



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
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Abstract:

Distinct subsets of $\gamma\delta$ 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. $\gamma\delta$ T cells are the predominant T cell subset in the peripheral blood of newborn calves, making this animal a useful model to study $\gamma\delta$ T cells. In this dissertation project, I tested the hypothesis that tissue-specific accumulation of $\gamma\delta$ T cells is controlled, in part, through selective homing (leukocyte trafficking). To begin, I characterized four TCR-defined subsets of bovine $\gamma\delta$ T cells: subsets 1, 2, 3, and 4. These cells were shown to segregate to different tissues and organs much like murine $\gamma\delta$ 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 $\gamma\delta$ T cells coexpressed 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 $\beta 7$ 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+ $\gamma\delta$ cells in some tissues may be mediated, in part, by $\alpha 4\beta 7$ /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 $\gamma\delta$ 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 $\gamma\delta$ T cells can be used to predict the ultimate tissue localization of these cells.

ANALYSIS AND CHARACTERIZATION OF TISSUE SPECIFIC
ACCUMULATION OF TCR-DEFINED $\gamma\delta$ T CELL SUBSETS
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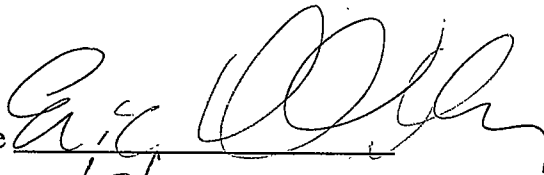
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ABSTRACT

Distinct subsets of $\gamma\delta$ 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. $\gamma\delta$ T cells are the predominant T cell subset in the peripheral blood of newborn calves, making this animal a useful model to study $\gamma\delta$ T cells. In this dissertation project, I tested the hypothesis that tissue-specific accumulation of $\gamma\delta$ T cells is controlled, in part, through selective homing (leukocyte trafficking). To begin, I characterized four TCR-defined subsets of bovine $\gamma\delta$ T cells: subsets 1, 2, 3, and 4. These cells were shown to segregate to different tissues and organs much like murine $\gamma\delta$ 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 $\gamma\delta$ 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 $\beta 7$ 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 $\alpha 4\beta 7$ /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 $\gamma\delta$ 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 $\gamma\delta$ 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 $\alpha\beta$ TCR. In the early 1980s, cDNAs encoding the $\alpha\beta$ 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 $\alpha\beta$ 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 $\alpha\beta$ and the $\gamma\delta$ (6). The $\alpha\beta$ TCR is composed of α and β chains, while the $\gamma\delta$ TCR is composed of γ and δ chains.

The genes encoding the $\alpha\beta$ and $\gamma\delta$ 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 $\alpha\beta$ 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 $\alpha\beta$ T cells to antigen is an exclusive process where the TCR of the lymphocyte only binds antigen on the surface of other cells. The $\alpha\beta$ 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 $\alpha\beta$ T cells, $\gamma\delta$ T cell binding to an antigen is not as well characterized. There are several major differences between the binding of antigen by $\gamma\delta$ T cells and $\alpha\beta$ T cells. Although some $\gamma\delta$ 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 $\alpha\beta$ T cells, most $\gamma\delta$ T cells can recognize native unprocessed antigen, as do antibodies. In an effort to better understand and characterize the differences of antigen recognition between $\gamma\delta$ and $\alpha\beta$ 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 $\gamma\delta$ 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 $\gamma\delta$ T cell receptor. CDR3 loop diversity may contribute to the ability of $\gamma\delta$ T cells to bind native antigen, similar to antibody, as opposed to $\alpha\beta$ T cell receptors, which must bind processed and presented antigen (9).

$\gamma\delta$ T cell roles in immune responses

The role and importance of $\gamma\delta$ 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 $\gamma\delta$ T cell-deficient mice. Although $\alpha\beta$ and $\gamma\delta$ 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 C δ gene is knocked out, which would normally be spliced to a VDJ δ gene, the VDJ δ sometimes alternatively fuses with a C α gene. This VDJ δ /C α union results in a cell expressing a chimeric $\alpha\delta$ chain and a γ chain that potentially carries the specificity of the original $\gamma\delta$ 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 $\gamma\delta$ 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 $\gamma\delta$ T cell functional studies have been done in which the $\alpha\beta$ 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 $\gamma\delta$ T cells express CD4 and CD8 which would also have been depleted. However, later studies using monoclonal antibodies (mAbs) against the $\alpha\beta$ T cell receptor validated several of these earlier studies (13-15).

Despite the problems associated with the use of $\gamma\delta$ T cell-deficient mice, studies on these mice strongly suggest several important functions of $\gamma\delta$ T cells. $\gamma\delta$ 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 $\alpha\beta$ 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).

$\gamma\delta$ T cells in viral infections

Most research on the role of T cells in controlling viral infections has focused on conventional $\alpha\beta$ T cells. In fact, most anti-viral T lymphocyte activity is attributed to $\alpha\beta$ T cells. However, recent work has shown that $\gamma\delta$ 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 $\gamma\delta$ T cells in infected tissue. However, $\gamma\delta$ T cells do not accumulate at the site of infection if $\alpha\beta$ 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 $\alpha\beta$ and $\gamma\delta$ 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 $\gamma\delta$ T cells in viral infections has come from research on Coxsackievirus (25, 26) and Herpes simplex virus (HSV) (27). In both settings, cytotoxic virus-specific $\gamma\delta$ T cells have been demonstrated. Interestingly, it has also been shown that $\gamma\delta$ 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 V γ 9/V δ 2 T cell clones, but none of the V δ 1 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 $\gamma\delta$ T cells in viral immuno-surveillance, and demonstrate the functional importance of different $\gamma\delta$ T cell subsets in response to viral infections.

$\gamma\delta$ T cells in bacterial infections

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

Murine (34, 35) and human (36, 37) $\gamma\delta$ T cells have been shown to respond to mycobacterial antigens. Originally, $\gamma\delta$ 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 $\gamma\delta$ 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 $\gamma\delta$ 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 $\gamma\delta$ T cells expressing V γ 9/V δ 2 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 $\gamma\delta$ T cell responses against mycobacterial antigens, no studies have been able to demonstrate the presence of mouse $\gamma\delta$ T cells reactive with prenyl pyrophosphate derivatives (43). Bovine $\gamma\delta$ T cells have also failed to respond to isopentenyl pyrophosphate in *in vitro* culture experiments (Wilson unpublished results).

$\gamma\delta$ T cells in parasitic infections

The importance of $\gamma\delta$ T cells in parasite infections has been well established in several different disease models. Although some research has shown a potential role for $\gamma\delta$ T cells in helminth infections (44-46), the vast majority of data on $\gamma\delta$ T cell involvement in parasitic infections comes from protozoal disease models. Studies in which $\gamma\delta$ and $\alpha\beta$ T cell-depleted mice were used have shown that mouse mortality is greatly increased in $\gamma\delta$ T cell-depleted mice following infection with *Toxoplasma gondii* (47). Although this study did not specifically demonstrate cytotoxic $\gamma\delta$ T cells specific for the parasite, it does demonstrate a role for $\gamma\delta$ T cells in parasite immunity. *Leishmania* infections have been shown to elicit strong antigen specific $\gamma\delta$ 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 $\gamma\delta$ T cells in culture (50). Interestingly, in human peripheral blood mononuclear cells (PBMCs) stimulated with an *L. amazonensis* lysate, a large number of $\gamma\delta$ T cells co-expressed the CD8 molecule (50), yet few $\gamma\delta$ T cells express this marker in peripheral blood (51).

Perhaps the most extensively studied example of $\gamma\delta$ T cells affording protection against parasitic disease is the case of malaria. Extensive work has been done that shows V γ 9 $\gamma\delta$ 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 $\gamma\delta$ T cell response (52). Elloso and coworkers (52) demonstrated that $\gamma\delta$ T cells specifically kill *Plasmodium* merozoites and this killing is dependent solely on the presence of $\gamma\delta$ 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 $\gamma\delta$ 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*. $\gamma\delta$ T cells from resistant cattle were shown to proliferate when stimulated *in vitro* with trypanosome antigens. Increased proliferation of some $\alpha\beta$ T cells was also seen in Trypano-resistant cattle; however, this proliferative response was much less vigorous than the proliferation of $\gamma\delta$ T cells. In contrast, negligible *in vitro* proliferative responses were seen in $\gamma\delta$ 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 $\gamma\delta$ T cells.

Lineage-specific markers of $\gamma\delta$ T cells

In the human and mouse systems, $\gamma\delta$ T cells are identified by expression of the $\gamma\delta$ TCR. Although $\gamma\delta$ T cells express a variety of other surface molecules, to date the TCR is the only known lineage-specific marker of human and mouse $\gamma\delta$ T cells. Conversely, ruminant $\gamma\delta$ 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 $\gamma\delta$ T cells. The T19 marker is now known as the WC1 family, and comprises a large group of related molecules found exclusively on $\gamma\delta$ T cells (58).

The WC1 marker is found only on ruminant and swine $\gamma\delta$ 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 $\gamma\delta$ T cell proliferation following the crosslinking of the WC1 molecule has also been published (64).

Other functionally important molecules on $\gamma\delta$ T cells

Some subsets of $\gamma\delta$ T cells have been shown to express functionally important molecules also found on $\alpha\beta$ T cells. $\gamma\delta$ T cells express CD3, and in the human the majority co-express CD2, CD5 and CD7 (65). In addition, small subsets of $\gamma\delta$ 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 $\gamma\delta$ T cells have been identified, one passing through the thymus and the other originating in the gut. Gut derived $\gamma\delta$ T cells express CD8 and comprise the majority of intraepithelial lymphocytes (51, 69, 70). However, unlike the CD8 $\alpha\beta$ heterodimer expressed on thymus-derived CD8⁺T cells, gut derived CD8⁺ $\gamma\delta$ T cells express a CD8 $\alpha\alpha$ 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).

$\gamma\delta$ T cell distribution

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

An intriguing characteristic of $\gamma\delta$ T cells is that they are found in different tissues and locations than $\alpha\beta$ T cells. The majority of $\alpha\beta$ T cells are found in secondary

lymphoid organs, whereas the majority of $\gamma\delta$ T cells are found in nonlymphatic sites such as the skin and mucosa. $\gamma\delta$ 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 $\gamma\delta$ 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 $\gamma\delta$ T cells (75, 76). The positioning of $\gamma\delta$ T cells at "portals of entry," such as skin and female reproductive tract, have prompted the idea that $\gamma\delta$ T cells are important in the first line of defense against invading pathogens (77). Indeed, the ability of the $\gamma\delta$ 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 $\gamma\delta$ T cells found at these sites are often composed of identical or very similar γ and δ TCR chains. For example, mouse $\gamma\delta$ 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 $\gamma\delta$ T cells are found in the blood, spleen, intestine, lung, liver, and mammary glands (reviewed in ref. 72).

