, a 36 kD molecule, was expressed predominantly on γδ T cells and a subpopulation of activated $CD8^+$ cells. ACT3, a 120 kD molecule, was expressed predominantly on $CD4^+$ cells in lectin stimulated cultures but appears on $WC1^+$ and $WC1^-$ cells following long term culture [5]. The IL-2Rα peptide (CD25) was expressed on activated αβ and γδ T cells and B lymphocytes [38]. ACT1, ACT17, and CD25 were expressed within 6 to 8 hrs after stimulation on all subpopulations of αβ and γδ T cells with the maximal level of expression evident by 24 hrs. Examination of the composition of cultures of PBMC during the first week of culture revealed γδ T cells could represent up to 90% of the cells at 3 to 6 days following stimulation with Con A. Two color FC analysis of the cultures during the first two weeks of culture (on conditioned medium [CM] containing IL-2 and other cytokines) showed the phenotypes of the γδ T cell subpopulations were stable: i.e., $WC1^+$ and $WC1^-$ subpopulations did not interconvert. This studies also showed that $CD4^+$ cells became the predominant population in most cultures maintained over two weeks on CM, with $WC1^+$ populations persisting at low concentrations (data not shown).

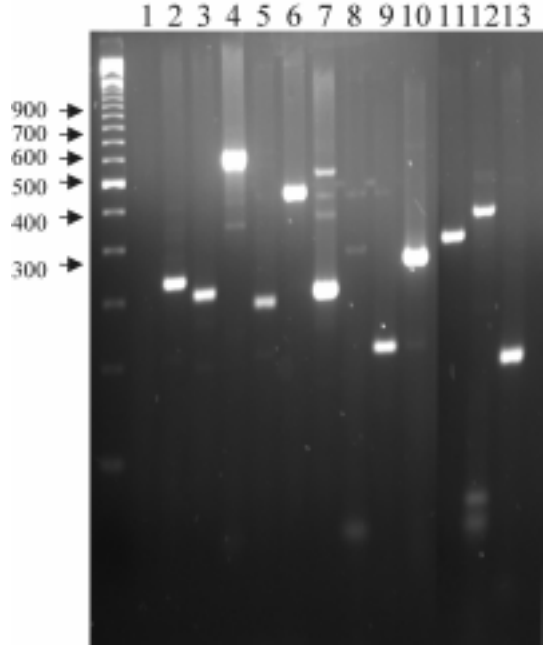

Fig. 5. Cytokine mRNA profile of PBMC stimulated with ConA for 24 hrs. 1 = IL-1, 2 = IL-2, 3 = IL-4, 4 = IL-6, 5 = IL-7, 6 = IL-10, 7 = TNF-α, 8 = iNOS, 9 = IFN-γ, 10 = GAP, 11 = IL-12, 12 = IL-15, 13 = GMCSF

Cytokine profile

Most recently, studies have been initiated to determine which cytokines were produced following stimulation with polyclonal activators. The studies have shown multiple cytokine genes were activated following 24 hrs stimulation with Con A: IL-2, IL-4, IL-6, IL-7, IL-10, IFN-γ, TNF-α, IL-12, IL-15, and GMCSF(Fig. 5).

Polyclonal activation with superantigens

In contrast, studies with staphylococcal enterotoxin C1 (SEC1) have shown differential patterns of activation of αβ and γδ T cells. Both αβ and γδ T cells showed the initial steps of activation as detected by the upregulation of the expression of MHC class II molecules and IL-2Rα (CD25). A proportion of $CD4^+$ cells increased in cell size and expressed the activation molecule ACT3 but did not proliferate, suggesting stimulation caused only partial activation. $WC1^+$ and $WC1^-$ γδ T cells did not increase in size nor proliferate. Only $CD8^+$ αβ T cells increased in cell size and proliferated. Activation was accompanied by a high level of expression of ACT3, an activation molecule that was expressed predominantly by $CD4^+$ cells following stimulation with Con A(data not shown).

Discussion

Early on, studies had been focused on the development and characterization of monoclonal antibodies (mAbs) specific for leukocyte differentiation molecules in ruminants. Further studies in cattle and sheep established that the γδ T cell population was actually comprised of two complex subpopulations with different phenotypes and patterns of distribution in peripheral blood and lymphoid tissues.

