Biochemical and functional analysis of a novel lineage-specific gd T cell surface antigen (GD3.5Ag)
by Ward McAlister Jones

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Veterinary Molecular Biology MONTANA STATE UNIVERSITY Bozeman, Montana
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Abstract:
Gamma-delta (γδ) T cell lymphocytes were discovered over a decade ago. Since that time, a great deal of research has been done to gain an understanding of them. Here, I define a novel γδ T cell lineage-specific antigen (GD3.5Ag) at the biochemical, regulatory, and functional level. GD3.5Ag is expressed on 90% of the bovine peripheral blood γδ T cell population. GD3.5Ag is approximately 230kD and considerable evidence suggests that it does not represent one of the known WC1 isotypes. GD3.5Ag and WC1 exhibit disparate sensitivity to V-8 protease digestion as well as to PNGase-F, O-sialoglycoprotein endopeptidase, and neuraminidase treatment. GD3.5Ag expression on the cell surface is extremely sensitive to low-dose chymotrypsin treatment, while WC1 is not. I show that large numbers of γδ T cells that express lower levels of GD3.5Ag are found in the spleen. Treatment of γδ T cells with PHA or ConA results in the downregulation of GD3.5Ag expression, mimicking the findings in the spleen. Functional analyses revealed that chymotrypsin removal of GD3.5Ag from the cell surface in a dose-dependent manner correlates well with the loss of ability to roll on 24hr activated endothelium. Also, polyclonal antibodies against GD3.5Ag partially inhibit γδ T cell interactions with 24hr activated endothelium. Although GD3.5Ag is not an E-selectin ligand itself, I confirmed that γδ T cells express a 250kD E-selectin ligand and possibly 200kD and 300kD E-selectin ligands. Additionally, I define three potentially relevant E-selectin ligands of 120kD, ~220kD, and 260kD on human lymphocytes.

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Ward McAlister Jones

This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

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Approved for the Major Department

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Head, Major Department

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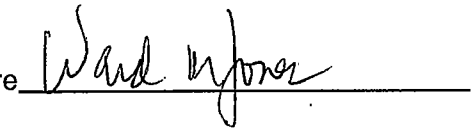
Signature 

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PREPARATION OF THE DISSERTATION

To assist the reader, I decided to include this section to describe how the dissertation was compiled and formatted. In the Introduction (Chapter 1), a general overview is given to provide sufficient background information for the understanding of subsequent chapters. The chapters that follow the Introduction also include an introductory section. The purpose for using this format is to allow those chapters that have been published or submitted for publication to be integrated into the dissertation in a logical manner. Chapters 2 and 4 represent data that has been published or submitted for publication. The manuscripts included in Chapters 2 and 4 , respectively, are as follows:

Jones, W. M., B. Walcheck, and M. A. Jutila. 1996. Generation of a new γδ T cell-specific monoclonal antibody (GD3.5): biochemical comparisons of GD3.5 antigen with the previously described workshop cluster 1 (WC1) family. *J. Immunol. 156:3772.*

Jones, W. M., G. M. Watts, M. K. Robinson, D. Vestweber, and M. A. Jutila. 1997. Comparison of E-selectin binding glycoprotein ligands on human lymphocytes, neutrophils, and bovine γδ T cells. Submitted to *J. Immunol.*

During the process of obtaining my degree, several collaborative projects within the lab were established. We performed experiments involving several individuals, including the study of E-selectin ligand and GD3.5Ag expression on bovine lymphocytes. Figures 9 and 20 are the result of these collaborative studies. Therefore, I would like to specifically acknowledge Gayle Watts and Karen Sipes for their assistance in generating Figures 9 and 20, respectively. In addition, I would like to acknowledge Martyn Robinson and Dietmar Vestweber for providing critical reagents (P11.4 chimera and ESL-1 polyclonal antibody). Finally, I would like to acknowledge Bruce Walcheck who generated the GD3.5 mAb.

I dedicate this thesis to my wife, Betsy Jones, and children, Krystal Fix and Erica Fix, for their unending patience in my endeavors to obtain this degree.

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ABSTRACT

Gamma-delta (γδ) T cell lymphocytes were discovered over a decade ago. Since that time, a great deal of research has been done to gain an understanding of them. Here, I define a novel γδ T cell lineage-specific antigen (GD3.5Ag) at the biochemical, regulatory, and functional level. GD3.5Ag is expressed on 90% of the bovine peripheral blood γδ T cell population. GD3.5Ag is approximately 230kD and considerable evidence suggests that it does not represent one of the known WC1 isotypes. GD3.5Ag and WC1 exhibit disparate sensitivity to V-8 protease digestion as well as to PNGase-F, O-sialoglycoprotein endopeptidase, and neuraminidase treatment. GD3.5Ag expression on the cell surface is extremely sensitive to low-dose chymotrypsin treatment, while WC1 is not. I show that large numbers of γδ T cells that express lower levels of GD3.5Ag are found in the spleen. Treatment of γδ T cells with PHA or ConA results in the downregulation of GD3.5Ag expression, mimicking the findings in the spleen. Functional analyses revealed that chymotrypsin removal of GD3.5Ag from the cell surface in a dose-dependent manner correlates well with the loss of ability to roll on 24hr activated endothelium. Also, polyclonal antibodies against GD3.5Ag partially inhibit γδ T cell interactions with 24hr activated endothelium. Although GD3.5Ag is not an E-selectin ligand itself, I confirmed that γδ T cells express a 250kD E-selectin ligand and possibly 200kD and 300kD E-selectin ligands. Additionally, I define three potentially relevant E-selectin ligands of 120kD, ~220kD, and 260kD on human lymphocytes.

CHAPTER 1

INTRODUCTION

Historical Background

A large portion of T cells found in circulation express the αβ T cell receptor (TCR), which is associated with the "invariant components" of the CD3 signalling complex (1). Importantly, the TCR recognizes, in conjunction with coreceptors CD8 or CD4, processed antigen that is presented in the context of MHC Class-I or MHC Class-II molecules, respectively (2). In 1984, cDNAs encoding the αβ TCR were identified. Initially, subtractive hybridization and differential screening revealed a cDNA clone that encoded the β chain molecule (3,4). Soon after this discovery, a second cDNA clone was identified and thought to encode the α chain (5). Paradoxically, this particular clone did not encode any potential N-linked glycosylation sites which were known to exist in the α chain (1). Subsequent studies uncovered the "true" α chain cDNA; the protein encoded by the original "α" chain cDNA was termed the "γ" chain (6-8).