This site-specific accumulation of $\gamma\delta$ 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 $\gamma\delta$ 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 $\alpha\beta$ 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 $\gamma\delta$ T cells leave the thymus "preprogrammed" to home to certain locations. This is well illustrated in the case of $V\gamma 5$ and $V\gamma 6$ $\gamma\delta$ T cells in the mouse. A wave of $V\gamma 5$ cells is known to leave the thymus and home to the epidermis at 14-17 days of fetal development. A second wave of $\gamma\delta$ T cells expressing $V\gamma 6$ 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 $V\gamma 5$ and $V\gamma 6$ 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 $\alpha 4 \beta 7$ integrin. Using Stamper Woodruff frozen section binding assays, it has been shown that anti- $\alpha 4 \beta 7$ function-blocking mAbs reduce lymphocyte binding 70%-90%. Similar inhibitory effects are seen in *in vivo* gut homing experiments (93). The role of $\alpha 4 \beta 7$ role in mucosal homing was proven when a nonmucosa binding lymphoma cell line was transfected with the $\alpha 4$ and $\beta 7$ genes. Following transfection, these cells avidly bound Peyer's patch high endothelial venules (HEV) (94). Later work demonstrated that the counter ligand for $\alpha 4 \beta 7$ 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 $\gamma\delta$ 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 $\gamma\delta$ T cells have also been shown to support the rolling of other neutrophils and bovine $\gamma\delta$ 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 $\gamma\delta$ 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, $\gamma\delta$ 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% $\gamma\delta$ T cells, whereas peripheral lymph nodes typically contain less than 6% $\gamma\delta$ T cells. This is in contrast to the bovine spleen, where $\gamma\delta$ T cells comprise a large proportion of the lymphocyte pool.

The bovine model holds several distinct advantages for the study of $\gamma\delta$ T cell homing and localization to different tissues and organs. The peripheral blood of young calves contains the highest proportion of $\gamma\delta$ T cells of any animal tested. Bovine $\gamma\delta$ T cells also express additional $\gamma\delta$ T cell lineage-specific markers. These characteristics, combined with the striking differences in the number of $\gamma\delta$ 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 $\gamma\delta$ T cells prompts three important questions. First, do TCR-defined $\gamma\delta$ T cell subsets exist in the cow as described in the mouse and human? Second, does organ-specific accumulation of $\gamma\delta$ T cells correlate to homing of specific subsets? Third, do $\gamma\delta$ T cell subsets differ in their expression of defined homing molecules, and is it possible to predict the migratory behavior of different $\gamma\delta$ 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 $\gamma\delta$ 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 $\gamma\delta$ T cell subsets occur in cattle. These results, describing the initial characterization of mAbs used to identify bovine TCR-defined $\gamma\delta$ T cell subsets, and $\gamma\delta$ T cell subset distribution, are contained in chapter two. Chapters three and four describe the direct attempt to define the adhesion profile of $\gamma\delta$ 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 $\gamma\delta$ T cell subsets defined by anti-T cell receptor for antigen antibodies. Immunology Letters, 64:39.

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CHAPTER 2

PREFERENTIAL TISSUE LOCALIZATION OF BOVINE $\gamma\delta$ T CELL SUBSETS
DEFINED BY ANTI-T CELL RECEPTOR FOR
ANTIGEN ANTIBODIESIntroduction

$\gamma\delta$ 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 $\gamma\delta$ T cells is that particular subsets, defined by their TCR, have been shown to localize in specific tissues. For example, mouse $\gamma\delta$ 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 $\gamma\delta$ T cell subsets are not completely known.

To test whether recruitment mechanisms are involved in the tissue-selective accumulation of $\gamma\delta$ 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 $\gamma\delta$ T cell population. Newborn calves offer useful animal models for studying $\gamma\delta$ T cells because 1) $\gamma\delta$ 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

outside antigenic influences, which alter the phenotype and behavior of circulating T cells. A shortcoming of the bovine model is that tissue-specific $\gamma\delta$ T cell subsets have not been well defined. Recent studies have started to address this shortcoming. A subset defined by a single C γ region has been found to predominate in the skin and a TCR-defined subset co-expressing CD8 has been described to predominate in the spleen and gut (10, 11).

Here, we expand upon the analysis of $\gamma\delta$ T cells in the bovine system by using three new anti- $\gamma\delta$ TCR-specific mAbs, GD3.8, GD197 and GD3.1, to distinguish four TCR-defined $\gamma\delta$ T cell subsets. These subsets specifically localized to various tissues and sites of inflammation, and responded differently to PHA stimulation. These data establish differences in $\gamma\delta$ T cell subsets relevant to proliferation and tissue accumulation.

Materials and Methods

Animals

Holstein calves were purchased from local producers and housed at the Montana State University large animal facilities at the Veterinary Molecular Biology Laboratory. Peripheral blood was collected into tubes by venipuncture and PBMCs were separated by centrifugation through Ficoll-Hypaque (Histopaque 1077; Sigma Chemical Co., St. Louis, MO). Bovine tissue samples were collected from animals upon necropsy.

Reagents

CACT61A is an IgM mAb that recognizes a pan epitope of the bovine $\gamma\delta$ TCR (12, 13). IL-A29 (American Type Culture Collection, Manassas, VA) is a mouse mAb that recognizes all molecularly characterized members of the Workshop Cluster 1 (WC1) family (a non TCR, lineage specific marker found on most bovine peripheral blood $\gamma\delta$ T cells). GD3.8, GD197, and GD3.1 mAbs were produced by immunization (i.p.) of BALB/c mice with 6×10^7 purified $\gamma\delta$ T cells in Gerbu adjuvant (Biotech, Poway, CA). Two subsequent immunizations were performed at 2 wk intervals. The mouse spleen was aseptically removed and spleen cells fused to SP2-0 hybridoma cells, as previously described (14). Hybridomas producing mAb that positively stained $\gamma\delta$ T cells by two-color flow cytometric (FCM) analysis were subsequently subcloned by limiting dilution and rescreened by FCM analysis. Monoclonal antibodies GD3.8 (pan TCR), GD197 (subset TCR), and GD3.1 (subset TCR) are characterized in this chapter.

Immunofluorescence staining and flow cytometric analysis

Immunofluorescence staining was carried out using standard protocols, as described earlier (15). For two-color analysis, fluorescein isothiocyanate (FITC)-conjugated mAbs were used after a conventional indirect stain of cells using phycoerythrin (PE)-labeled second stage (Jackson ImmunoResearch, West Grove, PA), as previously described (15). Cells were analyzed on a Becton Dickinson FACScan or FACSCalibur and data were collected from 10,000 cells and presented as contour plots.

Immunoprecipitation and cross-precipitation

$\gamma\delta$ T cells were purified to near homogeneity using E-selectin cDNA-transfected L cells, as previously described (16). Lymphocytes were labeled using NHS-LC-Biotin/PBS (550 mg/ml Pierce, Rockford, IL), as described earlier (15). Biotin-labeled $\gamma\delta$ T cell lysates were precleared using 5% rabbit serum and Protein G beads (Boehringer Mannheim Biochemicals, Indianapolis, IN) or anti-mouse IgM-agarose beads (Sigma, St. Louis, MO). Precleared lysate, in a volume of 100 μ l, was incubated with 15 mg of GD3.8, GD3.1, GD197, IL-A29 or CACT61A for 1 hr at room temperature. Antigen/antibody complexes were then precipitated with Protein G or anti-IgM beads overnight at 4°C. Antibody-bead complexes were collected through centrifugation and washed. Beads were mixed and boiled in standard 2X reducing or nonreducing sample buffer and run on an 8% SDS-PAGE gel and transferred to PVDF membrane (Bio-Rad, Hercules, CA). After transfer, membranes were washed briefly and then blocked with 7% BSA/TBST for 2 hr at RT. Streptavidin horseradish peroxidase conjugate (Amersham Life Science, Buckinghamshire, England) was diluted 1/5000 in sterile PBS and incubated with the membrane for 40 min. The membrane was

then washed extensively. ECL detection reagents were used according to manufacturer's recommendations (Amersham Life Science). Membranes were then exposed to X-OMAT film (Kodak, Rochester, New York), and the film developed.

Tissue collection/preparation

Lymphocytes were isolated from spleen and lymph nodes by finely mincing tissues with a razor blade. The samples were then dounced in a tissue homogenizer to release lymphocytes from the stromal elements. Cell homogenates were passed through Nytex fabric (Fairmont Fabrics, Hercules, CA) and the resulting single cell suspension was stained for FCM analysis. Lymph nodes were inflamed by injecting 500 units of mouse TNF α (Genentech, San Francisco, CA), which is also active on bovine lymphocytes, in 1 ml of sterile PBS directly into the subscapular or flank lymph node. Control nodes were injected with PBS alone. Four hours post injection, the calf was sacrificed and lymph nodes removed and prepared for FCM analysis, as described above.

Subset analysis of *in vitro* PHA-stimulated cells

Lymphocytes (5×10^6 /ml) were cultured in cRPMI (RPMI 1640, 10% FBS, 10 mM HEPES, 200 mM L-glutamine, with sodium pyruvate, nonessential vitamins, and Pen/Strep) with 1-6 mg/ml PHA (Sigma). Cultures were maintained at 100% humidity, 37°C and 10% CO₂. After 48 hrs of culture, cells were washed once and $\gamma\delta$ T cell subset composition determined by FCM analysis. Subset analysis of cells consisted of analyzing the entire lymphocyte population, as well as non-blast cells and blast cells. Blast cells were classified as those lymphocytes with increased forward- and side-light

scatter profiles when compared to the majority of lymphocytes in unstimulated controls. Non-blast cells were classified as those that did not increase in forward- and side-light scatter over unstimulated controls.

Statistical analysis

Results were analyzed using paired Student's *t* test. Significant *P* values are indicated.

Results

GD3.8, GD3.1, and GD197 bind specifically to $\gamma\delta$ T cells

MAb GD3.8 was found to bind exclusively to CACT61A (pan $\gamma\delta$ T cell) positive cells (Fig. 1A). No staining was seen on $\alpha\beta$ T cells, B cells, monocytes or neutrophils (data not shown). MAbs GD3.1 and GD197 reacted exclusively with CACT61A and GD3.8 reactive cells (data not shown and Fig. 1B and 1C). MAbs GD3.1 and GD197 recognized exclusive subsets of $\gamma\delta$ T cells, as well as an overlapping subset (Fig. 1D). MAbs GD3.1 and GD197 in combination did not react with all $\gamma\delta$ T cells stained with GD3.8 (data not shown). Using mAbs GD3.8, GD3.1 and GD197, four subsets of $\gamma\delta$ T cells were identified (Table 1). To determine the nature of the antigen recognized by GD3.8, GD3.1 and GD197, immunoprecipitation and cross-immunoprecipitation experiments were done. Biotin-labeled $\gamma\delta$ T cell detergent lysates were immunoprecipitated with GD3.8, GD197, GD3.1, CACT61A and IL-A29. CACT61A, GD3.8, GD197 and GD3.1 precipitated proteins of identical molecular mass under non-reducing conditions (80-87 kDa) and reducing conditions (37 and 47 kDa) (Fig. 2A and data not shown), correlating to the molecular mass of the bovine $\gamma\delta$ T cell receptor (7, 13). IL-A29 precipitated a protein of 220 kDa under both reducing and nonreducing conditions, as previously described (Fig. 2A and data not shown). In preclearing experiments, GD3.8 precleared proteins reactive with CACT61A, GD3.1, and GD197, whereas IL-A29-reactive proteins (WC1) were unaffected (Fig. 2B). These results demonstrate that GD3.8, GD197, and GD3.1 all recognize the same antigen as CACT61A, the bovine $\gamma\delta$ TCR.

