Analysis and characterization of tissue specific accumulation of TCR-defined [gamma delta] T cell subsets in the bovine system
by Eric Wilson

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Veterinary Molecular Biology
Montana State University

Abstract:
Distinct subsets of yS T cells, based on the expression of T cell receptor for antigen (TCR), have been shown to localize in different tissues of mice and humans. The mechanisms responsible for the tissue-selective accumulation of these cells are not known. y5 T cells are the predominant T cell subset in the peripheral blood of newborn calves, making this animal a useful model to study y5 T cells. In this dissertation project, I tested the hypothesis that tissue-specific accumulation of y5 T cells is controlled, in part, through selective homing (leukocyte trafficking). To begin, I characterized four TCR-defined subsets of bovine y5 T cells: subsets 1,2,3, and 4. These cells were shown to segregate to different tissues and organs much like murine yg T cells. Subset 1 accumulated in inflamed peripheral lymph nodes, and subset 4 was found primarily in mucosal sites and the spleen. The majority of subset 4 y5 T cells coexpressed CDS and CD2. CD8+γδ5 T cells did not efficiently accumulate in a site of artificial inflammation. Analysis of CD8-γδ T cells demonstrated these cells express E-selectin and GR antigen ligands, as well as homogeneously high levels of L-selectin. Conversely, most CD8+γδ T cells lacked these adhesion markers. CD8+γδ T cells expressed higher levels of the β7 integrin than CD8-γδT cells. In an ex vivo adhesion assay, peripheral blood bovine CD8+y8 T cells were shown to preferentially bind the mucosal addressin MAdCAM-1, indicating that accumulation of CD8+ γδcells in some tissues may be mediated, in part, by α4β7/MAdCAM-l binding. Indeed, CD8+γδ T cells were found to accumulate in sites expressing high levels of MAdCAM-1, such as the spleen, gut and mesenteric lymph nodes, but not in peripheral sites which did not express MAdCAM-1. These results indicate that circulating bovine yS T cells are composed of distinct TCR defined subsets that localize to distinct tissues, as has been described in other species. Additionally, in some cases the adhesive phenotype of γδ T cells can be used to predict the ultimate tissue localization of these cells.

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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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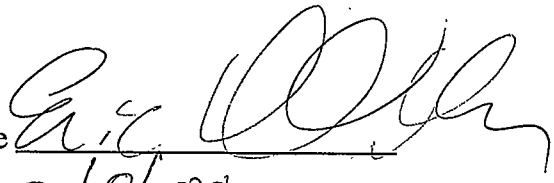
Signature 

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ABSTRACT

Distinct subsets of γδ T cells, based on the expression of T cell receptor for antigen (TCR), have been shown to localize in different tissues of mice and humans. The mechanisms responsible for the tissue-selective accumulation of these cells are not known. γδ T cells are the predominant T cell subset in the peripheral blood of newborn calves, making this animal a useful model to study γδ T cells. In this dissertation project, I tested the hypothesis that tissue-specific accumulation of γδ T cells is controlled, in part, through selective homing (leukocyte trafficking). To begin, I characterized four TCR-defined subsets of bovine γδ T cells: subsets 1, 2, 3, and 4. These cells were shown to segregate to different tissues and organs much like murine γδ T cells. Subset 1 accumulated in inflamed peripheral lymph nodes, and subset 4 was found primarily in mucosal sites and the spleen. The majority of subset 4 γδ T cells co-expressed CD8 and CD2. $CD8^{+}\gamma\delta$ T cells did not efficiently accumulate in a site of artificial inflammation. Analysis of $CD8^{-}\gamma\delta$ T cells demonstrated these cells express E-selectin and GR antigen ligands, as well as homogeneously high levels of L-selectin. Conversely, most $CD8^{+}\gamma\delta$ T cells lacked these adhesion markers. $CD8^{+}\gamma\delta$ T cells expressed higher levels of the β7 integrin than $CD8^{-}\gamma\delta$ T cells. In an *ex vivo* adhesion assay, peripheral blood bovine $CD8^{+}\gamma\delta$ T cells were shown to preferentially bind the mucosal addressin MAdCAM-1, indicating that accumulation of $CD8^{+}\gamma\delta$ T cells in some tissues may be mediated, in part, by α4β7/MAdCAM-1 binding. Indeed, $CD8^{+}\gamma\delta$ T cells were found to accumulate in sites expressing high levels of MAdCAM-1, such as the spleen, gut and mesenteric lymph nodes, but not in peripheral sites which did not express MAdCAM-1. These results indicate that circulating bovine γδ T cells are composed of distinct TCR defined subsets that localize to distinct tissues, as has been described in other species. Additionally, in some cases the adhesive phenotype of γδ T cells can be used to predict the ultimate tissue localization of these cells.