Although the α and β chain cDNAs had been identified, the function of the third "γ" chain remained unknown. Several investigators suggested that the γ chain was expressed in less mature thymocytes and represented an αβ T cell progenitor (9,10). Others suggested that cloned αβ T cells possessed "nonproductive γ rearrangements" and, therefore, γ genes played no role in αβ T cell function (8). In 1986, several groups described TCR heterodimers associated with the CD3 complex that contained the γ chain (11-13), and some suggested that the γ chain was associated with an

unidentified fourth chain (11,13). Finally, in 1987, the fourth unidentified gene, termed the "δ" chain, was discovered in studies looking at C, D, and J region gene rearrangements in $CD4^-$ and $CD8^-$ thymocytes (14,15). In addition, the human δ chain cDNA was discovered using subtractive hybridization (16). This new heterodimeric TCR, comprised of the γ and δ chain molecules, was found on a subset of $CD4^-$ and $CD8^-$ T cells that were collectively termed γδ T cells.

Determining the biological function of this novel T cell subset continues to generate a great deal of attention. Although the function of γδ T cells has not been completely defined, several investigators suggest that these cells are involved in the initial host response to infectious agents, contributing to what is referred to as "the first line of defense," which will be discussed later.

T cell receptor γ and δ chain: Lineage-specific γδ T cell marker

As described earlier, γδ T cells received their namesake from the γ and δ TCR gene products on their cell surface. Therefore, γδ T cells can be identified by mAbs directed against the TCR. As found for αβ T cells, the TCR on γδ T cells is associated with an array of proteins referred to as the CD3 complex. The complex is similar to CD3 on αβ T cells and consists of γ, δ, ε, and ζ CD3 proteins on most cells (17) though some reports indicate that epithelial γδ T cells do not express ζ chain proteins (18-21). αβ T cell TCR function involves the recognition of peptide in the context of Class I or Class II MHC. The $CD4^+$ αβ T helper cells recognize antigen expressed with MHC Class II, while $CD8^+$ T cytolytic cells recognize antigen in conjunction with Class I MHC (22). Both CD4 and CD8 are referred to as coreceptors assisting in the TCR recognition of MHC complexes (22). γδ T cells appear to have a less restricted antigen recognition event. Interestingly, most γδ T cells lack CD4 or CD8 and apparently are not restricted by MHC Class-I or Class-II recognition. Although some γδ T cells have been isolated that react

with classical MHC I and II, many appear to react with other nonclassical MHC gene products (23-26).

Recently, some reports indicate that γδ T cell antigen recognition may be independent of antigen processing, suggesting a direct recognition event (27,28). It has been suggested that γδ TCRs are more closely related to immunoglobulins than to αβ TCRs based upon CDR3 length analysis (27,29). γδ T cells demonstrate greater diversity in CDR3 length than do αβ T cells. It is thought that αβ T cells demonstrate limited variability in CDR3 length because of MHC-restricted interactions with antigen presenting cells (APCs). Therefore, CDR3 length variability may explain the apparent lack of MHC restriction demonstrated by some γδ T cells. The authors suggest that this may mediate γδ T cell cytotoxicity in the absence of APCs with a direct response to antigen (18,27).

Several reports suggest that γδ T cells recognize unconventional antigens, such as heat shock proteins (HSP) and nonpeptide antigens (18,23,28,30-36). Very small (<1kD) protease-resistant mycobacterial antigens are capable of activating γδ T cell subsets (32-34). In addition, human γδ T cell lines have been shown to respond to PPD and recombinant HSP (36). More recently, γδ T cell response to heat-killed mycobacteria has been attributed to small, nonpeptide, phosphate-containing antigens (28,35). Specifically, a 1-3kD phosphate-containing carbohydrate has been identified as the stimulatory component in mycobacterium (28,37). Therefore, γδ T cells have the capacity to respond to rather unusual or unconventional antigens.

Workshop Cluster 1 (WC1): Lineage-specific γδ T cell marker

In 1986, MacKay et al. described a unique, lineage-specific, γδ T cell marker in sheep (38). This cell surface marker (T19) is found on as many as 60% of ovine peripheral blood lymphocytes (PBLs). In comparison, only 1-3% of lymph node (LN)

lymphocytes and thymocytes express T19 (38). Later reports show that ovine $T19^+$ cells comprising 30-60% of PBLs are positive for the γδ TCR and negative for CD4 and CD8 (39).

T19 can be distinguished from TCRs by SDS-PAGE analysis. The T19 antigen migrates under reducing and nonreducing conditions at ~215kD and ~300kD, while γδ TCRs demonstrate a migratory pattern of approximately 40kD under reducing conditions and 70-75kD under nonreducing conditions (39). The γδ TCR and T19 appear at different times during fetal and neonate development. Cells expressing γδ TCR are detected at 40 days gestation while $T19^+$ cells are not detected until 15 days later (39). The percentage of γδ TCR^+ cells in all tissues is higher than the percentage of $T19^+$ cells, indicating that some γδ TCR-bearing T cells are $T19^-$ (39).

Soon after the discovery of T19 in sheep, several groups identified a similar antigen on bovine γδ T cells (40-43). Many mAbs were generated against this antigen and are now clustered into a group collectively called Workshop Cluster 1 (WC1) (44). As in the sheep, bovine WC1 is characterized by ~210kD and ~300kD bands (43). Additionally, as described for T19 in sheep, the percentage of $WC1^+$ cells in tissues is lower than the percentage of γδ TCR^+ cells (43,45).

By 1992, it became apparent that the multitude of mAbs that recognized WC1 were widely variable relative to the percentage of the population stained in a single individual. This prompted studies looking for the DNA that codes for WC1. A full length cDNA clone of WC1 was reported in bovine by Wijngaard et al. (46). Sequence analysis revealed a predicted protein containing a N-terminal leader peptide with a transmembrane region located in proximity to the C-terminus (46). In addition, 17 potential N-linked glycosylation sites are located in the extracellular domain. The extracellular domain contains 11 homologous regions that demonstrate strong identity to the scavenger receptor cysteine-rich (SRCR) family (46). In this same report,

Southern blots indicate that bovine genomic DNA contains several similar sequences, suggesting the potential for a WC1 gene family. Goats, pigs, sheep, and horse genomes appear to present similar complexity, while man and rodents appear to have fewer related sequences (46).