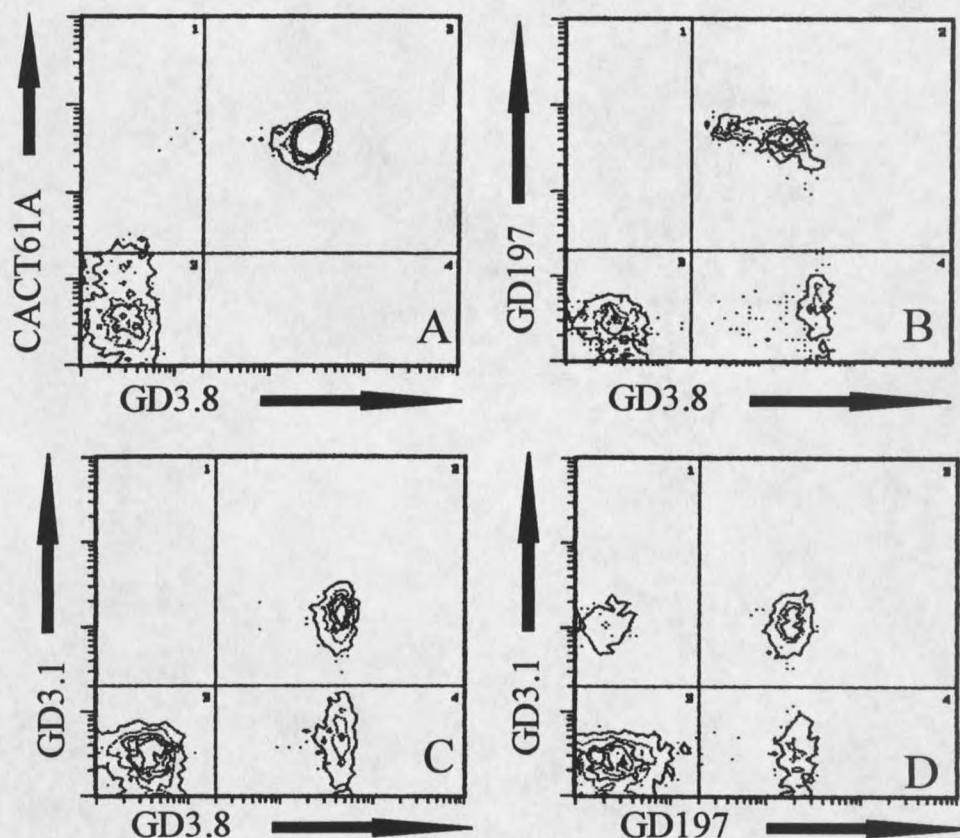


Figure 1

MAb GD3.8 recognizes bovine $\gamma\delta$ T cells; mAbs GD197 and GD3.1 recognize mutually exclusive as well as overlapping subsets of $\gamma\delta$ T cells. Two-color flow cytometric analysis was done as described in Materials and Methods. Panel A represents cells stained with GD3.8 (pan $\gamma\delta$ TCR) and CACT61A (pan $\gamma\delta$ TCR). Panel B represents cells stained with GD3.8 and GD197 (subset $\gamma\delta$ TCR). Panel C represents cells stained with GD3.8 and GD3.1 (subset $\gamma\delta$ TCR). Panel D represents cells stained with GD3.1 and GD197. The quadrants reflect the upper level of background fluorescence obtained with negative control antibodies. Data is representative from 10 repeats.

Splenic $\gamma\delta$ cells are predominantly subset 4 and express CD8

In surveys of bovine tissues, splenic $\gamma\delta$ T cells were found to express GD3.1 and GD197 much less frequently than $\gamma\delta$ T cells from peripheral blood (data not shown). This suggested a large number of cells falling into subset 4 (GD3.8+) preferentially

accumulate in the spleen. This was demonstrated by staining with both mAbs GD197 and GD3.1 in one channel of fluorescence and GD3.8 in the other, in a two-color FCM analysis. This staining procedure confirmed that a large percentage of the splenic $\gamma\delta$ T cells did, in fact, fall into subset 4. Of six calves tested, subset 4 $\gamma\delta$ T cells averaged 23% of the $\gamma\delta$ T cells in peripheral blood; however, in the spleen these cells represented 52% of the $\gamma\delta$ T cells. This difference was statistically significant ($P < 0.02$). Splenic lymphocytes were further analyzed by two-color FCM analysis to determine if CD8 expression correlated with any of the TCR-defined $\gamma\delta$ T cell subsets. Staining with GD3.8 showed approximately 50% of splenic CD8 T cells also expressed the $\gamma\delta$ TCR, confirming earlier reports that $\gamma\delta$ T cells make up a large percentage of the total CD8-positive T cell population in the spleen (11). Approximately, 85% of the CD8-positive splenic $\gamma\delta$ T cells fell into subset 4 (data not shown).

Table 1

The GD3.8, GD3.1, and GD197 mAbs define four different $\gamma\delta$ T cell subsets

MAb	Staining		
	GD3.8	GD3.1	GD197
Subset 1	+	+	-
Subset 2	+	-	+
Subset 3	+	+	+
Subset 4	+	-	-

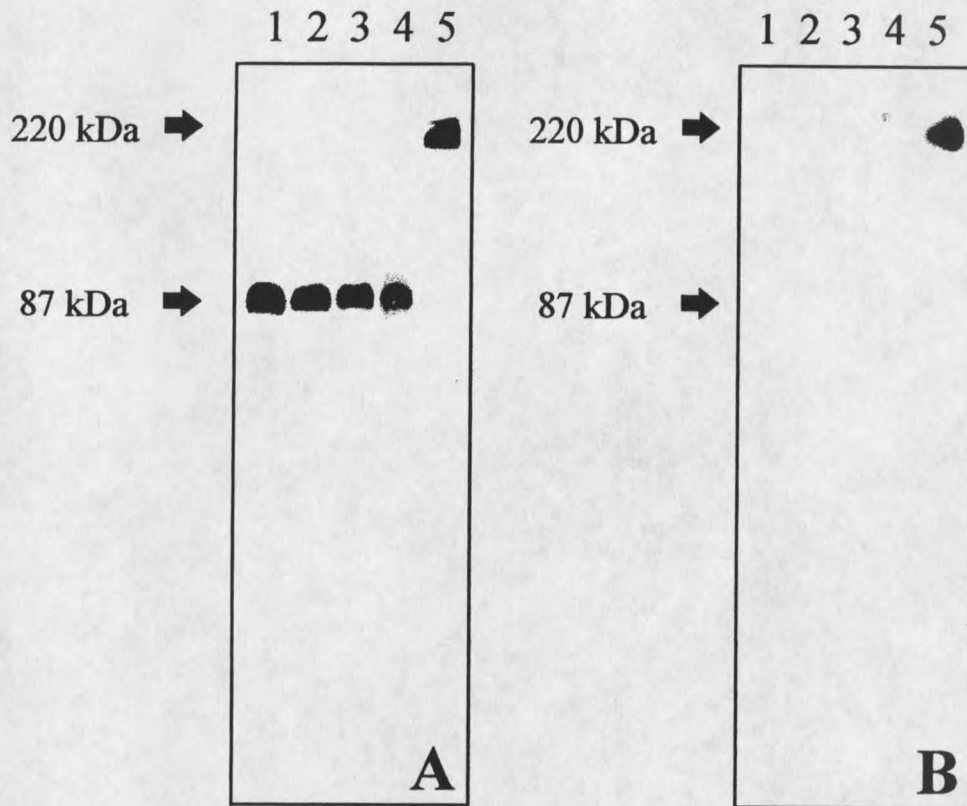


Figure 2

GD3.8, GD3.1, GD197 and CACT61A immunoprecipitate similar 80-87 kDa molecules when analyzed under nonreducing conditions. Immunoprecipitation analyses were performed as described in Materials and Methods. In panel A, biotin-labeled $\gamma\delta$ T cell detergent lysates were immunoprecipitated with GD3.8, GD197, GD3.1, CACT61A, and IL-A29, and individual immunoprecipitates were run on a non-reducing 8% SDS-PAGE gel, which are shown in lanes 1-5, respectively. In panel B, the biotin-labeled detergent lysates were precleared with GD3.8, as described in Materials and Methods, prior to immunoprecipitation with GD3.8, GD197, GD3.1, CACT61A, and IL-A29, which are represented in lanes 1-5, respectively.

Subset 1 cells accumulate in some sites of inflammation

All uninflamed tissues studied showed greater numbers of GD197-reactive cells than GD3.1-reactive cells. Conversely, in various sites of inflammation, this ratio was reversed. As an illustration of these results, four hours following the injection of $\text{TNF}\alpha$, peripheral lymph nodes contained approximately the same ratio of $\gamma\delta$ T cells as control nodes (8.1% and 8.8%, respectively). The ratio of GD197 and IL-A29-positive cells to GD3.8-positive cells remained constant as well. However, the ratio of GD3.1-positive cells in the $\text{TNF}\alpha$ -inflamed nodes was consistently higher (Table 2). This increase in the number of GD3.1-positive cells in inflamed nodes over control nodes was statistically significant ($P < 0.05$). These results suggested that cells stained with mAb GD3.1 were specifically retained in, or recruited to, inflamed lymph nodes. The percentage of GD197-positive cells did not increase in the inflamed nodes. This indicated that either a selective accumulation of subset 1 cells (GD3.8⁺, GD197⁺, GD3.1⁺) had occurred or, a selective increase of subset 3 (GD3.8⁺, GD197⁺, GD3.1⁺), in concert with a selective decrease of subset 2 (GD3.8⁺, GD197⁺, GD3.1⁻) had occurred. Importantly, in either scenario, there appeared to be a shift in the subset composition of $\gamma\delta$ T cells in inflamed lymph nodes.

Effect of mitogen stimulation on $\gamma\delta$ T cell subsets

To determine whether mitogen stimulation could drive the selective expansion of one or more of the $\gamma\delta$ T cell subsets, perhaps mimicking what occurs in the spleen or inflamed tissues, mixed lymphocyte populations were treated with 1-6 $\mu\text{g/ml}$ of PHA, cultured for 48 hrs and then analyzed by flow cytometry. Subset analysis of the blast cell population, identified by its distinctive forward- and side-light scatter profiles,

showed that little change occurred in the frequency of the four $\gamma\delta$ T cell subsets identified by GD3.1, GD197, and GD3.8 (Fig. 3A). Surprisingly, when the non-blast cells were analyzed, subset 4 increased in percentage as a function of the dose of PHA (Fig. 3B). In fact, following treatment with 5 $\mu\text{g/ml}$ of PHA, the percentage of subset 4 increased in the non-blast cell population by almost 300%.

Table 2

Comparison of the staining of mAbs GD3.8, GD3.1, GD197, and IL-A29 in control and $\text{TNF}\alpha$ -inflamed lymph nodes

Tissue	Percent staining of each antibody ^a			
	GD3.8	GD3.1	GD197	IL-A29
PBS node (%)	100	44 $\pm$ 6.3*	57 $\pm$ 4.7	56 $\pm$ 4.0
$\text{TNF}\alpha$ node (%)	100	62 $\pm$ 5.7*	60 $\pm$ 3.0	55 $\pm$ 4.6

^aLymph nodes were injected with PBS alone or $\text{TNF}\alpha$ in PBS. Numbers represent the percentage of $\gamma\delta$ T cells stained as a function of total $\gamma\delta$ T cells $\pm$ S.E. of the mean. Data represents the averages from six calves tested.