CHAPTER 1

IMMUNE RESPONSE AND THE T CELL RECEPTOR

Introduction

Immune responses are initiated following exposure to foreign antigenic substances, often infectious agents such as bacteria, viruses and parasites. The infected individual responds to this antigen with a wide array of immune cells including neutrophils, macrophages, natural killer cells, and T and B lymphocytes. These broad classifications of immune cells can be subdivided into defined cellular subsets. Each leukocyte subset serves a distinct, yet sometimes overlapping, role in the immune response and each has evolved to function optimally in its discrete role.

Cell mediated immunity was first described by the Nobel laureate Elias Metchnikoff in a paper describing phagocytic cells of marine invertebrates (1). It has since become apparent that the immune system is infinitely more complex than Metchnikoff imagined, with all immune cells working in concert to produce a highly functional immune system. T lymphocytes are particularly important both for their role in modulating the immune system response and for their capacity to recognize and kill infected cells. The majority of circulating lymphocytes express T cell receptors for antigen (TCR). The "traditional" T cell expresses an αβ TCR. In the early 1980s, cDNAs encoding the αβ TCR were identified through subtractive hybridization, with the β chain of the TCR being identified first (2, 3). Soon after the discovery of the β chain,

another chain was cloned. At the time it was assumed that this was the α chain of the αβ TCR (4). However, it was later recognized that the supposed α chain was in fact a new, TCR-like, gene later named the γ chain (5). After several years of confusion concerning these genes, it was eventually determined that two different types of T cell receptors exist: the αβ and the γδ (6). The αβ TCR is composed of α and β chains, while the γδ TCR is composed of γ and δ chains.

The genes encoding the αβ and γδ T cell receptors have a germ-line organization that is similar to the multigene organization of immunoglobulin (Ig) genes. As in Ig genes, separate V, D, and J gene segments rearrange to form functional genes, encoding antigen binding proteins. The αβ TCR has been shown to bind only processed antigen, displayed in the context of MHC class I or MHC class II molecules. The MHC I and MHC II molecules are recognized by two other accessory T cell antigens, CD8 and CD4 respectively. The binding of αβ T cells to antigen is an exclusive process where the TCR of the lymphocyte only binds antigen on the surface of other cells. The αβ TCR recognizes a complex consisting of a proteolytically derived peptide, which is bound into a specialized groove of an MHC class I or MHC class II protein on antigen presenting or target cells (reviewed in ref. 7).

In contrast to αβ T cells, γδ T cell binding to an antigen is not as well characterized. There are several major differences between the binding of antigen by γδ T cells and αβ T cells. Although some γδ T cells express CD4 and CD8 molecules, the majority do not, making antigen recognition in the context of traditional MHC molecules impossible. In direct contrast to αβ T cells, most γδ T cells can recognize native unprocessed antigen, as do antibodies. In an effort to better understand and characterize the differences of antigen recognition between γδ and αβ T cells, detailed analyses of TCR and Ig complementarity determining region 3 (CDR3) have been performed. CDR3 region variability has recently been proposed to be the principal source of antigen

specificity for γδ T cells and Ig. Molecular analysis of CDR3 loops has demonstrated the CDR3 lengths of both α and β chains to be nearly identical. In contrast, CDR3 lengths of δ chains are long and extremely variable in their structure, as are Ig heavy chains. Conversely, γ chains are shorter and exhibit less diversity, as do Ig light chains (8). These results demonstrate molecular similarities between Ig molecules and the γδ T cell receptor. CDR3 loop diversity may contribute to the ability of γδ T cells to bind native antigen, similar to antibody, as opposed to αβ T cell receptors, which must bind processed and presented antigen (9).

γδ T cell roles in immune responses

The role and importance of γδ T cells in the immune system has long been a point of controversy and contention. Earlier it was thought that the role of these cells would be quickly elucidated through the generation of γδ T cell-deficient mice. Although αβ and γδ T cell-deficient mice have been useful in understanding the function of these cells, the hope that this technology would answer all questions concerning the role of these cells has proven overly optimistic. This is largely due to the plasticity and overlapping nature of the immune system. In addition, although researchers have successfully knocked out genes, the immune system has sometimes "functionally" replaced them. For example, it has been shown that when the Cδ gene is knocked out, which would normally be spliced to a VDJδ gene, the VDJδ sometimes alternatively fuses with a Cα gene. This VDJδ/Cα union results in a cell expressing a chimeric α/δ chain and a γ chain that potentially carries the specificity of the original γδ TCR. This chimeric TCR has been shown to be prominent in TCR δ gene-deficient mice (10). The generation of mice deficient in both γ and δ TCR chains could add additional insight to the role of γδ T cells. Another common method to functionally deplete cells is to flood

the system with antibodies against specific cells allowing the animal to essentially knock out a cell type through complement lysis. Several γδ T cell functional studies have been done in which the αβ T cells have been eliminated, usually by the addition of anti-CD4 and -CD8 antibodies (11, 12). These studies do not address the fact that some γδ T cells express CD4 and CD8 which would also have been depleted. However, later studies using monoclonal antibodies (mAbs) against the αβ T cell receptor validated several of these earlier studies (13-15).