A plethora of mAbs to WC1 are clustered into two groups according to their reactivity to two different WC1 cDNA clones transfected into mouse L-cells (47). Several mAbs react with both WC1.1 and WC1.2 L-cell transfectants, while other antibodies react with either transfectant, but not both. Simultaneous reports indicated that WC1.1 and WC1.2 can be differentiated into two groups by apparent molecular weights of 205 and 215kD, respectively (48). Subsequently, a third cDNA was isolated (WC1.3) which shares significant nucleic acid identity with the first two cDNAs (49). Upon comparison, all three cDNAs share approximately 90% nucleic acid identity. Interestingly, a portion of the first SRCR domain, the most N-terminal, demonstrates only 50% identity among the three cDNA clones (49). Thus, this portion of the molecule could provide a unique N-terminal domain for each isotype, thereby directing its function and suggesting one reason that multiple WC1 species exist. Most importantly, mAbs IL-A29 and CC15 stain all three cDNA transfectants, providing pan-markers for the three known WC1 isotypes (49).

Although molecular biological techniques and mAbs have somewhat elucidated WC1 structure and tissue distribution, little is known about its function. MacKay et al. suggest that WC1 may play a role in tissue-specific homing, since WC1 is lost once some $\gamma\delta$ T cell populations enter certain tissues (39). Taking it one step further, Wijngaard et al. suggest that expressing different WC1 genes may provide a mechanism for tissue-specific homing (49). However, direct evidence supporting a role for WC1 in homing has not been shown.

Some authors speculate that WC1 may play a role similar to CD4 and CD8 on αβ T cells (49,50), whereas others suggest that WC1 does not "substitute" for these antigens (51). Binding of WC1 with mAbs augments proliferation of γδ T cells under certain circumstances (51). More specifically, cell-bound IL-A29 specifically enhances γδ T cell proliferation in autologous mixed leukocyte reactions. Additionally, immobilized IL-A29 enhances γδ T cell proliferation when cells are treated with anti-CD3 mAb, suggesting that WC1 engagement may augment TCR signalling events (51). Another report indicates that WC1 signals cell-cycle arrest, but not apoptosis (52). In this report, anti-WC1 antibodies induce cell-cycle arrest in G0/G1 in a dose-dependent manner that is reversible upon removal of the antibody (52). The antibody-induced cell arrest could be overcome by treating with anti-CD3 mAb. Therefore, engagement of the TCR and WC1 may provide an enhanced stimulatory event while immobilized WC1 alone signals cell-cycle arrest.

Other lineage-specific antigens

In our lab, we (my colleagues and I) have endeavored to generate mAbs against bovine γδ T cell surface antigens by injecting mice with purified γδ T cells. During this process, as expected, many mAbs against γδ TCR and WC1 were generated. However, one of the mAbs generated in a fusion piqued our interest. Preliminary results suggested that this mAb (GD3.5) stained a unique population of γδ T cells and recognized a unique antigen by Western Blotting. This antigen (GD3.5Ag) is described in Chapters 2 and 3.

γδ T cell distribution and function

Even though many laboratories have performed elegant studies of the function of γδ T cells, a clear consensus of their importance has not been established. We and others

hypothesize that their tissue distribution may lend some clues to their function. In ruminants, γδ T cells comprise 60-80% of the peripheral blood lymphocyte (PBL) population (45,50), but do not appreciably accumulate in the organized lymphatic tissues, such as the lymph node (39,43,53). Studies have demonstrated considerable homing of bovine γδ T cells into epithelial tissues, such as the skin and gut (50,53,54). In addition to epithelial tissues, bovine γδ T cells home to sites of inflammation. For example, intradermal injection of PPD results in accumulation of γδ T cells at the inflammatory site (55).

Many studies have been conducted in mice describing γδ T cells in association with extralymphoid sites, including the mucosal epithelial of the gut, tongue, vaginal areas and the skin (56-58). Interestingly, tissue localization of these cells seems to correlate with TCR gene usage in some cases (31,58,59). For example, murine γδ T cells expressing the Vγ5 Vδ1 TCR migrate to the skin (30,58,60). Therefore, localization may be either linked to or correlate with TCR gene usage (58,59). Several authors have hypothesized that specific homing receptors are expressed by the different T cell subsets directing their tissue-specific homing (30,58).

Human γδ T cell tissue localization is different in comparison to the bovine and mouse. Human γδ T cells are found in lymphoid as well as nonlymphoid tissues and are not as tightly associated with epithelial tissues as seen in the mouse and bovine models (35,53). Human TCR gene usage has been associated with circulating γδ T cell populations (35,61,62). Vγ9 Vδ2 γδ T cells represent a large subset of human peripheral blood γδ T cells (35,62). Importantly, this γδ T cell subset binds small molecular mass, non-protein antigens (32,35). Hence, it has been suggested that these cells respond quickly to antigen, performing as sentinels (35).

Since γδ T cells locate in areas where invading pathogens would first be encountered (portals of entry into the body) in what appears to be "directed" localization of γδ T

cells to epithelial tissues, many hypothesize that γδ T cells play a major role in "the first line of defense" and, in fact, recent reports support this hypothesis. In studies using γδ and αβ TCR-knockout mice, the role of the γδ T cells in malarial infection appears to be during initial protection (63). γδ TCR-deficient mice exhibit early, acute malaria, but eventually clear the infection. In contrast, αβ-deficient mice exhibit chronic, almost subclinical malaria (63). Other reports indicate that γδ T cells may play a role in eradication of intracellular bacterial pathogens, such as *Listeria* and *Mycobacteria* species (28,64,65). γδ T cells are crucial in reducing bacterial growth during early infection (28). Additionally, αβ T cells appear to play a role in bacterial eradication in the later stages of infection (28,65). Therefore, γδ T cell function during these bacterial infections appears to be similar to their response during malarial infections. Lastly, γδ T cells are involved in protection against viral infections (66,67). In one report, cytotoxic γδ T cells were shown to recognize and lyse HSV-infected target cells (66).