Data from these studies indicated the TCR1 determinants were expressed on Vγ or Cγ segments restricted in usage to $WC1^+$ γδ T cells. The finding of the subsets of $WC1^-$ γδ T cells positive for the TCR1-N6 determinant indicated the determinance might be expressed on more than one Vγ or Cγ segments. Ongoing studies with MacHugh at the International Livestock Research Institute (ILRI) on analysis of V and C segment usage by γδ T cells support this contention. The data have shown the mAbs with the broadest specificity reacted with determinants on the δ chain of TCR1 [13] and the mAbs with narrow specificity with determinants most likely expressed on the Cγ5 or Vγ gene products [28] (MacHugh, Davis *et al*. Manuscript in preparation). The pattern of expression of

these determinants suggested, at this juncture, that V-gene defined subsets of the TCR1 expressed by $WC1^-$ γδ T cells have not yet been identified, except for a subset that expressed TCR1-N6. The pattern of expression of TCR1-$N6^+$ and TCR1-$N7^+$ cells also suggested expansion of the $WC1^+$ population of γδ T cells, in the course of evolution, included selective usage of a subset of TCR1 Vγ, Jγ, and Cγ genes. The molecular studies suggested no mAbs were identified that reactive with Cγ and Vγ gene products used by $WC1^-$ γδ T cells. A recent study reported by Hein and Dudler [25] provided additional data that supports this contention. Recent studies of the thymus, using a mAb specific for the TCR1 δ chain [33], had shown γδ T cells comprise ~7% of thymocytes. Of particular interest, these recent studies have revealed both $WC1^+$ and $WC1^-$ γδ T cells express CD2 and CD6 (Fig. 1). This was a significant new finding, which suggested the two populations originated from a common precursor early in development and that expression of CD2 and CD6 stopped on $WC1^+$ cells during maturation. The data also suggested that expression of TCR1-N6, -N7, and -N6/N7 were also associated with maturation of $WC1^+$ γδ T cells and that expression of WC1 might occur after expression of these families of the γδ TCR.

The pattern of distribution was similar in the mucosal epithelium with the main difference being in abundance. $WC1^-$ cells were abundant whereas $WC1^+$ cells were sparsely distributed in the epithelium. Both populations were present in low concentration in the lamina propria [33, 48]. Several types of studies have been conducted to elucidate the function of γδ T cells. These have included investigations on the response to polyclonal activators, superantigens, and also investigations on the immune response to antigens derived from pathogens. Studies have shown activated cells expressed IL-2Rα, MHC class II and additional activation molecules recently identified in our laboratory: ACT1, ACT2, ACT3, ACT4, ACT13, ACT14, ACT16, and ACT17 [17]. Both ACT2 and ACT3 were expressed on thymocytes [46]. ACT2 was also constitutively expressed on γδ T cells in the gut epithelium and mammary secretions [30, 32]. The human equivalents of these molecules had not been identified. ACT16 appeared later with maximal expression evident by 24-48 hrs [17].

Further studies are needed to determine which cytokines are produced by the each population of cells. The cytokine profile of SEC1 stimulated cells differed, indicating the difference in proliferative responses most likely was associated with absence of cytokines essential for activation and proliferation of γδ T cells. γδ T cells could represent a significant part of the proliferating population in bulk cultures following stimulation with *Mycobacterium paratuberculosis*(*M. paratuberculosis*) [9, 10] as well as crude preparations and recombinant antigens derived from *Babesia bovis*(*B. bovis*) [5]. Efforts to establish antigen-reactive cell lines have shown clones with CD4, CD8, and γδ T cell subpopulation phenotypes could be obtained from bulk cultures. It had been possible to maintain CD4 and CD8 positive clones on rIL-2 and CM, but not γδ T cell clones, suggesting that additional cytokines must be present to support proliferation. We have shown that γδ T cells proliferated in the presence of human rIL-12. These studies have also shown IL-2 may inhibit IL-12 activity similar to what had been noted with human γδ T cells [7]. Others have reported that IL-15, a cytokine with similar activity to IL-2, supported γδ T cells in culture .

The functional significance of the proliferative response of γδ T cells in antigen-stimulated cultures remains to be elucidated. Data obtained thus far, however, showed cells present in both the $WC1^+$ and $WC1^-$ populations of γδ T cells possessed immunoregulatory activity [8, 41, 42]. Investigation of the factors governing the proliferative response to *Staphylococcus aureus*(*S. aureus*) with lymphocytes derived from peripheral blood and mammary secretions have revealed the existence of a subpopulation of $WC1^-$ $CD8^+$ γδ T cells that coexpressed the activation molecule, ACT2. The $CD8^+$ $ACT2^+$ subpopulation was present in low frequency in peripheral blood and relatively high frequency in mammary secretions [41]. Previous experiments have shown this subpopulation downregulated the MHC-restricted response of $CD4^+$ T cells to heat-killed *S. aureus* [41, 42]. *In vitro* studies have shown the proliferative response to heat-killed *S. aureus* was low when the concentration of γδ $CD8^+$ $ACT2^+$ T cells in the culture were high. The available evidence indicated that the γδ $CD8^+$ $ACT2^+$ T cells were responsible for the low response to heat-killed *S. aureus*. $CD4^+$ cells isolated from peripheral blood and mammary secretions exhibited a depressed response to *S. aureus* only when mixed with 'CD4-depleted' preparations of γδ $CD8^+$ $ACT2^+$ T cells obtained from mammary secretions. $CD8^+/ACT2^-$ cells from the mammary gland and peripheral blood had no effect on $CD4^+$ cells.