Despite the problems associated with the use of γδ T cell-deficient mice, studies on these mice strongly suggest several important functions of γδ T cells. γδ T cells have been shown to enhance B cell function (16), stimulate isotype switching in B cells (17), improve IgA responses (18), present antigen to αβ CD4 cells (19), and help to maintain the integrity of the intestinal epithelia (20). Perhaps most impressively, TCR δ gene deletion mutants succumb to *M. tuberculosis* infection, an infection easily tolerated by immunocompetent controls (21). These functions are most probably mediated directly through cell contact as well as indirectly through secreted cytokines (22).

γδ T cells in viral infections

Most research on the role of T cells in controlling viral infections has focused on conventional αβ T cells. In fact, most anti-viral T lymphocyte activity is attributed to αβ T cells. However, recent work has shown that γδ T cells are also stimulated by and play an active role in viral infections (reviewed in ref. 23). Studies of murine influenza and Sendai virus have shown large influxes of γδ T cells in infected tissue. However, γδ T cells do not accumulate at the site of infection if αβ T cells are first depleted by the addition of anti-CD4 and anti-CD8 antibodies (11, 12). These results suggest that a cooperative effort involving αβ and γδ T cells, acting in concert, is important in

regulating the progression of the immune response to these viruses (24). Other data showing a more active role for γδ T cells in viral infections has come from research on Coxackievirus (25, 26) and Herpes simplex virus (HSV) (27). In both settings, cytotoxic virus-specific γδ T cells have been demonstrated. Interestingly, it has also been shown that γδ T cells are capable of directly binding unprocessed HSV glycoprotein (27). Work by Wallace, *et al* (28) has shown that 40% of the TCR-defined subset Vγ9/Vδ2 T cell clones, but none of the Vδ1 T cell clones, isolated from the blood of HIV-seronegative donors are capable of lysing HIV infected cells. These data strongly suggest a role for γδ T cells in viral immuno-surveillance, and demonstrate the functional importance of different γδ T cell subsets in response to viral infections.

γδ T cells in bacterial infections

The importance of γδ T cells in the immune defense against various bacteria has been well documented. Although some data exist showing a role for γδ T cells in extra-cellular bacterial infections (29, 30), most reports in the literature deal with γδ T cell responses to intracellular bacteria such as *Mycobacterium* and *Listeria*. Studies of *Listeria* infection in mice have shown that large numbers of γδ T cells accumulate in the peritoneum of infected animals (31, 32). Interestingly, the absence of γδ T cells did not significantly alter the pathogen load, but did result in altered pathology in the infected tissues with γδ T cell knockout mice developing non-characteristic liver lesions (32, 33).

Murine (34, 35) and human (36, 37) γδ T cells have been shown to respond to mycobacterial antigens. Originally, γδ T cell lines against *Mycobacterium* were isolated from blood and tissues of BCG vaccinated donors (36) and patients with leprosy (37). However, it later became apparent that many γδ T cell clones isolated from persons with no known contact with *Mycobacteria* reacted strongly with mycobacterial antigen *in vitro*

(38), showing that prior mycobacterial antigen priming was not necessary to obtain antigen-specific cells.

The target of most *Mycobacteria* activated γδ T cells in humans is not PPD or Hsp 65, as originally believed (39), but rather non-protein carbohydrate lectins. These "non-traditional" antigens are prenyl pyrophosphate derivatives, usually isopentenyl pyrophosphate (40), and are bound exclusively by human γδ T cells expressing Vγ9/Vδ2 chains (41). These compounds can be found in both microbial and mammalian cells (42), and it has been proposed that cells specific for these antigens respond to a class of antigens shared by a number of pathogens as well as damaged or stressed host cells (6). Although mice and humans have been shown to produce strong γδ T cell responses against mycobacterial antigens, no studies have been able to demonstrate the presence of mouse γδ T cells reactive with prenyl pyrophosphate derivatives (43). Bovine γδ T cells have also failed to respond to isopentenyl pyrophosphate in *in vitro* culture experiments (Wilson unpublished results).