In general, γδ T cells appear to provide protection against intracellular viral, bacterial, and parasitic pathogens, thus supporting the "first line of defense" hypothesis. In addition, their tissue distribution is consistent with "the first line of defense" theory. Finally, unlike αβ T cells, their ability to recognize unprocessed antigens may suggest that these cells provide a rapid response against invading pathogens (18,27).

Lymphocyte trafficking

To get to specific tissues, lymphocytes follow very distinct steps in a process called trafficking. The study of lymphocyte trafficking greatly enhances our understanding of lymphocyte function. It is known that expression of specific receptors and their ligands regulates lymphocyte trafficking of discrete T cell subsets. αβ T cells originate from

the hematopoietic stem cells of the bone marrow and develop to maturity in the thymus. Although mature, these cells are naive in relation to exposure to antigen and expression of cell-surface antigens. For instance, these cells express peripheral lymph node homing receptor (L-selectin) and the naive cell-surface marker CD45RA. Naive αβ T cells recirculate through secondary lymphoid tissues, such as PLN, MLN, and Peyer's patches, using very specific molecular interactions.

Naive αβ T cells can bind to specific sites in the PLN using the lymphocyte homing receptor L-selectin. In the PLN, specialized high-endothelial venules (HEV) express peripheral node addressin (PNAd) which is recognized by surface-expressed L-selectin (68). This interaction can be inhibited by functionally blocking L-selectin or PNAd. Specifically, anti-L-selectin mAbs inhibit binding of lymphocytes to PNAd (69). Additionally, mAb MECA-79, which recognizes PNAd, blocks binding of lymphocytes to PLN HEV (70). Therefore, L-selectin plays a key role in naive αβ T cell trafficking into the PLN via binding PNAd expressed on HEVs.

Recirculation of naive αβ T cells through the gut-associated secondary lymphoid tissues, such as the MLN and Peyer's patches, involves a more complex homing mechanism. As described above, PNAd is expressed on PLNs, which plays a major role in lymphocyte homing into PLN. In experiments using L-selectin-deficient mice, lymphocyte homing to PLN was significantly inhibited while inhibition of homing to MLN and Peyer's patch was less severe (71). Thus, a significant portion of homing to gut-associated secondary lymphoid tissues occurs in the absence of L-selectin. Importantly, low levels of L-selectin ligand (MECA-79-reactive molecules) are expressed in MLNs and Peyer's patches, indicating that L-selectin binding can occur (72,73). However, L-selectin alone is not the only naive lymphocyte ligand that can mediate rolling on MLNs and Peyer's patches (73,74). The integrin α4β7 can initiate rolling on MLNs or Peyer's patches via binding mucosal addressin cell-adhesion

molecule (MAdCAM-1) (72,73). Thus, homing of naive αβ T cells to gut-associated lymphoid tissues can be initiated by L-selectin and α4β7 interactions with ligands expressed on these tissues (72-74). In fact, it has been proposed that rolling on L-selectin ligands, in Peyer's patch, is too fast to allow cell arrest and α4β7 serves as a "bridge" between rolling and cell arrest by significantly reducing rolling velocity (73,74). Furthermore, Peyer's patch-HEVs express a unique form of MAdCAM-1 which possesses ligands for both L-selectin and α4β1 (74,75).

Once the cells have homed to a particular tissue, they must either perform their function or continue to recirculate. Naive cells that enter the secondary lymphoid tissue and encounter antigen are retained in the tissue where they undergo conversion to memory/effector T cells. If, however, the naive cell does not encounter antigen, it must recirculate throughout the body to continue its search for foreign antigen. The naive cells exit the lymphoid tissue via the efferent lymph. Here, through the lymphatic system, the naive cells are returned to the blood by way of the thoracic duct where they can recirculate through the body and once again home to the secondary lymphoid tissues (76,77).

As opposed to naive T cells, memory T cells express CD45RO and varying levels of L-selectin (72,78). In addition, several adhesion molecules, such as cutaneous leukocyte-associated antigen (CLA), α4β1, and α4β7 are expressed on memory cells (72,79,80). Tissue-specific homing receptor expression on these cells appears to be dependent upon the site where naive to memory conversion occurs (72,81-83). For example, memory T cell production in PLN correlates with CLA upregulation or L-selectin on some memory T cells (83). Importantly, CLA^+ lymphocytes represent the skin-homing T cell population (79,84). Therefore, conversion to memory in PLN correlates with skin or PLN homing T cells.

Memory T cells can migrate through the secondary lymphoid tissues (73,76), but

unlike naive T cells, they also have the capacity to migrate into extralymphoid tissues. In sites of inflammation, where upregulation of various adhesion molecules occurs, memory T cells have the capacity to bind the endothelium and enter. For example, memory cells expressing $\alpha 4\beta 1$ (VLA-4) can bind vascular cell adhesion molecule-1 (VCAM-1), which is expressed by endothelium at sites of inflammation (68,73,85). In addition, memory cells expressing CLA have the capacity to bind E-selectin, allowing their migration into cutaneous sites of inflammation (79,84,86). Recently, several reports state that recruitment of specific memory T cell subsets is possible. E- and P-selectin are expressed by inflamed endothelium and are capable of initiating leukocyte/endothelial cell rolling interactions. Importantly, Th1 cells bind to these vascular selectins, while Th2 cells do not (87,88). Thus, Th1 cells can be specifically recruited into inflammatory sites where E- and P-selectin are expressed.

In other extralymphoid sites, such as the lamina propria, memory cells have the capacity to bind MAdCAM-1 via $\alpha 4\beta 7$. Interestingly, naive cells cannot bind this form of MAdCAM-1 due to its lack of L-selectin ligand expression and lower level of naive T lymphocyte $\alpha 4\beta 7$ expression (73,89). Therefore, memory cells can home to lamina propria, while naive T cells cannot.