An asterisk (*) denotes statistical significance at $P < 0.05$ for the difference between PBS and $\text{TNF}\alpha$ nodes.

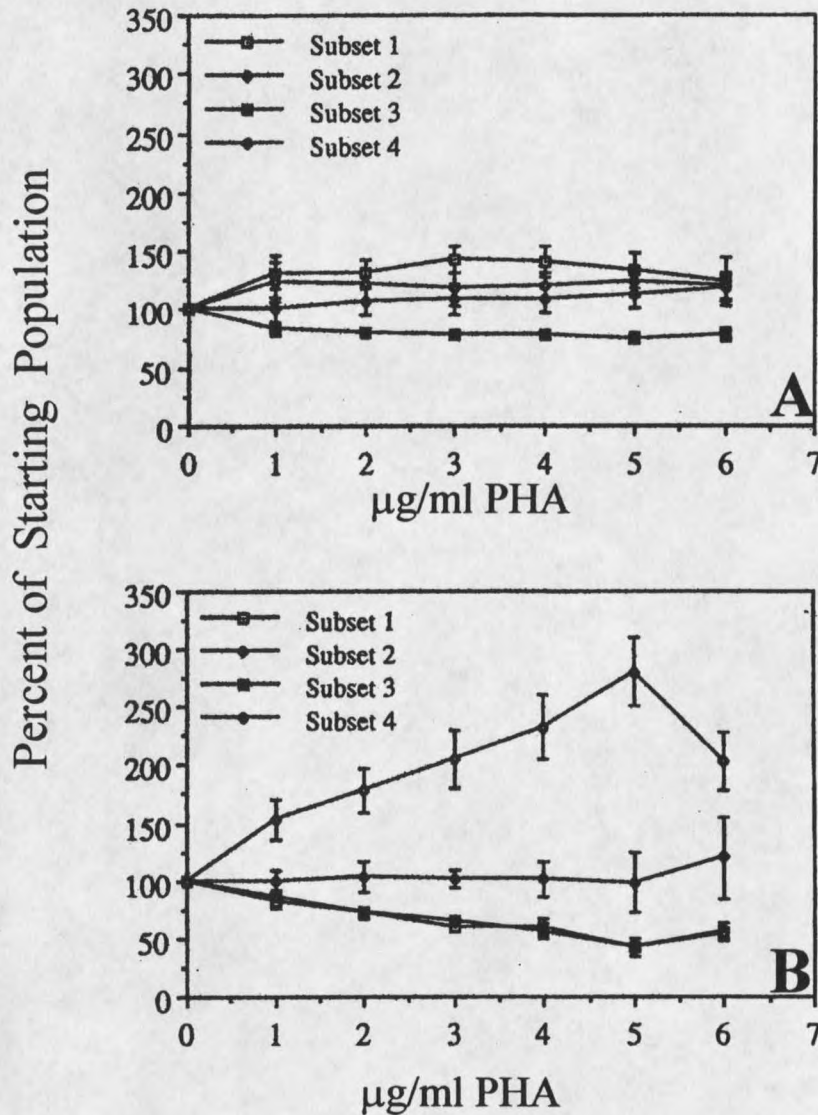


Figure 3

Subset 4 cells are resistant to blast formation in PHA-stimulated cultures. Fig. 3A and 3B show subset analysis of blast and non-blast cells respectively, following 48 hrs of culture in different concentrations of PHA, as described in Materials and Methods. Blast cells were identified by an increase in forward- and side-light scatter over unstimulated controls. Y axis shows the percent of the starting population of each subset, while the X axis shows the µg/ml of PHA used in the 48 hr PBMC cultures. Graphs represent the average of four repeats and error bars show the standard error of the mean.

Discussion

$\gamma\delta$ T cells operate differently from $\alpha\beta$ T cells in many ways. For instance, in humans and mice it has been shown that $\gamma\delta$ T cells, which localize in normal and inflamed tissues, are often nonpolymorphic in their TCR representation, and distinct TCR-defined $\gamma\delta$ T cell subsets predominate in specific tissues (4-6). The mechanisms involved in $\gamma\delta$ T cell tissue accumulation and retention are currently being studied. However, the study of $\gamma\delta$ T cells is difficult because these cells represent a small minority of the T cell population in the peripheral blood of mice and humans. On the other hand $\gamma\delta$ T cells can represent the majority of the T cell population in the peripheral blood of young calves.

In the past, a major limitation of the study of bovine $\gamma\delta$ T-cells has been the lack of reagents for immunoprecipitation, flow cytometric, and immunohistological analyses. This chapter characterizes new mAbs that add unique reagents to the earlier panels which have recently been described (12, 13, 17, 18). Using these mAbs, four $\gamma\delta$ T cell subsets based on TCR expression were phenotypically distinguished. The primary objective of this study was to determine if bovine $\gamma\delta$ T cells localized based on TCR usage to specific tissues, as has been shown in mice and humans (1, 2, 5, 19-22), using these newly characterized mAbs. Subsets 2 and 3 (GD3.8⁺, GD197⁺, GD3.1⁻ and GD3.8⁺, GD197⁺, GD3.1⁺, respectively) make up the major population in peripheral blood and in most uninflamed tissues, such as the skin and lymph nodes. Subset 4 (GD3.8⁺ GD197⁻, GD3.1⁻), which constitutes a minor population in the blood, comprises over 50% of the $\gamma\delta$ T cells in the spleen. Following TNF α injection into lymph nodes, a reversal of the frequency of GD3.1⁺ to GD197⁺ cells occurs, which is potentially due to the selective accumulation of subset 1 (GD3.8⁺, GD197⁻ GD3.1⁺).

This suggests that these cells may represent an inflammation-specific population: one selectively recruited to, or retained in, sites of inflammation. In support of this latter finding, selective accumulation of subset 1 also occurs in tissues of a protozoal (*Cryptosporidium parvum*-infected gut) disease model (Mitchell Abrahamson, unpublished results).

Of cells that respond to PHA and proliferate (blast cells) in an *in vitro* mitogen-induced proliferation assay, no change in the levels of the four $\gamma\delta$ T cell subsets from time zero through day two was evident. In contrast, some subset 4 cells have a unique resistance to blast cell formation, which results in a significant increase in the percentage of these cells in the resting lymphocyte population following PHA treatment. The physiological significance of this observation is unclear, but it illustrates a dramatic functional difference in the ability of this subset to respond to PHA cross-linking of the TCR. A similar functional distinction of a $\gamma\delta$ T cell subset has not been previously defined in any system.

Currently, studies are underway using these new, TCR-specific, markers to further define the functional properties of bovine $\gamma\delta$ T cells. For example, it can now be determined whether molecular mechanisms responsible for T cell recruitment (expression of homing receptors, for example) or retention (expression of adhesion molecules for extracellular matrices, for example) within tissues are differentially expressed on the different subsets. These mAbs can be used to sort cells to study their cytokine production profiles or other functional activities.

In summary, using these newly described $\gamma\delta$ T cell TCR-specific mAbs has enabled the characterization of four distinct subsets of $\gamma\delta$ T cells. The characterization of these subsets has shown that their accumulation and blast response to PHA are regulated independently. Importantly, these results show that TCR-defined subsets are not simply cells composed of different TCR gene products. Rather, bovine TCR-defined $\gamma\delta$ T cell

subsets may be functionally different and exhibit distinct homing and recirculation patterns as previously demonstrated in the murine and human systems.

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CHAPTER 3

A CIRCULATING BOVINE $\gamma\delta$ T CELL SUBSET, WHICH IS FOUND IN LARGE
NUMBERS IN THE SPLEEN, ACCUMULATES INEFFICIENTLY IN AN
ARTIFICIAL SITE OF INFLAMMATION: CORRELATION WITH LACK OF
EXPRESSION OF E-SELECTIN
LIGANDS AND L-SELECTIN

Introduction

Though considerable effort has been devoted to the study of $\gamma\delta$ T cells, there are many aspects of this population that are still poorly understood. One intriguing attribute which has come from studies in mice and humans, is that discrete subsets of $\gamma\delta$ T cells, defined by their TCR usage, specifically accumulate in certain tissues and organs of the body. For example, mouse $\gamma\delta$ T cells associated with epidermis (1, 2) and the mucosal epithelia of vagina, uterus, and tongue (3) utilize a TCR repertoire consisting of distinct pairs of γ and δ gene products. What accounts for this TCR-restricted, tissue-specific $\gamma\delta$ T cell accumulation is not known. One study suggests that some $\gamma\delta$ T cell subsets exhibit subtle differences in the expression of homing and recruitment-associated molecules (4), yet the *in vivo* relevance of these observations has not been shown.

Ideally, to evaluate recruitment mechanisms, circulating cells should be examined. This is technically difficult in mice, because of their small blood volume, and

in adult humans, due to their minimal number of $\gamma\delta$ T cells (5). In contrast, young cattle have elevated numbers of $\gamma\delta$ T cells in circulation which are easily isolated for study (6-8). Furthermore, newborn calves can be used, thus minimizing complications which follow antigenic stimulation of T-cells, such as the generation of activated/memory cells. The shortcomings in studying bovine $\gamma\delta$ T cells are 1) reagents defining subsets based on TCR usage are only now being developed, and 2) until recently, there has been no evidence that bovine $\gamma\delta$ T cell subsets exhibit differential tissue tropism. These shortcomings have recently been addressed. For example, it has been shown that bovine $\gamma\delta$ T cells can be divided into subsets based on TCR usage, as well as by the expression of other surface markers, and that at least some of these subsets differentially accumulate in certain tissues (9-12). Specifically, a TCR-associated population, which is also characterized by CD2 and CD8 co-expression, is a minor subset of circulating cells, but is the predominant population in the spleen (10, 12).

We have previously shown that most circulating bovine $\gamma\delta$ T cells avidly bind selectins (13), molecules important in the initial step of migration into a variety of tissues, including skin, lymph nodes, and Peyer's patches (14-16). There have been no reports suggesting that selectins are important in the accumulation of T cells in the spleen. This dissertation describes a circulating $\gamma\delta$ T cell subset defined by CD8 co-expression that does not efficiently accumulate in an extra-lymphoid site of inflammation, whereas CD8-negative $\gamma\delta$ T cells do. The lack of accumulation to inflammatory tissue correlates with inefficient binding of this subset to E-selectin and the recently described bovine GR antigen. This subset is also largely deficient in L-selectin expression. This is the first demonstration of a $\gamma\delta$ T cell subset which exhibits a defined tissue tropism, having a unique adhesion molecule expression profile.

Materials and Methods

Animals

Holstein calves were purchased from local producers and housed at the Montana State University Large Animal Facilities at the Veterinary Molecular Biology Laboratory. Bovine tissue samples were collected from animals upon necropsy.