γδ T cells in parasitic infections

The importance of γδ T cells in parasite infections has been well established in several different disease models. Although some research has shown a potential role for γδ T cells in helminth infections (44-46), the vast majority of data on γδ T cell involvement in parasitic infections comes from protozoal disease models. Studies in which γδ and αβ T cell-depleted mice were used have shown that mouse mortality is greatly increased in γδ T cell-depleted mice following infection with *Toxoplasma gondii* (47). Although this study did not specifically demonstrate cytotoxic γδ T cells specific for the parasite, it does demonstrate a role for γδ T cells in parasite immunity. *Leishmania* infections have been shown to elicit strong antigen specific γδ T cell

responses in both mice (48, 49) and humans (50). Studies involving patients diagnosed with clinical leishmaniasis have shown that stimulating cells with promastigote (an extracellular stage of the parasite) lysates resulted in a dramatic increase of γδ T cells in culture (50). Interestingly, in human peripheral blood mononuclear cells (PBMCs) stimulated with an *L. amazonensis* lysate, a large number of γδ T cells co-expressed the CD8 molecule (50), yet few γδ T cells express this marker in peripheral blood (51).

Perhaps the most extensively studied example of γδ T cells affording protection against parasitic disease is the case of malaria. Extensive work has been done that shows Vγ9 γδ T cells are specifically activated by *Plasmodium* merozoites (52-55). *Plasmodium* merozoites are an extra-erythrocytic stage of the parasite (56), suggesting that killing of the parasite is dependent on the recognition of native rather than processed antigen. In contrast, intracellular stages of the parasite do not elicit a γδ T cell response (52). Elloso and coworkers (52) demonstrated that γδ T cells specifically kill *Plasmodium* merozoites and this killing is dependent solely on the presence of γδ T cells. Parasite lysis was shown to be totally dependent on T cell/merozoite contact, and was not dependent on the presence of any accessory cells (52).

In the context of this dissertation, perhaps the most pertinent example of the role of γδ T cells in parasitic infections is the report by Flynn and Sileghem (57). This study compared two different species of cattle, one susceptible to and one resistant to infection with *Trypanosoma congolense.* γδ T cells from resistant cattle were shown to proliferate when stimulated *in vitro* with trypanosome antigens. Increased proliferation of some αβ T cells was also seen in Trypano-resistant cattle; however, this proliferative response was much less vigorous than the proliferation of γδ T cells. In contrast, negligible *in vitro* proliferative responses were seen in γδ T cells isolated from Trypano-susceptible cattle. These data suggest that resistance to some bovine pathogens is influenced or dependent on the host's ability to activate γδ T cells.

Lineage-specific markers of γδ T cells

In the human and mouse systems, γδ T cells are identified by expression of the γδ TCR. Although γδ T cells express a variety of other surface molecules, to date the TCR is the only known lineage-specific marker of human and mouse γδ T cells. Conversely, ruminant γδ T cells express several lineage-specific markers. The first was discovered by Charles Mackay in 1986 (58). This 220 kD marker, initially termed T19, was found exclusively on $CD2^-$, $CD8^-$, and $CD4^-$ lymphocytes, which were known at the time as null cells. Null cells have since been determined to be γδ T cells. The T19 marker is now known as the WC1 family, and comprises a large group of related molecules found exclusively on γδ T cells (58).

The WC1 marker is found only on ruminant and swine γδ T cells (59). Southern blot analysis has shown that humans carry genes for the WC1 family of molecules; however, no expressed protein has been detected (59). Recently, another lineage specific marker called GD3.5 has been defined in the cow. This marker exhibits many similar characteristics to the WC1 marker, but has been shown to be biochemically distinct (60). The function of these molecules has not yet been determined. Several labs have proposed a role in lymphocyte homing or trafficking (58), while others have demonstrated that crosslinking WC1 induces a reversible growth arrest of the cell cycle at the G0/G1 interface (61-63). Conversely, data showing γδ T cell proliferation following the crosslinking of the WC1 molecule has also been published (64).

Other functionally important molecules on γδ T cells

Some subsets of γδ T cells have been shown to express functionally important molecules also found on αβ T cells. γδ T cells express CD3, and in the human the majority co-express CD2, CD5 and CD7 (65). In addition, small subsets of γδ T cells have been identified which co-express CD4 or CD8 molecules (51, 65, 66). Although rare in peripheral blood, $CD4^+\gamma\delta$ T cells have been shown to be common in fetal liver (66). $CD4^+\gamma\delta$ T cells are not cytotoxic and have been shown to produce a variety of cytokines including IL-2, IL-4, IL-5, TNF-α, IFN-γ, and GM-CSF (67, 68).