Similarities in the trafficking of memory $\alpha\beta$ T cells and $\gamma\delta$ T cells

Upon initial observation, it becomes apparent that memory $\alpha\beta$ T cells and $\gamma\delta$ T cells possess the ability to home to nonlymphatic epithelial tissues. In addition, $\gamma\delta$ T cells and memory $\alpha\beta$ T cells express CD45RO, possessing a memory-like phenotype (90). Importantly, memory $\alpha\beta$ T cells and $\gamma\delta$ T cells are "primed" for antigen recognition and respond more rapidly then naive $\alpha\beta$ T cells; however, $\alpha\beta$ T cells recognize antigen in the context of MHC while some $\gamma\delta$ T cells recognize antigen in the absence of antigen processing and MHC restriction (27,28). In addition, both subsets appear to be

surveying the portals of entry into the body thereby providing protection from invading pathogens.

In cattle, young animals possess large numbers of circulating γδ T cells (43). As the animals mature, these numbers drop dramatically (43). Young animals do not possess a large repertoire of memory αβ T cells, therefore, these animals would need an initial defense mechanism to compensate for the slow response to infection due to the lack of memory αβ T cells. It is tempting to propose that perhaps the higher number of γδ T cells in young animals are needed until a sufficient repertoire of memory αβ T cells can be generated. Thus, later in life, the more responsive memory T cells could respond faster and to a wider variety of antigens thereby requiring fewer γδ T cells. In fact, it has been suggested that γδ T cells and αβ T cells complement and in some cases slightly compensate for each other (28). Therefore, we suggest that αβ T cells become more like γδ T cells upon conversion to the memory phenotype. Both subsets migrate to epithelial tissues and possess a heightened response to antigen. Although these subsets do possess some unique features, perhaps their complementary and potentially compensatory functions and rapid response would allow more efficient immune responses in older animals which possess fewer γδ T cells.

As stated above, memory αβ T cells and γδ T cells are capable of entering extralymphoid tissues. In some cases, expression of specific molecules, such as E-selectin, can initiate recruitment of γδ T cells (98) and specific memory αβ T cell subsets (87,88). For example, as discussed above, memory Th1 αβ T cells preferentially bind E-selectin (87,88). Thus, the ligands expressed on lymphocytes that bind this vascular selectin are important.

Leukocyte ligands for vascular E-selectin

Leukocyte adhesion to endothelium is sometimes initiated by an inflammatory response. The expression of multiple adhesion molecules on endothelial cell surfaces and their interactions with leukocyte ligands results in the recruitment of leukocytes into an inflamed site. Leukocyte adhesion and transmigration through the endothelium into inflammatory sites has been described as a multistep process (72,91,92). These events are very complex and include initial tethering and rolling interactions between the leukocyte and endothelium followed by tight adhesion to the endothelium and finally transmigration into the underlying tissue (91,92). Many of the initial adhesion events between leukocytes and endothelial cells require a unique group of molecules called selectins (68,72,93,94). To date, three proteins have been identified and classified as selectins: CD62E or E-selectin, CD62P or P-selectin, and CD62L or L-selectin. The selectins are defined by an NH_2-terminal Ca^{2+}-dependent (C-type) lectin domain, an EGF-like region, several consensus repeats and a transmembrane region with a cytoplasmic tail (68,94,95).

E-selectin is expressed on endothelial cells while P-selectin is expressed on endothelium and platelets. L-selectin is expressed on leukocytes and is characterized by a different molecular mass depending upon the expressing cell type. The two vascular selectins support interactions of myeloid cells and unique subsets of T cells, including memory cells and $\gamma\delta$ T cells (79,96-100). P-selectin is stored preformed in Weibel-Palade bodies and α-granules of endothelial cells and platelets, respectively (101-104). Upon stimulation with a variety of activators; P-selectin is rapidly expressed within minutes on the cell surface (103,104). In contrast, E-selectin upregulation upon stimulation with cytokines or LPS requires transcriptional activation exhibiting peak expression 4-6 hours after stimulation (105-108). Therefore, P-selectin expression is capable of mediating rolling interactions minutes after stimulation while

E-selectin interactions occur hours after stimulation. Additionally, E-selectin expression is variable depending upon species and anatomic location (109).

Recent in vitro results indicate that leukocyte rolling on chronically inflamed endothelium appears to be independent of E- or P-selectin, suggesting novel adhesion systems that mediate leukocyte/endothelial cell interactions (85,109,110). Because of their critical role in the initial leukocyte tethering to endothelium, many investigators have devoted a great deal of time to defining the ligands for each selectin on various target cells.

E-, L-, and P-selectin ligands have been described in several systems. All three selectins bind to both sialyl Lewis a (SLe^a) and sialyl Lewis x (SLe^x) tetrasaccharide structures which are expressed by many different cell types (111-117). Specific high molecular mass glycoproteins present these carbohydrates in appropriate fashion to support cell/cell adhesion (113,118).

E-selectin ligands on myeloid cells have been well defined. Early reports have demonstrated that L-selectin can bind to E-selectin. In vitro reports have shown that neutrophil adhesion to inflamed endothelium, which expresses E-selectin, is blocked by anti-L-selectin mAb (119-121). In addition, adhesion of neutrophils on E-selectin-transfected L-cells was inhibited by both anti-E-selectin and anti-L-selectin mAb, suggesting that E-selectin and L-selectin may represent a receptor/counterreceptor pair (119). Of interest, neutrophil but not lymphocyte L-selectin is sLe^x positive and only neutrophil L-selectin binds E-selectin-transfected L1.2 cells (122). Extensive analyses, including reprecipitation experiments, have revealed that neutrophil L-selectin binds to E-selectin (123). More specifically, an affinity-purified E-selectin ligand of 80-90kD was reprecipitated with anti-L-selectin mAb, revealing that soluble L-selectin binds to E-selectin.

Another neutrophil E-selectin ligand, called ESL-1, has recently been described.

ESL-1, a 150kD ligand under reducing conditions and 130kD under nonreducing conditions, was affinity-isolated from mouse myeloid cells (124,125). In these reports, immobilized E-selectin/Ig chimera immunoprecipitated a 150kD ligand from metabolically labeled PMN lysates. In addition, a weak 250kD ligand was also purified from mature neutrophils. Later reports described two additional but weaker E-selectin ligands of 130kD and 230kD on mouse neutrophils, which were also reactive with a P-selectin chimera (125). Furthermore, these authors indicated that human neutrophils possess similar ligands (125).