Reagents

IL-A29 (American Type Culture Collection, Rockville, MD) is a mouse mAb that recognizes all molecularly characterized members of the Workshop Cluster 1 (WC1) family (17). GD3.8, GD197, and GD3.1 are mouse anti-TCR mAbs that define four bovine $\gamma\delta$ T cells subsets (12). GD3.5 is a mouse mAb that recognizes a $\gamma\delta$ lineage-specific molecule found on more than 90% of circulating bovine $\gamma\delta$ T cells (18). GR284 is a mouse mAb that binds to the GR antigen, similar to the previously described GR113 (19). DREG 56 is a mouse mAb against human L-selectin that cross-reacts with bovine L-selectin (20). CC58 (anti-CD8) and CC42 (anti-CD2) (21) are mouse mAbs provided by Chris Howard (Institute for Animal Health, Compton, UK). PE-conjugated anti-human IgG, PE- and FITC-conjugated anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA), and avidin-conjugated CyChrome (PharMingen, San Diego, CA) were used. Porcine E-selectin chimera (P11.4) was kindly provided by M. Robinson (Celltech Therapeutics Ltd., Berkshire, UK) and used as previously described (22). EL86.10 is a mouse mAb that binds human E-selectin and was used as an isotype matched negative control.

Lymphocyte preparation, E-selectin binding assays, and flow cytometric analysis

Peripheral blood was collected into sodium heparin anticoagulant tubes by venipuncture and PBMC were purified by Ficoll-Hypaque (Sigma Diagnostics, St. Louis, MO) gradient centrifugation. In some assays, purified mononuclear cells were incubated for 30 min at 37°C in T-175 flasks (Nunc, Naperville, IL) containing DMEM (Sigma Diagnostics) to remove monocytes. The nonadherent lymphocyte population was washed in HBSS (Life Technologies, Grand Island, NY) prior to use in the functional and flow cytometric assays. Two approaches were used to measure E-selectin binding, as described in previous reports (13,22). Briefly, in the first method the adhesion of bovine lymphocytes to E-selectin cDNA transfected mouse L cells was measured (13). This assay has been well characterized and lymphocyte binding has been shown to be specifically mediated via the transfected E-selectin molecule, with minimal lymphocyte binding to nontransfected control cells (13). In this method, E-selectin binding cells were purified from whole lymphocyte preparations by incubation in T-175 flasks coated with 7×10^6 adherent E-selectin cDNA L cell transfectants. Unbound cells were discarded, and bound lymphocytes were released by treatment with 2 mM EDTA/PBS (13). The procedure was then repeated a second time, and samples of cells before the adhesion steps and after each EDTA/PBS wash were analyzed by flow cytometry. In the second approach, an E-selectin/immunoglobulin chimera was used in flow cytometric assays to study the phenotype of the selectin binding population (22). In previous studies we showed that a porcine E-selectin/human IgG chimera worked well in flow cytometric assays of bovine $\gamma\delta$ T cells (22). In this assay, peripheral blood lymphocytes were incubated for 1 hr on ice with supernatant fluids from CHO transfectants producing the E-selectin chimera. Binding of the chimera was revealed by

a PE-labeled anti-human IgG second stage antibody. Specificity controls have included irrelevant chimeras (human CD4/IG), EDTA treatment, and function blocking mAbs to reverse or block chimera binding (22, 23). In this dissertation, chimera binding was combined with three-color flow cytometric analysis, as described below.

GR antigen binding of $\gamma\delta$ T cells was determined by incubating PBMC in platelet-derived, soluble GR antigen, as previously described (19). Briefly, bovine platelets were collected and activated with thrombin for 4 hr at 37°C, debris was removed by high speed centrifugation and the clarified supernatant fluid was used as a source of GR antigen. Bovine PBMC were incubated in platelet supernatant fluid for 30 min at 37 °C. After the incubation period, the treated leukocytes were washed and stained for flow cytometric analysis to detect bound GR antigen and determine the phenotype of the binding population.

Flow cytometric analysis was performed as follows. Single-color analyses were done with the mAb reagents and E-selectin chimera, as previously described (22). The following procedure was followed for three-color flow cytometric analysis of cells. Bovine PBMC were resuspended in HBSS containing Ca^{++} Mg^{++} (HBSS- $\text{Ca}^{++}\text{Mg}^{++}$) at 1×10^7 cells/ml. Biotin-labeled GD3.8 (anti-pan $\gamma\delta$ T cell mAb) was added along with the E-selectin chimera. The cells were incubated on ice for 1 hr, washed in PBS containing 5% horse serum (Sigma Diagnostics) (PBS-HS), and resuspended in HBSS- $\text{Ca}^{++}\text{Mg}^{++}$. The cells were then incubated with FITC-conjugated anti-CD8, avidin-CyChrome and PE-labeled anti-human IgG (preabsorbed and exhibiting minimal reactivity with bovine and mouse Ig). After 30 min on ice, the cells were washed in PBS-HS and then analyzed using a BD FACSCalibur (Becton Dickinson Immunocytometry Systems, Mountain View, CA). FL1 (FITC), FL2 (PE), and FL3 (CyChrome) detectors were used and the FACSCalibur was calibrated using Calibright beads (Becton Dickinson). Compensation was set manually using single-color stains of

the various fluorochromes. In assays where it was necessary to analyze small subsets of cells, live gate acquisition was utilized to acquire sufficient cell numbers for statistical analysis. Data from up to 20,000 cells was acquired and reported in two-dimensional dot plots or histograms. Negative controls included 1) single-color stains, 2) irrelevant isotype-matched antibody stains, and 3) second-stage reagent controls. Experiments were done to ensure that the anti-human Ig second stage reagents did not cross-react with the mouse mAbs and that the CyChrome second stage reagent was specific for the biotin-labeled GD3.8 mAb. Marker placement for statistical analysis was determined by placing the marker outside the upper limit of background staining of control antibodies. There was no evidence that the CyChrome reagent bound nonspecifically, as previously described for some reagents conjugated to this fluorochrome (24, 25).

Generation of inflammatory populations of bovine lymphocytes

Experiments were carried out to determine the phenotype of $\gamma\delta$ T cells which entered an extra-lymphoid site of inflammation. The following method was used to generate a fluid-phase inflammatory population of leukocytes, which could be easily sampled and analyzed by flow cytometry. Briefly, plastic balls (4 cm diameter) with multiple holes (wiffle balls) were sterilized by autoclaving and surgically inserted under the dermis of one month old calves. The balls became encapsulated within one week and for the next three weeks the center of the balls remained as a fluid which could be sampled by a hypodermic needle and syringe. To induce an overt leukocyte response in the wiffle balls PHA alone (250 μ g/ml), a PHA-stimulated lymphocyte supernatant fluid, or LPS (10 μ g/ml) was injected into the fluid filled balls 10 days post surgery. Fluid was withdrawn from the site 24 hrs following injection of the inflammatory agents. Previous studies have shown that this method leads to the accumulation of all leukocyte types,

including $\gamma\delta$ T cells, into the inflammatory site (Wilson E., unpublished results). PBMC in the fluid were separated by centrifugation through Ficoll-Hypaque and analyzed by flow cytometry.

Statistical analysis

Results were analyzed using paired Student's *t* test. Significant *P* values are indicated.

Results

Identification of the CD8-positive $\gamma\delta$ T cell subset

Previous studies have shown that the major population of $\gamma\delta$ T cells in the bovine spleen express CD8 and CD2 (10, 12); whereas these markers are found on only a minor subset of $\gamma\delta$ T cells in the blood (ranging from 2-31%, mean 5%). We have also characterized mAbs against $\gamma\delta$ TCRs that help further define this population of cells (12). As shown in Figure 1, the majority of GD3.1 and GD197 (TCR subset-specific anti- $\gamma\delta$ T cell mAbs) staining cells did not co-express CD8. However, approximately 50% of the GD197- and GD3.1-negative $\gamma\delta$ T cells expressed CD8. Thus, most of these cells have a unique TCR profile.

The CD8⁺ $\gamma\delta$ T cells also expressed CD2, but lacked expression of the putative homing molecules WC1 and GD3.5 (Fig. 2). For simplicity, this unique subset of $\gamma\delta$ T cells will be referred to as CD8⁺ $\gamma\delta$ T cells throughout the rest of this chapter.

CD8⁺ $\gamma\delta$ T cells do not accumulate in appreciable numbers at sites of inflammation

To determine if CD8⁺ $\gamma\delta$ T cells accumulate in inflammatory tissues, plastic wiffle balls were inserted under the skin of a calf, allowed to become encapsulated, and then inflammatory reactions were induced with PHA, a PHA/lymphocyte supernatant fluid, or LPS, as described in the Materials and Methods. The advantage of this method is that the center of the ball remains fluid, and can be sampled with a hypodermic needle and syringe. The cells can then be stained for multi-color flow cytometric analyses, which are required to identify CD8⁺ $\gamma\delta$ T cells in a mixed population. Phenotypic analyses of

the $\gamma\delta$ T cells in inflammatory lesions showed that the frequency of $CD8^+\gamma\delta$ T cells was far higher in the peripheral blood (Fig. 3A) than in the inflammatory fluid (Fig. 3B). These results show that $CD8^+\gamma\delta$ T cells have a greatly reduced capacity to enter into these artificial sites of inflammation. Perhaps just as dramatic, $\gamma\delta$ T cells that lacked CD8 were preferentially found in the inflammatory site. Thus, the accumulation patterns of two different $\gamma\delta$ T cell subsets correlate with either expression of or lack of CD8.

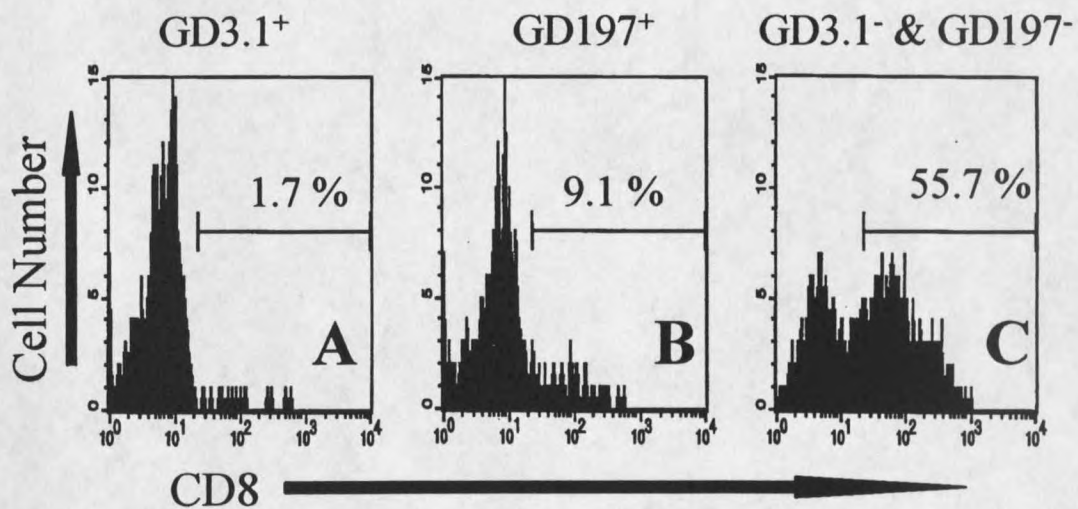


Figure 4

$CD8^+\gamma\delta$ T cells are restricted to a TCR-defined subset of $\gamma\delta$ T cells. Three-color flow cytometry was done using the GD3.1, GD197, and GD3.8 anti- $\gamma\delta$ T cell TCR specific mAbs versus anti-CD8, as described in the Materials and Methods. Histograms were generated by gating on both $\gamma\delta$ T cells, as determined by mAb GD3.8 staining (FL3), and the subset markers indicated above each panel (FL2). CD8 expression of each cell population is shown in the X axis (FL1). Values shown in each panel represent the percent of cells co-expressing CD8 as determined by the indicated marker reflecting the upper level of FL1 background fluorescence. Data are representative of five individual experiments.