Studies with antigens derived from *M. paratuberculosis, M. bovis, B. bovis,* and *Fasciola hepatica* showed the role of $WC1^+$ γδ T cells in immune responses might be quite complex. Depletion and add back experiments with *M. paratuberculosis* showed $WC1^+$ γδ T cells downregulated the proliferative response of $CD4^+$ cells to antigen and that this effect was modulated by $CD8^+$ cells [8, 10]. With *B. bovis* and *F. hepatica*, $WC1^+$ cells tended to proliferate to a greater extent than $CD4^+$ cells in cultures maintained by cycles of antigen stimulation and culture in the presence of CM. Whether this reflects a greater capacity to proliferate in the presence of cytokines in the medium or a direct inhibitory effect on the capacity of $CD4^+$ cells to proliferate in response to antigen remains to be clarified. It is evident that removal of γδ T cells early in cultures leads to greater proliferation of $CD4^+$ cells and facilitates cloning

[6].

Studies with *M. bovis* have provided evidence that *in vivo*, $WC1^+$ γδ T cells may be the first cells to be recruited to the site of a lesion induced by injection of PPD .

Few studies have been conducted to analyze the mechanisms regulating activation and proliferation of γδ T cells in ruminants. It was not yet known whether antigen recognition through the TCR is sufficient for activation and the development of effector activity or whether additional signals mediated through accessory molecules were required. Although some unique antigens have been identified that reacted specifically with the γδ TCR in other species [31], none have been identified in ruminants. Studies to date have focused on determining if γδ T cells could be activated by cross-linking the TCR with antibody to the ε chain of the TCR complex and antibodies to the γδ TCR. Studies by Baldwin *et al*. [24, 40] showed that γδ cells proliferated in cultures of monocyte depleted PBMC in culture plates coated with anti-CD3. Their data suggested that proliferation was enhanced in culture plates coated with suboptimal concentrations of anti-CD3 and anti-WC1 in a dose dependent manner [24]. Baldwin and associates also showed $WC1^+$ γδ T cells were activated and proliferated in response to a membrane associated molecule on macrophages and a soluble product released by irradiated monocytes present in cultures comprised of irradiated PBMC and monocyte-depleted lymphocytes, autologous mixed leukocyte reaction (AMLR) [40]. We have confirmed that γδ cells could be activated with anti-CD3 mAb. However, efforts to demonstrate enhancement of proliferation with several anti-WC1 mAbs have not been successful. In addition, preliminary studies with anti-δ chain mAb have not been successful alone or in combination with anti-WC1 mAbs, suggesting that unidentified accessory molecules might be important in TCR driven activation of $WC1^+$ γδ T cells. Preliminary studies have confirmed monocyte/macrophages stimulate $WC1^+$ γδ T cells *in vitro*. Studies have not yet been conducted to determine if activation involved membrane bound and/or soluble factors. However, studies with hrIL-12 showed IL-12 might be one of the stimulatory factors .

In summary, we have charaterized the immune system in ruminants and pigs, especially the characterization of γδ T cells. It is now clear that the γδ T cell population was comprised of two complex subpopulations that differ in phenotype and distribution in peripheral blood and tissues. The population that was positive for WC1 was unique to ruminants and pigs and appeared to be a population that had undergone expansion in the course of evolution of these groups of animals. The WC1 molecule has been cloned and characterized. The first counter-receptor for WC1 has been identified and shown to be expressed on macrophages and dendritic cells. Although the function of both populations of γδ T cells remain to be determined, progress has been made in identifying factors involved in activation and proliferation of γδ T cells. Some information has been obtained on the capacity of γδ T cells to produce cytokines.

To fully delineate the regulatory and effector activities of γδ T cells in ruminants, it will be essential to detail the capacity of γδ T cell subpopulations to produce regulatory cytokines and determine which membrane molecules are involved in activation and function. With ruminants (and also pigs), it will be essential to characterize the unique population that expresses the WC1 molecule as well as the WC1 negative population that more closely resembles the population identified in other species.

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