Since E- and P-selectin bind the same tetrasaccharides, it is possible that E- and P-selectin would bind the same glycoprotein expressing these carbohydrates. Supporting this possibility are recent reports indicating that PSGL-1 (P-selectin glycoprotein ligand-1), a known P-selectin ligand (126-130), can also serve as an E-selectin ligand (124,129-134). Specifically, transfection of CHO cells with PSGL-1 cDNA and a combination of various glycosyltransferase cDNAs conferred binding to E- and P-selectin, indicating that PSGL-1/E-selectin interactions occur (134). In addition, tyrosine sulfation of PSGL-1 seems to be required for binding to P-selectin, but not E-selectin (134). These results suggest that the PSGL-1 protein backbone provides the support upon which post-translational modifications confer selectin binding specificity.

In contrast to neutrophils, far less is known about the glycoprotein ligands for the vascular selectins expressed by lymphocytes. As stated before, E- and P-selectin support interactions of unique subsets of T cells, including memory cells and $\gamma\delta$ T cells (79,96-100). An appropriate post-translationally modified form of PSGL-1 is likely to be the lymphocyte ligand for P-selectin (99,135); however, the lymphocyte ligands for E-selectin are unknown. Lymphocyte PSGL-1 does not appear to interact appreciably with E-selectin (97,136,137), as has been shown with neutrophil PSGL-1 (124,129-134). Indeed, O-sialoglycoprotease treatment of lymphocytes, which

hydrolyzes PSGL-1, blocks their binding to P-selectin, but not E-selectin (97,136,137). Of importance, mAb HECA-452 recognizes the E-selectin-binding human lymphocytes, but HECA-452 staining does not correlate with the P-selectin-binding subset, further distinguishing the two (84,86,136). The HECA-452 epitope is a carbohydrate structure called cutaneous lymphocyte-associated antigen (CLA) that decorates many different cell surface ligands (79) and correlates with E-selectin binding (84,86). This antigen is associated with cutaneous T lymphocytes and HECA-452 specifically blocks the interaction of CLA+ T cells with E-selectin-transfected COS cells (79,84,86). In addition, purified CLA coated onto glass supports specific binding of E-selectin-transfected L1.2 cells to CLA (86). Thus, in humans, CLA is capable of binding E-selectin. In initial reports describing HECA-452-reactive antigens, Picker et al. showed that it reacts with 125kD and 200kD glycoproteins on T lymphocytes (79). Which of the different T lymphocyte CLA glycoproteins serve as the predominant E-selectin binding molecule is unknown at this time.

Recently, we showed that virtually all bovine γδ T cells in newborn animals have the capacity to bind E-selectin (96). In contrast, αβ T cells acquire this binding capacity only after conversion to a memory phenotype (79,84,100). We isolated a 250kD E-selectin-binding molecule from γδ T cells by affinity isolation (96) using purified, recombinant E-selectin immobilized on Sepharose beads by a nonblocking anti-E-selectin mAb. Silver staining of the column fractions revealed a 250kD ligand expressed by these cells. In addition, using an in vitro shear-dependent recirculating loop assay developed while studying neutrophil interactions (138,139), we have shown that bovine γδ T cells bind E- and P-selectin avidly and in newborn calves γδ T cells are the only lymphocyte population that bind to E-selectin (98).

In pursuit of the bovine E-selectin ligands, we have proposed that lineage-specific cell-surface antigens may represent selectin ligands. More specifically, GD3.5Ag and

WC1, which share biochemical characteristics with other adhesion ligands, may bind E-selectin.

Summary

Historically, lineage-specific cell-surface markers have been shown to be functionally important. Since the discovery of the γδ T cell, definition of γδ T cell lineage-specific markers continues to be a topic of interest. Unfortunately, in the past ten years, only two lineage-specific markers have been described on γδ T cells (WC1 and TCR). Here, I will describe a new lineage-specific γδ T cell marker (GD3.5Ag) and determine if this marker is an E-selectin ligand.

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CHAPTER 2

GENERATION OF A NEW γδ T CELL-SPECIFIC MONOCLONAL ANTIBODY (GD3.5): BIOCHEMICAL COMPARISONS OF GD3.5 ANTIGEN WITH THE PREVIOUSLY DESCRIBED WC1 FAMILY

Introduction

Although gamma/delta (γδ) T cells were discovered a decade ago, their specific biological function has yet to be fully defined. Some suggest that these cells contribute to the initial host response to various infectious agents, which include parasitic, bacterial, and viral pathogens, forming what is referred to as "the first line of defense" (1-3). This hypothesis is consistent with their anatomical location at the portals of entry in nonlymphatic tissues, such as the skin and gut (4-6). In studies using γδ or αβ T-cell-deficient mice, the role of γδ T cells in malarial infection appears to occur during initial host responses against the liver-stage of parasite development (1). In another report, $\beta_2$microglobulin-deficient mice infected with live avirulent mycobacteria appear to respond with increased numbers of γδ T cells when compared to normal mice indicating that these cells may possess an early compensatory function in the absence of MHC I molecular expression (2). Further reports indicate that γδ T cells respond to and destroy virally infected target cells (3).

Although γδ T cells that react with classical MHC I and II have been isolated, they appear to react with other nonclassical MHC gene products, possibly providing a faster and more encompassing immunological response (7-10). Several reports suggest that γδ T cells may recognize "unconventional" antigens, such as nonpeptide (carbohydrate) antigens and heat-shock proteins (11-16). Recent reports indicate that γδ T cell antigen recognition may be independent of antigen processing, suggesting a direct recognition event (17).

Historically, lineage-specific cell surface markers have been indispensable in describing various cell functions. In the case of γδ T cells, only one non-TCR lineage-specific marker (SBU-T19), originally described in sheep, has been defined (4,18). This marker, later demonstrated in cattle, goats, pigs, horses, humans and rodents, is collectively called WC1 (6,19-26). Bovine genomic DNA contains several similar sequences to WC1, suggesting the potential for a WC1 gene family (23). Three WC1 isotypes have been identified to date (23,26) and the IL-A29 monoclonal antibody recognizes all three native species, including gene products of the 3 WC1 cDNAs. At the protein level, bovine WC1 exhibits bands at approximately 215kD and 300kD under reducing conditions (recognized by IL-A29); while under nonreducing conditions, the smaller band migrates somewhat faster (200kD) (4,6,22,23,25,27). WC1 is clearly distinguishable from γδ TCR which migrates at approximately 45kD under reducing and 70-75kD under nonreducing conditions. Unfortunately, little is known about the function of WC1. Some speculate that WC1 may play a role similar to CD4 and CD8, while others suggest the expression of different WC1 isotypes may provide a mechanism for tissue-specific homing (4,23,26,28).