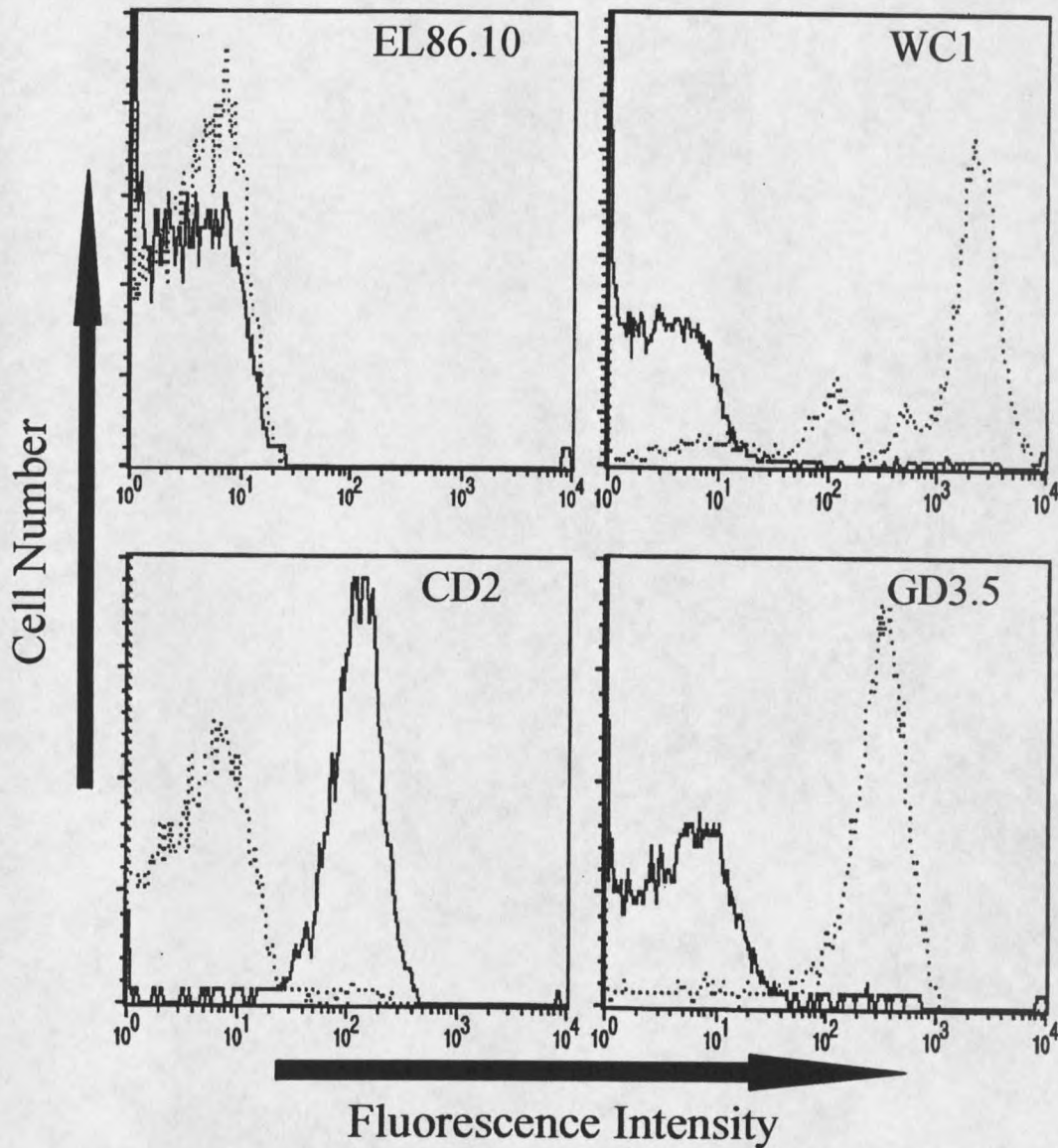


Figure 5

Expression of CD2, WC1 and GD3.5 antigen on CD8⁺ and CD8⁻ γδ T cells. Three-color flow cytometric analysis of γδ T cells was performed, as described in Materials and Methods. In all plots, dashed lines represent gated CD8⁻ γδ T cells. Solid lines represent CD8⁺ γδ T cells. Each histogram represents the FL2 channel fluorescence with the marker indicated in the upper right corner. Data are representative of five individual experiments.

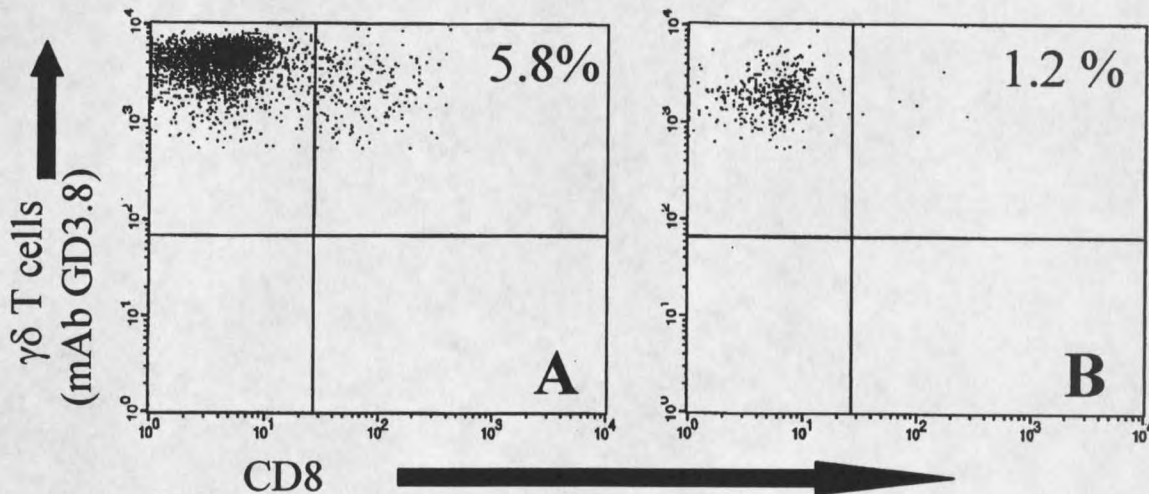


Figure 6

CD8⁺γδ T cells do not efficiently enter sites of inflammation. Two-color flow cytometric analysis was performed on γδ T cells obtained from: A) peripheral blood and B) PHA-induced fluid phase inflammatory lesion, as described in Materials and Methods. The percent of CD8⁺γδ T cells in each population is listed in the upper right quadrant. Plots show gated γδ T cells as determined by a positive stain with mAb GD3.8. Data are representative of three individual experiments.

The CD8⁺γδ T cell subset does not efficiently bind E-selectin or GR antigen and has low, yet heterogeneous, expression of L-selectin

E- and L-selectin are adhesion molecules important in directing lymphocytes to lymph nodes and sites of inflammation. L-selectin is expressed by the lymphocyte,

whereas E-selectin is expressed by endothelial cells. In previous studies, we found that up to 90% of the circulating $\gamma\delta$ T cells in most calves avidly bind E-selectin and are L-selectin positive (13, 26). The GR antigen is expressed on chronically inflamed endothelium and bovine platelets, and is believed to function as a homing molecule for bovine $\gamma\delta$ T cells, the majority of which express ligands for GR antigen (19). Experiments were performed to determine if the inability of CD8 $^{+}\gamma\delta$ T cells to accumulate in inflammatory lesions correlated with a low expression of L-selectin or interactions with E-selectin and the GR antigen.

Two approaches were used to examine the binding interaction of $\gamma\delta$ T cells with E-selectin. In the first assay, mixed populations of bovine lymphocytes were sequentially passed over monolayers of E-selectin transfected L cells. After each adhesion step, the monolayers were washed to remove nonadherent cells and the adherent cells were released for flow cytometric analysis by incubation in EDTA/PBS to reverse the selectin-mediated binding event. The frequency of CD8 $^{+}\gamma\delta$ T cells was dramatically reduced after only one adhesive interaction with monolayers of E-selectin expressing L cells, and then remained constant during successive adhesion steps (Fig. 4). Reciprocal experiments were also done to determine if non-adherent cells were enriched for CD8 $^{+}\gamma\delta$ T cells. Mixed populations of bovine lymphocytes were sequentially passed over monolayers of E-selectin transfected L cells and the non-adherent cells collected after each round of binding. As expected, these experiments produced a cell population enriched for CD8 $^{+}\gamma\delta$ T cells (data not shown). This suggested that the majority of CD8 $^{+}\gamma\delta$ T cells did not efficiently bind E-selectin whereas CD8 $^{-}\gamma\delta$ T cells did bind.

Three-color flow cytometric analysis using an E-selectin/Ig chimera, $\gamma\delta$ T cell-specific mAbs, and anti-CD8 was then done to directly examine the phenotype of the E-selectin binding population. CD8 $^{+}\gamma\delta$ T cells represented a minor population in the

peripheral blood (approximately 5% of the total $\gamma\delta$ T cell pool). Interestingly, few of these cells were stained by the E-selectin/Ig chimera, whereas most of the CD8 $\gamma\delta$ T cells were stained (Fig. 5A). Pooled data from multiple experiments show a significant difference ($P < 0.01$) in the capacity of CD8 $^{+}$ versus CD8 $\gamma\delta$ T cells to bind E-selectin (Fig. 5B).