Two different lineages of γδ T cells have been identified, one passing through the thymus and the other originating in the gut. Gut derived γδ T cells express CD8 and comprise the majority of intraepithelial lymphocytes (51, 69, 70). However, unlike the CD8αβ heterodimer expressed on thymus-derived $CD8^+$T cells, gut derived $CD8^+\gamma\delta$ T cells express a CD8αα homodimer (51, 71); furthermore, $CD8^+\gamma\delta$ T cells exhibit cytotoxic effects similar to $CD8^-CD4^-\gamma\delta$ T cells and $CD8^+\alpha\beta$ T cells (67).

γδ T cell distribution

γδ T cells have been found in all vertebrates thus far examined (reviewed in ref. 65). The percentage of γδ T cells in the pool of total lymphocytes in these species varies greatly. Human peripheral blood contains between 0.5-16% γδ T cells (51, 72), mouse 0.5-2.0% (73), chicken 15% (74), and cattle 15-70% (75). Much of our knowledge of γδ T cells comes from research done in the mouse and human, which has been hampered in some respects due to the small numbers of γδ T cells in these systems.

An intriguing characteristic of γδ T cells is that they are found in different tissues and locations than αβ T cells. The majority of αβ T cells are found in secondary

lymphoid organs, whereas the majority of γδ T cells are found in nonlymphatic sites such as the skin and mucosa. γδ T cells for the most part are excluded from secondary lymphoid organs (75, 76). This non-lymphoid localization is well illustrated in the bovine system where γδ T cells often constitute as much as 60% of the circulating lymphocytes. By contrast, only 1-6% of the lymphocytes typically found in the thymus, lymph nodes and Peyer's patches are γδ T cells (75, 76). The positioning of γδ T cells at "portals of entry," such as skin and female reproductive tract, have prompted the idea that γδ T cells are important in the first line of defense against invading pathogens (77). Indeed, the ability of the γδ T cell to recognize native unprocessed antigen, combined with the positioning of these cells, makes them well suited for a rapid assault on invading pathogens. Interestingly, the TCRs of γδ T cells found at these sites are often composed of identical or very similar γ and δ TCR chains. For example, mouse γδ T cells associated with the epidermis (78, 79) and the mucosal epithelia of the vagina, uterus, and tongue (73) utilize a TCR repertoire consisting of distinct pairs of γ and δ gene products which express virtually no diversity, in terms of γ gene usage. Other distinct TCR-defined subsets of γδ T cells are found in the blood, spleen, intestine, lung, liver, and mammary glands (reviewed in ref. 72).

This site-specific accumulation of γδ T cell subsets is potentially mediated by one or a combination of the following mechanisms. 1) Selective trafficking of T cell subsets: cells expressing different adhesion molecule profiles may home to tissues expressing appropriate endothelial cell ligands, resulting in the accumulation of distinct subsets of cells in various tissues. 2) Selective retention of T cell subsets: all subsets of cells may migrate equally well into a tissue; however, some subsets may be selectively retained in tissues through cellular retention mechanisms. 3) Selective expansion of T cell subsets: subsets may migrate equally well to all tissues, but once within a site, specific subsets may be activated and result in proliferation and clonal expansion of specific TCR-defined

subsets. Although the accumulation of TCR-defined γδ T cell subsets in specific organs and tissues has been extensively studied, the mechanisms by which this occurs remains enigmatic. This dissertation focuses on the role of lymphocyte homing in the accumulation of cell subsets to different organs and tissues.

Lymphocyte homing and recirculation

Proper immune function is dependent on the effective homing and recirculation of lymphoid cells. Naive αβ T cells generally recirculate from the blood into secondary lymphoid organs on a continuous basis. This process of recirculation provides lymphocytes access to the lymphoid organs of the body that collect and present antigen to cells. As T lymphocytes encounter antigen, those cells that have the appropriate T cell receptors for antigen bind, become activated, proliferate, and develop into memory cells. Conversion of a naive cell into a memory cell involves a number of phenotypic and functional changes that distinguish the cell from its naive counterparts.

Memory cells exhibit different trafficking behaviors than naive cells, often trafficking to extralymphoid sites such as skin and inflamed joints (reviewed in 80). Defined subsets of memory cells may also exhibit different trafficking patterns than other subsets of memory cells. The tissue-selective homing of lymphocytes is believed to aid in the efficiency of the immune response, by ensuring that cells which have contacted antigen in a particular tissue efficiently migrate back to that same tissue (81). This is well illustrated by memory lymphocytes from mucosal sites, which preferentially migrate back to mucosal sites when reinfused into an animal (82, 83). A similar phenomenon occurs with cells isolated from peripheral sites (83).