We have sought the identification of additional lineage-specific markers to better study γδ T cell function. We chose the bovine model because γδ T cells represent a major portion of the circulating T cell pool in neonates; thus, their biological function may be

more easily studied. We have raised many specific monoclonal antibodies against purified γδ T cells with most being shown to recognize either WC1 or TCR. In this report, we provide the characterization of one antibody (GD3.5) that recognizes a γδ T cell-restricted surface antigen that is distinct from IL-A29-reactive WC1 and TCR. GD3.5 stains approximately 90% of the circulating γδ T cell population. SDS-PAGE analysis indicates that GD3.5 antigen migrates slightly slower than WC1 under nonreducing conditions and does not exhibit a 300kD band. Crossprecipitation experiments indicate that mAb GD3.5 and mAb IL-A29 immunoprecipitate distinct molecules that exhibit distinct proteolytic digestion profiles. Glycosidase and endopeptidase treatment further characterize and distinguish GD3.5 antigen and WC1 demonstrating unique profiles. Interestingly, GD3.5 antigen and WC1 are sensitive to O-sialoglycoprotease treatment indicating that these molecules possess sialomucin-like characteristics. In addition, GD3.5 is exquisitely sensitive to low-dose α-chymotrypsin, while WC1 is resistant to equivalent protease concentrations.

Materials and Methods

γδ T cell purification and ^{125}I labeling

Blood was obtained by jugular venipuncture from Holstein calves housed at the Montana State University Large Animal Facility. Lymphocytes were purified by Histopaque gradient centrifugation (Sigma Chemical Co., St. Louis, MO). Lymphocytes were resuspended in HBSS and platelets were removed by Percoll gradient centrifugation (Sigma Chemical Co., St. Louis, MO) with subsequent incubation of lymphocytes in T175 flasks for 30min at 37^{o}C to allow monocytes to adhere and be removed from suspension. γδ T cell lymphocytes were purified from whole lymphocytes by incubation for 30min at RT in T175 flasks coated with 7 x 10^{6} adherent E-selectin L-cell transfectants.

Remaining unbound cells were discarded and bound γδ T cells lifted with 2mM EDTA (29). This purification protocol provided >90% pure γδ T cells (29). 1 x 10^8 purified γδ T cells were $Na^{125}I$ (ICN Biomedicals, Inc., Irvine, CA)/lactoperoxidase-labeled according to published methods (30) and lysed for 30min on ice with NP40 lysis buffer (2% NP-40, 100mM NaCl, 1mM $MgCl_2$, 1mM $CaCl_2$, 5mM NaN_3, 10mM Hepes, and protease inhibitors: pepstatin A, 1,10-phenanthroline, phenylmethylsulfonyl fluoride, benzamidine, antipain, leupeptin, and chymostatin (Sigma Chemical Co., St. Louis, MO).

mAbs and FACS analysis

Monoclonal antibodies used included IL-A29 (anti-WC1) and two mouse mAbs CC21 (145kD B-cell marker) and CC42 (αβ T cell-CD2) that recognize bovine and caprine lymphocytes (kindly provided by Chris Howard, Institute for Animal Health, Compton, UK). In house mAbs, GD3.5, GD3.8, and GD197 were produced by immunization (i.p.) of Balb/C mice with 6 x 10^7 purified γδ T cells in Gerbu Adjuvant (Biotech, Poway, CA). Mouse serum was screened by FACS analysis to determine reactivity with γδ T cells after two weeks. Two subsequent immunizations were performed as described at two week intervals. After the third immunization, mouse spleens were aseptically removed 72hrs later and fused as described (31). Fusion wells containing hybrids that positively stained γδ T cells by FACS analysis were subsequently subcloned and screened by FACS analysis. mAb production resulted in various γδ T cell-positive mAbs including GD3.5, GD197 (γδ TCR), and GD3.8 (pan-γδ TCR). (B. Walcheck and M.A. Jutila, unpublished observations; and Wilson, E. et al., manuscript in preparation).

FACS analysis was performed on ice as follows: cells were blocked with 2% horse serum/PBS for 10min, washed, and incubated with appropriate primary Ab for 30min. Cells were washed and incubated with appropriate FITC-conjugated or PE-conjugated

goat α-mouse secondary Ab (Jackson ImmunoResearch, West Grove, PA) for 30min, washed again, and analyzed. Two-color analysis was performed using PE-conjugated secondary Ab followed by a 10min block with 10% mouse serum and subsequent 30min incubation with FITC-conjugated second-stage Ab. All analyses were performed using a FACScan flow cytometer (Becton Dickinson).

Western Blot

Purified γδ T cells were lysed with NP-40 lysis buffer (see above), mixed with nonreducing loading buffer and electrophoresed through 8% polyacrylamide gels. Gels were transferred to nitrocellulose (NC) overnight. Resulting NC was washed, blocked for 30min with horse serum, washed again, and placed in a Miniblotter 25 (Immunetics, Cambridge, MA). Lanes were loaded with GD3.5 or IL-A29 supernatants and incubated for 30min. Primary antibody was washed away and α-mouse alkaline phosphatase-conjugated second stage (Sigma Immuno Chemicals, St. Louis MO) applied for 30min. The NC was washed and removed from Miniblotter 25 and developed to desired intensity using Nitro Blue Tetrazolium and 5-Bromo-4-Chloro-3-Indolyl Phosphate (Sigma Chemical Co., St. Louis, MO) in AP buffer (100mM Tris pH 9.5, 100mM NaCl, and 5mM $MgCl_2$).