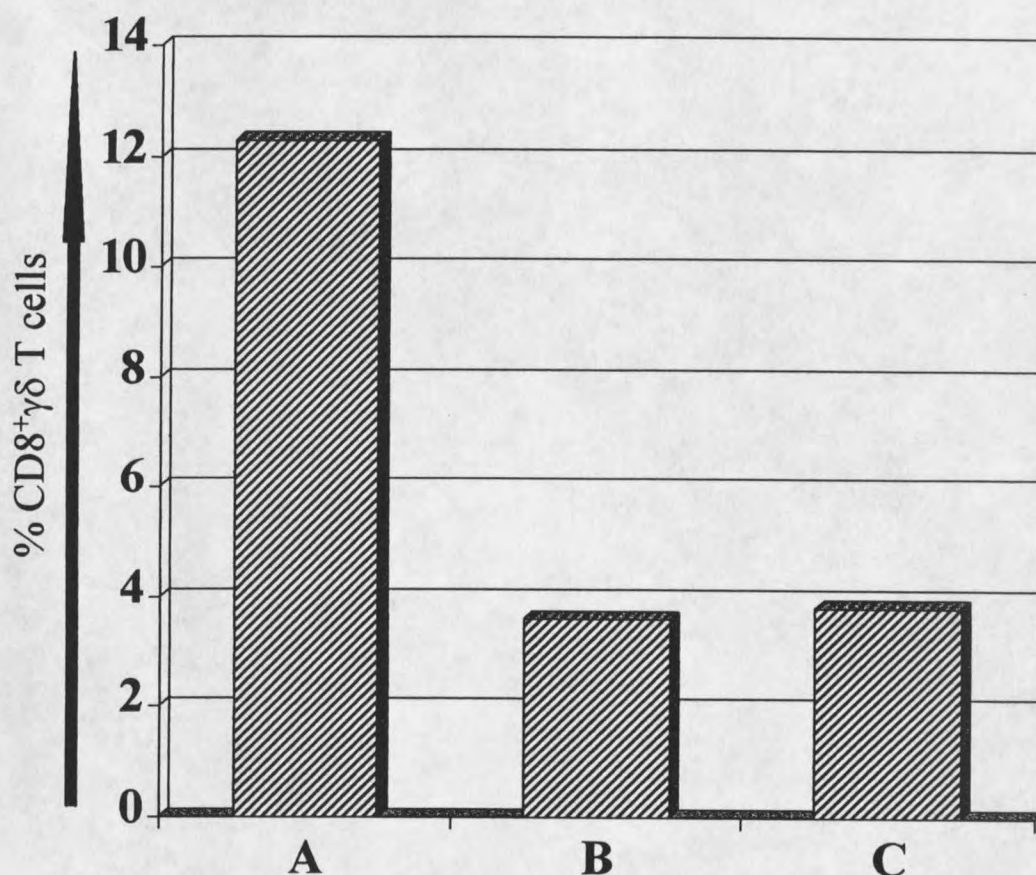


Figure 7
 CD8 $^{+}$ $\gamma\delta$ T cells do not efficiently bind E-selectin transfectants. Percentages of CD8 $^{+}$ $\gamma\delta$ T cells in the lymphocyte populations are shown from a representative experiment: (A) represents the percentage of CD8 $^{+}$ $\gamma\delta$ T cells in the non-adherent lymphocyte population after 30 min of incubation on plastic to remove macrophages and other non-specifically adherent cells; (B) represents the percentage of CD8 $^{+}$ $\gamma\delta$ T cells in the same lymphocyte population after one round of adherence to E-selectin transfected monolayers; (C) represents the percentage of CD8 $^{+}$ $\gamma\delta$ T cells in the same lymphocyte population after two rounds of adherence to E-selectin-transfected monolayers as described in Materials and Methods. Data are representative of five individual experiments.

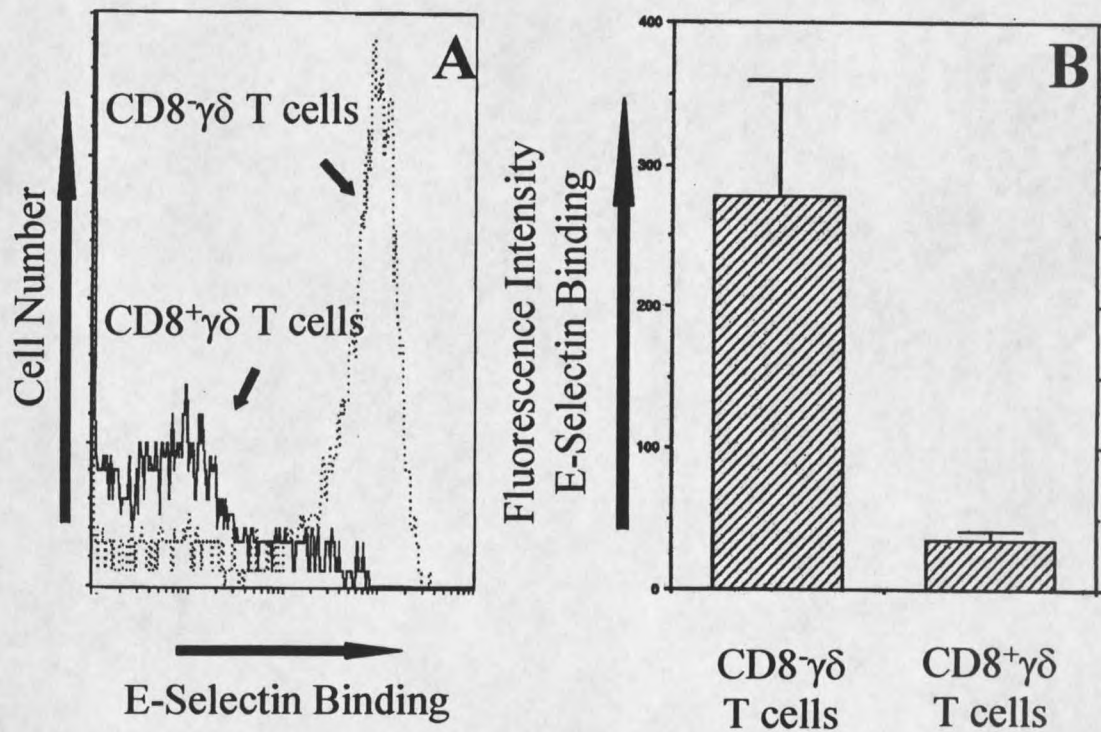


Figure 8

$CD8^{+}\gamma\delta$ T cells do not efficiently bind E-selectin chimera. Three-color flow cytometric analysis was performed, as described in Materials and Methods. Panel A represents E-selectin chimera staining of $CD8^{-}\gamma\delta$ T cells and $CD8^{+}\gamma\delta$ T cells, and panel B represents data pooled from five experiments. Y axis of graph shows mean fluorescence intensity. Error bars represent standard error of the mean.

Flow cytometry was also used to study other molecules important in the trafficking of $\gamma\delta$ T cells to sites of inflammation. Three-color analysis was done to determine the expression of L-selectin on $CD8^+$ and $CD8^- \gamma\delta$ T cells, and the ability of these cells to bind the GR antigen. Heterogeneous expression of L-selectin was observed on $CD8^+ \gamma\delta$ T cells; the majority of these cells expressed variable amounts of L-selectin. Conversely, L-selectin expression was uniformly high on $CD8^- \gamma\delta$ T cells (Fig. 6A). Analysis of GR antigen ligands, via an assay in which lymphocytes bind soluble GR antigen, showed that the GR antigen binding follows the same general pattern as L-selectin expression. These results indicate that very few $CD8^+ \gamma\delta$ T cells expressed GR antigen ligands, whereas $CD8^- \gamma\delta$ T cells showed high expression of the ligands (Fig. 6B).

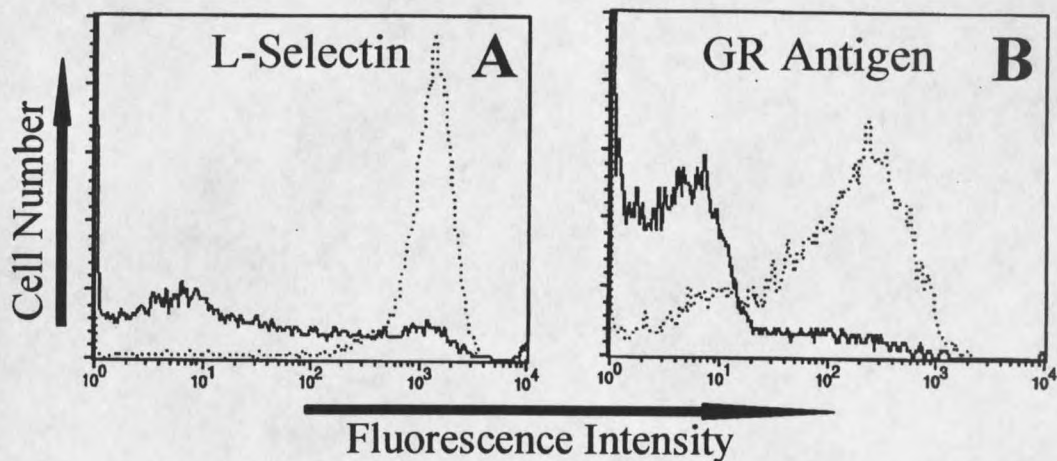


Figure 9

$CD8^+ \gamma\delta$ T cells express low, yet heterogeneous, levels of L-selectin and GR antigen ligand. Three-color FACS analysis was performed as described in Materials and Methods. Panel A shows anti-L-selectin staining of $CD8^+$ and $CD8^- \gamma\delta$ T cells. Panel B shows GR antigen ligand expression on $CD8^+$ and $CD8^- \gamma\delta$ T cells. In all plots, dashed lines represent gated $CD8^- \gamma\delta$ T cells. Solid lines represent $CD8^+ \gamma\delta$ T cells. Histograms are representative of five experiments.

Discussion

One of the fascinating aspects of $\gamma\delta$ T cells is their unique tissue tropism. Like human and murine $\gamma\delta$ T cells, bovine $\gamma\delta$ T cells can be separated into TCR-defined subsets that selectively accumulate in certain tissues (9-12). Here, it is demonstrated that CD8⁺ bovine $\gamma\delta$ T cells, which are found in large numbers in the spleen, do not accumulate in appreciable numbers in sites of inflammation. In contrast, bovine $\gamma\delta$ T cells, which lack CD8, selectively accumulate at these sites.

Two molecular mechanisms could account for this selective tissue accumulation: selective retention and proliferation or selective recruitment. To date, there have been few examples supporting either possibility in the context of tissue-specific $\gamma\delta$ T cell accumulation. To enter a tissue, a leukocyte follows a multi-step process which involves initial recognition of the vascular endothelium, followed by rolling along the vessel wall, tight adhesion and stopping, and eventually transendothelial migration (27). Adhesion molecules expressed by the T cell and endothelium regulate each of these steps. Members of the selectin family of adhesion molecules, which includes L-selectin on leukocytes, E-selectin on endothelium, and P-selectin on endothelium and platelets, are important in regulating rolling interactions. Recently, we defined another molecule, the GR antigen, that is expressed by chronically activated endothelial cells and supports rolling of different leukocyte subsets (19). We have not yet confirmed whether the GR antigen is a new molecule or a different form of a previously characterized molecule.

Bovine $\gamma\delta$ T cells avidly bind E-selectin, P-selectin, and the GR antigen, and these interactions are thought to be important in regulating the entry of these cells into different inflammatory lesions (13, 19, 23). $\gamma\delta$ T cells also express L-selectin. Leukocyte L-selectin is important in the homing of lymphocytes to lymph nodes (20, 28), and in the trafficking of neutrophils and other leukocytes to sites of inflammation

(29-31). Interestingly, L-selectin is expressed at three to five times the level on the majority of $\gamma\delta$ T cells as compared to other lymphocytes (26), where it is localized at the tips of microvilli and mediates both binding to endothelium in lymph nodes and other immobilized $\gamma\delta$ T cells (32, 33).