Although memory cells have been shown to exhibit well defined trafficking patterns, in some cases naive T cells also display unique trafficking profiles. For

example, some γδ T cells leave the thymus "preprogrammed" to home to certain locations. This is well illustrated in the case of Vγ5 and Vγ6 γδ T cells in the mouse. A wave of Vγ5 cells is known to leave the thymus and home to the epidermis at 14-17 days of fetal development. A second wave of γδ T cells expressing Vγ6 appears a few days later and homes to the reproductive tract and the tongue (84). Although the mode of accumulation and the function of the Vγ5 and Vγ6 cells in these sites is currently unclear, the efficiency with which these cells, as well as memory cells, accumulate in distinct tissues is striking and suggests a vital function for these cells in their respective locations.

The accumulation of distinct leukocyte subsets into tissues involves a multistep process (85, 86, 87). In extra-lymphoid sites, leukocyte accumulation generally occurs when endothelial cells lining the venules respond to inflammatory mediators, leading to expression of adhesion molecules on their cell surfaces. Circulating leukocytes expressing the cognate ligand recognize these newly expressed adhesion molecules, bind to them, and then roll along the vessel wall. If an appropriate secondary signal is detected, the rolling cell tightly adheres to the endothelial cell lining, usually through an integrin-mediated event. The tightly adhered cell may then migrate into the underlying tissue if the necessary signals are detected (85-89). In the recirculation of lymphocytes through lymph nodes, a very similar process occurs. However, in the trafficking of lymphocytes to lymph nodes, vascular adhesion molecules are generally constitutively expressed (90, 91).

Many chemokines have been identified as factors that induce rapid arrest and transendothelial migration of rolling lymphocytes. Chemokines are a large family of cytokines characterized by their capacity to induce the directional migration and activation of leukocytes. Acting on cells through the binding of specific receptors on the cell surface, chemokines have been shown to be involved in the regulation of leukocyte

homing. Chemokines can possess exquisite leukocyte subset specificity, with some acting on myeloid and others on lymphoid cell subsets (reviewed in ref. 92). Differential sensitivity to chemokines may enable a second level of discrimination in the recruitment of lymphocytes to tissues. The adhesion molecule profile of a cell combined with chemokine sensitivity is believed to result in the elegant homing patterns seen *in vivo*.

Lymphocyte homing to mucosal tissues

The homing of memory and/or activated lymphocytes to mucosal tissues is known to be mediated primarily by the α4β7 integrin. Using Stamper Woodruff frozen section binding assays, it has been shown that anti-α4β7 function-blocking mAbs reduce lymphocyte binding 70%-90%. Similar inhibitory effects are seen in *in vivo* gut homing experiments (93). The role of α4β7 role in mucosal homing was proven when a nonmucosa binding lymphoma cell line was transfected with the α4 and β7 genes. Following transfection, these cells avidly bound Peyer's patch high endothelial venules (HEV) (94). Later work demonstrated that the counter ligand for α4β7 is mucosal addressin cell adhesion molecule-1 (MAdCAM-1) (95, 96). MAdCAM-1 has been shown to be the principal mucosal addressin (90, 97)It is expressed at high levels on the HEV of the Peyer's patches, mesenteric lymph nodes, as well as on venules of the lamina propria. Inhibition of MAdCAM-1 by function-blocking mAbs results in a 70-95% reduction in lymphocyte trafficking into Peyer's patches (90).

Lymphocyte homing to peripheral nodes

The homing of lymphocytes to peripheral lymph nodes was originally characterized as being dependent on the lymphocyte homing molecule L-selectin (98). L-selectin has also been shown to be important in the homing of cells to some mucosal sites (99, 100). The importance of lymphocyte homing via L-selectin has been demonstrated both by antibody blocking studies, as well as by using L-selectin-deficient mice (101). The best characterized endothelial cell ligands for L-selectin are peripheral node addressins (PNAds) (90). Blocking PNAds with the mAb MECA 79 has been shown to reduce lymphocyte binding to peripheral lymph node HEVs by up to 90% (102).

Lymphocyte homing to the spleen

The spleen contains more lymphocytes than all the lymph nodes in the body combined (103-106). Despite the large numbers of lymphocytes present in the spleen, very little is known about the molecular mechanisms involved in lymphocyte homing and recirculation to this organ. Lymphocytes are known to exit the blood flow in the marginal zone, where minor vessels deposit blood in a loose network of cells. Lymphocytes then generally migrate to the white pulp of the spleen (107-109). Splenic architecture lacks HEV, which are important in facilitating lymphocyte migration to the lymph node in some species. Thus, an alternative entry point into splenic white pulp is necessary. Evidence proposed by Lyons and Parish (109) suggests that marginal zone macrophages constitutively express adhesion molecules, which regulate lymphocyte entry into the white pulp of the spleen in much the same manner as endothelial cells mediate entry into the paracortex of the lymph node.