Immunoprecipitation/Crossprecipitation

To eliminate nonspecific protein interactions, ^{125}I-labeled lysates were incubated for 1hr at RT with 5% rabbit serum and then incubated at 4°C with protein G beads (Boehringer Mannheim Biochemicals, Indianapolis, IN). After overnight incubation, protein G beads were removed and lysates were used in subsequent procedures. Precleared lysates were incubated with 75ug/ml GD3.5 or IL-A29 for 1hr at RT. Ab/Ag complexes were precipitated with protein G at 4°C overnight. Several subsequent

clearing steps with protein G were performed on the lysates to insure removal of all Ab/Ag complexes. IL-A29 and GD3.5 mAbs were added to lysates which had been cleared of GD3.5 and WC1 Ab/Ag complexes, respectively. Ab/Ag complexes were precipitated as described earlier and mixed with standard 2x reducing or nonreducing loading buffer, boiled for two minutes, and loaded onto 8% SDS-polyacrylamide gels. Gels were electrophoresed and dried at 80°C. Gels were exposed 1-2 weeks in a phosphorimaging storage cassette, and images were developed using a phosphorimager (both from Molecular Dynamics).

V-8 protease peptide mapping

^{125}I-labeled γδ T cell lysates, described above, were prepared and immunoprecipitated with IL-A29 and GD3.5. IL-A29 or GD3.5 immunoprecipitates were mixed with reducing buffer, boiled for two minutes, cooled to RT and centrifuged to remove beads. Supernatants were aliquoted (25ul) and incubated with 10, 50, and 100ng of V-8 protease for 40min at RT, loaded, and electrophoresed. Gels were dried at 80°C and exposed/developed using the MD phosphorimager, as described above.

Enzymatic deglycosylation of GD3.5 antigen and WC1

^{125}I-labeled γδ T cell lysates, described above, were prepared and immunoprecipitated with 30ug of IL-A29 or GD3.5. Immunoprecipitates were boiled for 3min after adding 80ul 50mM pH 6.0 phosphate buffer, 20ul 1% SDS, and 20ul .5M β-mercaptoethanol (Sigma Chemical Co., St. Louis, MO) and cooled to RT. After cooling, 35ul 200mM pH 6.0 phosphate buffer, 5ul 100mM $CaCl_2$, and 40ul 10% NP40 (Sigma Chemical Co., St. Louis, MO) were added to each reaction which were then centrifuged and the supernatants aliquoted (35ul) for treatment with 0.2U PNGase F (Boehringer Mannheim Biochemicals, Indianapolis, IN), 9.6ug O-sialoglycoprotein endopeptidase/O-

sialoglycoprotease (Accurate Chemical & Scientific Corp., Westbury, NY), or 2mU neuraminidase (Boehringer Mannheim Biochemicals, Indianapolis, IN). Aliquots of GD3.5 antigen and WC1 were adjusted to pH 6.0 (control and neuraminidase) or pH 7.4 (PNGase F and O-sialoglycoprotease) with 15ul of 500mM phosphate buffer, mixed with appropriate amount of enzyme, and incubated overnight at 37°C (13-15hrs). Reactions were stopped with 6x reducing loading buffer, boiled for 3min and electrophoresed. Gels were dried at 80°C and exposed/developed using the MD phosphorimager, as described above.

Chymotrypsin Sensitivity Assay

Lymphocytes were purified as described above. Cells ($1x10^7$) were resuspended in HBSS with Ca^{2+} and Mg^{2+} and 0, 62.5, 125, or 250 mU α-chymotrypsin (Sigma Chemical Co., St. Louis, MO)/ml and incubated for 1hr at 37°C (32). After completion of incubation, cells were washed with 2% horse serum/PBS and analyzed by single-color indirect FACS analysis for GD3.5 antigen and WC1 using PE-conjugated second stage, as described earlier.

Results

Surface expression of the GD3.5 antigen on bovine lymphocytes

GD3.5 was originally identified by flow cytometric analysis as a γδ T cell-specific staining monoclonal antibody (See Materials and Methods mAb production and see below). Using two-color FACS analysis, we determined the staining characteristics of GD3.5 on bovine peripheral blood lymphocytes. Cells were stained with GD3.5, IL-A29 (WC1), CC21 (B-Cell) or CC42 (αβ T cell), followed by a PE secondary Ab, and then stained with FITC-labeled GD3.5 or GD3.8 mAb. As shown in Figure 1A, GD3.5 stained 91.8% of

the total GD3.8 (pan-TCR) γδ T cell population. In comparison, panel 1B shows that IL-A29 stained 78% of the total γδ T cell population. Figure 1C indicates that a substantial percentage of the GD3.5-positive population was WC1 negative. Additionally, single-color FACS analysis further supported the two-color data indicating that GD3.5 stains approximately 90% of the total γδ T cell population while IL-A29 stained 70-80% of the total γδ T cell population (data not shown). To further demonstrate that GD3.5 specifically stained γδ T cells, cells were stained with B-cell (CC21) or αβ T cell (CC42) mAbs. Figure 1D and 1E illustrate that GD3.5 stained a population of lymphocytes that were distinct from both B cells (CC21) and αβ T cells (CC42). Furthermore, using the distinguishing light-scatter profiles of neutrophils and monocytes, gated FACS analysis of these cell populations in whole bovine leukocyte preparations revealed that mAbs GD3.5 and GD3.8 did not stain either cell population (data not shown).

Using single-color FACS analysis of peripheral blood, crossreactivity was determined using dog, horse, human, sheep, pig, and rabbit. GD3.5 crossreacted only with the sheep lymphocytes (data not shown).

Western Blot and immunoprecipitation analysis of the GD3.5 antigen

Western blot analysis was done to determine a Mr for the GD3.5 antigen. As shown in lanes 3 and 4 of Figure 2, GD3.5 recognized a single band migrating at 220-240kD under nonreducing conditions. Interestingly, using this technique sometimes resulted in a single GD3.5-reactive band at approximately 80kD which is likely due to the exquisite sensitivity of GD3.5 antigen to proteolysis (data not shown and see Figure 7). In comparison, lanes 5 and 6 show bands recognized by IL-A29 (anti-WC1) at 200-220kD and 300kD under nonreducing conditions. Therefore, under these conditions, GD3.5 antigen was electrophoretically distinct from IL-A29 antigen (WC1).