Several homing associated molecules are present in the spleen, and are expressed on macrophages, dendritic cells, and sinus lining endothelial cells in the marginal zone. Each of these adhesion molecule expressing cells could theoretically function to mediate lymphocyte traffic to the splenic white pulp. In the murine system, large amounts of MAdCAM-1 are expressed in the marginal zone (110). However, short term homing assays have not shown any decrease in the number of lymphocytes homing to the spleen following the injection of blocking mAbs against either MAdCAM-1 or its ligand $\alpha 4\beta 7$ (110). This study, although widely referenced, measured only the total number of lymphocytes entering the spleen, and did not address the possibility that the entry of some lymphocyte subsets was blocked by anti-MAdCAM-1 or anti-$\alpha 4\beta 7$ mAbs. Paradoxically, when antibodies against VLA-4, VCAM-1, and L-selectin were used to study lymphocyte accumulation to the spleen (all known to block homing to either lymph nodes or to sites of inflammation), lymphocyte numbers in the spleen actually increased (99, 111).

Based on the limited data available for lymphocyte trafficking to the spleen, it appears that homing may be regulated by adhesion molecules unique from those in other lymphoid organs. The possibility also exists that splenic homing occurs through characterized adhesion molecules in conjunction with unique chemokine receptor profiles. For example, integrin mediated adhesion may cause a cell to be retained in the spleen long enough for the cell to respond to chemokines. The response of the lymphocyte to chemokines may then result in the directed migration to distinct areas of the spleen. The combination of differential adhesion molecules and chemokine receptor expression could serve to achieve the distinct cell accumulation patterns of lymphocytes observed in the spleen.

Lymphocyte trafficking to sites of inflammation

Lymphocytes constitutively recirculate through secondary lymphoid sites; however, infection or other stress often necessitates the rapid accumulation of lymphocytes into extra-lymphoid tissues. The migration of T cells into non-lymphoid sites is dependent on a number of different trafficking molecules, including E-, P-, and L-selectin. Selectins possess carbohydrate lectin-binding domains; adhesion is mediated through the interaction of these domains with specific carbohydrate moieties on target cells (112, 113).

E-selectin is an endothelial cell-expressed adhesion molecule that mediates leukocyte/endothelial cell adhesion of neutrophils (114) as well as some memory lymphocytes (115-117). It has also been shown that most γδ T cells from newborn calves avidly bind E-selectin (118), demonstrating that previous exposure to antigen is not an absolute requirement to induce E-selectin ligands on lymphocytes. Upregulated on endothelial cells in response to cytokines such as TNF-α and IL-1 (reviewed in ref. 119), E-selectin has been shown to be important in directing lymphocyte migration to inflamed skin (118, 120-122).

P-selectin is expressed both on activated endothelial cells and platelets (reviewed in ref. 119). P-selectin is stored in the granules of platelets and the Weibel-Palade bodies of endothelial cells, and can be rapidly translocated to the cell surface to mediate the rolling of lymphocytes expressing appropriate carbohydrate ligands (123). Similar to E-selectin, P-selectin is capable of facilitating the migration of subsets of memory T cells to the skin (116, 120), as well as DTH reactions (124).

As discussed above, L-selectin is important in the homing of cells to peripheral lymph nodes. L-selectin has also been shown to be involved in the trafficking of lymphocytes to some sites of inflammation (101). Although L-selectin trafficking to lymph nodes has been shown to be mediated primarily via PNAds, the L-selectin ligands mediating homing to sites of inflammation are not well characterized.

Potential ligands for L-selectin in inflammatory sites include PNAds, which are expressed in some inflammatory lesions (125), and E-selectin, which has been shown to serve as an L-selectin ligand (126). Human neutrophils and bovine γδ T cells have also been shown to support the rolling of other neutrophils and bovine γδ T cells, respectively (127, 128). This leukocyte/leukocyte rolling has been demonstrated to be totally dependent on L-selectin and partly dependent on PSGL-1 (129). L-selectin mediated leukocyte/leukocyte rolling is thought to contribute to the magnitude of the inflammatory response (80).

In summary, both the constitutive and inducible homing of lymphocytes is dependent on the array of adhesion molecules expressed on the surface of a cell. Through differential expression of adhesion molecules, leukocyte subsets accumulate in distinct tissues with striking efficiency. The array of adhesion molecules on the surface of a cell reflects the general site where a memory cell first encountered antigen, and is suggestive of where a circulating cell will ultimately leave the circulation. In short, the array of adhesion molecules expressed on the surface of a circulating cell is indicative of where it has been and where it is going.

The study of cell adhesion molecules may ultimately make it possible to direct lymphocyte subsets to specific sites or tissues by manipulating adhesion molecule expression. Furthermore, by blocking adhesion molecules it may be possible to inhibit the accumulation of specific leukocyte subsets, thus potentially allowing manipulation of

the immune response through regulation of one of the most basic of immunological functions, cell accumulation.

Rationale and aim of research

It has been proposed that γδ T cells serve as an immunological first line of defense, since they are found predominantly in mucosal and epithelial associated tissues. As discussed above, these cells are comprised of distinct TCR-defined subsets in the mouse and human systems. Interestingly, γδ T cells are often excluded from secondary lymphoid organs. This is best illustrated in the cow, where the peripheral blood lymphocyte pool often contains greater than 50% γδ T cells, whereas peripheral lymph nodes typically contain less than 6% γδ T cells. This is in contrast to the bovine spleen, where γδ T cells comprise a large proportion of the lymphocyte pool.

The bovine model holds several distinct advantages for the study of γδ T cell homing and localization to different tissues and organs. The peripheral blood of young calves contains the highest proportion of γδ T cells of any animal tested. Bovine γδ T cells also express additional γδ T cell lineage-specific markers. These characteristics, combined with the striking differences in the number of γδ T cells in blood and lymphoid organs, makes the bovine an ideal model to study organ- and tissue-specific lymphocyte accumulation.

The organ-specific accumulation of γδ T cells prompts three important questions. First, do TCR-defined γδ T cell subsets exist in the cow as described in the mouse and human? Second, does organ-specific accumulation of γδ T cells correlate to homing of specific subsets? Third, do γδ T cell subsets differ in their expression of defined homing molecules, and is it possible to predict the migratory behavior of different γδ T

cell subsets based on an their adhesive phenotype? Through investigating these questions, I hope to address the hypothesis that tissue specific accumulation of TCR-defined γδ T cell subsets is controlled in part by selective homing.

In the following chapters, my efforts to answer the above mentioned questions will be outlined. First I developed the tools to perform these studies and determined if tissue-specific accumulation of γδ T cell subsets occur in cattle. These results, describing the initial characterization of mAbs used to identify bovine TCR-defined γδ T cell subsets, and γδ T cell subset distribution, are contained in chapter two. Chapters three and four describe the direct attempt to define the adhesion profile of γδ T cell subsets and correlate the accumulation of these subsets to tissues expressing defined endothelial cell ligands.

In the process of accomplishing my research objectives it was necessary to establish collaborations with several individuals. These collaborators were helpful in providing necessary reagents, invaluable suggestions, and assistance with manuscript preparations. Below are the journal citations for the data contained in this thesis.

Wilson, E., B. Walcheck, W.C. Davis, and M.A. Jutila. 1998. Preferential tissue localization of bovine γδ T cell subsets defined by anti-T cell receptor for antigen antibodies. Immunology Letters, 64:39.

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Wilson, E., M. Briskin and M.A. Jutila. Manuscript in preparation. A TCR-defined subset of bovine γδ T cells expresses high levels of functional β7 integrin and preferentially accumulates in sites expressing MAdCAM-1: A mechanism for tissue specific γδ T cell subset accumulation.

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CHAPTER 2

PREFERENTIAL TISSUE LOCALIZATION OF BOVINE γδ T CELL SUBSETS DEFINED BY ANTI-T CELL RECEPTOR FOR ANTIGEN ANTIBODIES

Introduction

γδ T cells are believed to be an important first line of defense against a variety of pathogens, and participate in the regulation of Th1 and Th2 type cytokine responses (1-3). An interesting feature of γδ T cells is that particular subsets, defined by their TCR, have been shown to localize in specific tissues. For example, mouse γδ T cells associated with epidermis and the mucosal epithelia of the gut, vagina, uterus and tongue utilize a TCR repertoire consisting of distinct pairs of γ and δ genes (4-6). The mechanisms accounting for the selective tissue accumulation of γδ T cell subsets are not completely known.

To test whether recruitment mechanisms are involved in the tissue-selective accumulation of γδ T cells, circulating T cells should be analyzed; however, this can be problematic in mice and humans due to the minimal, and in the context of humans, highly variable γδ T cell population. Newborn calves offer useful animal models for studying γδ T cells because 1) γδ T cells are the predominant T cell subset in peripheral blood (7-9), and 2) studies can be done before the animal has encountered extensive