Here, we demonstrate that CD8 $^{+}$ $\gamma\delta$ T cells in bovine blood have a greatly diminished capacity to bind E-selectin and soluble GR antigen. CD8 $^{+}$ $\gamma\delta$ T cell binding to cell surface expressed E-selectin, as well as the extent of soluble E-selectin chimera binding to the $\gamma\delta$ T cell, were measured by flow cytometry. The first approach is more physiologically relevant, since E-selectin mediates cell-cell binding. However, this assay cannot distinguish between a lack of ligand, or simply reduced expression of ligand below the threshold needed to support cell-cell binding. The flow cytometric assay measured quantitative differences in ligand expression; from these studies it was determined that the majority of the CD8 $^{+}$ $\gamma\delta$ T cells lacked the capacity to bind E-selectin, suggesting that the majority of CD8 $^{+}$ $\gamma\delta$ T cells do not express E-selectin ligands. Flow cytometry also confirmed that most CD8 $^{+}$ $\gamma\delta$ T cells lack ligands for soluble GR antigen.

E-selectin is a lectin which binds carbohydrate ligands on target cells (34). It is likely that the GR antigen is a lectin as well (19). Selectin-binding carbohydrates are modified to their functional form by fucosyltransferases, enzymes that catalyze the final step in ligand synthesis (35, 36). Therefore, lack of selectin binding could be due to lack of the appropriate carbohydrates or the protein backbone they are found on. Analysis of selectin binding carbohydrates on bovine cells is not straightforward, since reagents that recognize human ligands (HECA 452, for example) do not cross react with bovine counterparts. However, we are in the process of sorting enough CD8 $^{+}$ and CD8 $^{-}$ $\gamma\delta$ T cells to analyze mRNA for specific fucosyltransferases that are required to construct selectin carbohydrates, an approach which is beyond the scope of this initial study.

We have partially characterized a glycoprotein on $\gamma\delta$ T cells, which is decorated

by appropriate carbohydrates and binds E-selectin, using the E-selectin chimera described here (22). The E-selectin binding glycoprotein is approximately 250 kD, but we have not yet generated specific monoclonal antibodies against the protein portion, thus we cannot address whether it is lacking on the CD8⁺γδ T cells. However, the data strongly supports the possibility that the protein backbone of the ligand could be missing. Strikingly, CD8⁺γδ T cells are not stained by mAbs directed against a number of different large molecular mass surface glycoproteins. For example, these cells lack WC1 (M_r 180-210 kD), GD3.5 antigen (M_r 230 kD), and L-selectin (M_r 90 kD). Although we have no evidence that any of these molecules bind E-selectin or GR antigen (22), it may be that the expression of the protein component of the selectin ligand is regulated in a similar fashion.

Though bovine CD8⁺γδ T cells lack certain antigens, it is important to point out that it is a very selective deficiency. For example, these cells express the functionally important CD18 (M_r 90 kD), CD5 (M_r 67 kD), CD44 (M_r 90 kD) (E. Wilson, unpublished results), and CD2 (M_r 45-58 kD) (10) antigens, in addition to CD8. Thus, CD8⁺γδ T cells exhibit a unique pattern of gene expression, differing from other γδ T cells in the expression of several proteins, including molecules important in leukocyte recruitment.

It is not known if this unique adhesion molecule phenotype of CD8⁺γδ T cells is developmentally or environmentally regulated. Work in mice has shown two different lineages of γδ T cells, one passing through the thymus and the other originating in the gut and comprising the majority of intraepithelial lymphocytes (37, 38). In the mouse, gut derived γδ T cells express CD8. However, unlike the CD8αβ heterodimer expressed on thymus-derived T cells, the gut derived CD8 cells express the CD8αα homodimer (39). These cells have been shown to play an important role in protection against pathogens of the gut (40). In contrast, bovine CD8⁺γδ T cells express the CD8αβ

heterodimer (41). These CD8 $\alpha\beta$ $\gamma\delta$ T cells may represent yet another lineage of $\gamma\delta$ T cells, or could possibly represent the bovine lineage equivalent to the mouse CD8 $\alpha\alpha$ $\gamma\delta$ T cell. If CD8 $^{+}\gamma\delta$ T cells do represent a separate lineage of cells they may never express high levels of L-selectin, GR antigen or E-selectin ligands, as seen on CD8 $^{-}\gamma\delta$ T cells. Conversely, some $\gamma\delta$ T cells may respond to environmental stimuli that induce changes in gene regulation of some surface proteins. It may be that the expression of CD2 and CD8 genes in $\gamma\delta$ T cells correlates with the suppression of E-selectin ligand and L-selectin, as well as, WC1 gene transcription.

What accounts for the preferential accumulation of CD8 $^{+}\gamma\delta$ T cells in the gut and spleen is still not understood. Immunohistological studies show that splenic CD8 $^{+}\gamma\delta$ T cells are selectively localized to the red pulp (10). To date, there is no evidence for a role of selectins in the homing of T cells to the spleen. In fact, studies in L-selectin-deficient mice have shown that the spleen of these mice contains more lymphocytes than their wild type litter mates (42). E- and P-selectin-deficient mouse studies have reported similar results, showing no decrease in the number of splenic lymphocytes (43, 44). Studies in humans have also shown a population of splenic lymphocytes that do not express L-selectin (45). Thus, the lack of expression of L-selectin and selectin ligands does not inhibit migration to the spleen.

T cells can enter the spleen by two routes: migration across vessels in the marginal zone or accumulation via blood in the red pulp of the spleen (46). Potentially, all $\gamma\delta$ T cells enter the spleen by one or both of these routes; however, the CD8 $^{+}\gamma\delta$ T cells remain in the spleen, whereas the CD8 $^{-}\gamma\delta$ T cells may rapidly re-enter the circulation. Support for this possibility has come from experiments in which we have examined the homing of bovine $\gamma\delta$ T cells in mice. When bovine $\gamma\delta$ T cells are injected into the circulatory system of mice they exhibit the same pattern of tissue distribution as

they do in cattle; i.e., few cells in lymph nodes versus many in the spleen (26). This xenogeneic system, though clearly artificial, allows one to test whether selective recruitment of CD8 $\gamma\delta$ T cells occurs in the spleen. In short term homing assays, preferential homing of bovine CD8 $\gamma\delta$ T cells to the mouse spleen was not detected (data not shown). It may very well be that homing to bovine spleen is regulated differently, or that there is insufficient homology between mouse and bovine molecules to support the splenic homing of CD8 $\gamma\delta$ T cells. If selective localization of CD8 $\gamma\delta$ T cells to the spleen does occur it may be mediated through integrin associated adhesion, or by as of yet uncharacterized adhesion molecules.

Work by Haru-Hisa *et al* (47) suggests a functional role for the WC1 molecule, demonstrating that crosslinking WC1 induces a reversible G0/G1 growth arrest. This cell cycle arrest is mediated through reduced expression of the transcription factor E2F1 (48), apparently resulting in cell cycle arrest through the interruption of the IL-2 signaling pathway (49). IL-2 mediates cell proliferation through the high affinity IL-2 receptor found on activated but not resting T cells (50). Considering these results, it is possible that an, as of yet, unidentified WC1 ligand binds splenic WC1 $\gamma\delta$ T cells, resulting in G0/G1 growth arrest of IL-2 responsive CD8 $\gamma\delta$ T cells. Conversely, IL-2 responsive CD8 $\gamma\delta$ T cells, which do not express WC1, would not undergo cell cycle arrest, and proliferate in response to IL-2 signaling. This scenario would explain the large numbers of CD8 $\gamma\delta$ T cells compared to CD8 $\gamma\delta$ T cells found in the spleen, through the selective expansion of WC1 $\gamma\delta$ T cells. Work is currently underway to analyze the cell cycle phase of blood and spleen WC1 $^+$ and WC1 $\gamma\delta$ T cells to better determine if the splenic microenvironment results in a G1/G0 growth arrest of WC1 $\gamma\delta$.

In summary, this report provides a clear example of tissue-specific $\gamma\delta$ T cell subset accumulation correlating with a functional adhesive phenotype. TCR-defined $\gamma\delta$ T cell subset localization has been well documented and intensively studied. However,

previous reports have not demonstrated a defined adhesive phenotype correlating to the localization of these cells *in vivo*. Although no positive correlation between an adhesive phenotype and CD8⁺γδ T cell accumulation has been identified, the lack of or low expression of defined homing molecules on these cells, including E-selectin ligands, GR antigen ligands, and L-selectin, suggests that these molecules are not involved in the selective accumulation of CD8⁺γδ T cells to the spleen. Conversely, our data does demonstrate a positive correlation between expression of GR antigen ligands, E-selectin ligands, and L-selectin on CD8⁺γδ T cells, and their accumulation in sites of inflammation.

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CHAPTER 4

A TCR-DEFINED SUBSET OF BOVINE $\gamma\delta$ T CELLS EXPRESSES HIGH LEVELS
OF FUNCTIONAL $\beta 7$ INTEGRIN AND PREFERENTIALLY ACCUMULATES IN
SITES EXPRESSING MAdCAM-1: A MECHANISM FOR TISSUE SPECIFIC
 $\gamma\delta$ T CELL SUBSET ACCUMULATION

Introduction

Considerable effort has been devoted to the study of $\gamma\delta$ T cells. However, there are many aspects of this population that are still poorly understood. One intriguing attribute demonstrated in studies with mice, humans, and cattle is that discrete subsets of $\gamma\delta$ T cells, defined by their TCR usage, specifically accumulate in certain tissues and organs (1-6). What accounts for this TCR-restricted, tissue-specific $\gamma\delta$ T cell accumulation is not well characterized. Here it is demonstrated that the tissue localization of a TCR-restricted subset of bovine $\gamma\delta$ T cells, which co-expresses CD8 and CD2 (for simplicity this subset will be referred to as CD8⁺ $\gamma\delta$ T cells throughout the rest of the chapter), is, in part, predicted by adhesion molecules expressed on their cell surface. CD8⁺ $\gamma\delta$ T cells express low levels of adhesion molecules (E-selectin ligands and L-selectin) that are important in lymphocyte recruitment to sites of inflammation. The low levels of E-selectin ligands and L-selectin on CD8⁺ $\gamma\delta$ T cells correlate with the inability of these cells to accumulate in inflamed tissues (6).

Though CD8⁺ $\gamma\delta$ T cells are not found in appreciable numbers at sites of

inflammation, large numbers are found both in intestinal lamina propria and the spleen (5-7). We have yet to define a positive correlation of a tissue-specific adhesive phenotype with the tissue-specific tropism of this subset. A variety of vascular adhesion molecules are expressed in mucosal tissues. Of these, the mucosal vascular addressin (MAdCAM-1) exhibits the greatest tissue-specific restriction in its expression and function. MAdCAM-1, which binds to the $\alpha 4\beta 7$ integrin on lymphocytes (8-10), is selectively expressed in mucosal tissues, such as Peyer's patches, intestinal lamina propria, and mesenteric lymph nodes (MLN) (8, 11, 12). MAdCAM-1 is also expressed in the marginal zone of the mouse spleen, although a functional role for splenic MAdCAM-1 has not yet been demonstrated (13). The $\alpha 4\beta 7$ integrin has been shown to be differentially expressed on various lymphocyte subsets, with high expression of $\alpha 4\beta 7$ correlating with the tropism of cells for mucosal sites (14-16).

Although the preference of some subsets of $\gamma\delta$ T cells for mucosal sites has been well documented (17), differential expression and/or function of the $\alpha 4\beta 7$ integrin on subsets of $\gamma\delta$ T cells has not been described. Likewise, the importance of MAdCAM-1 in the homing of $\gamma\delta$ T cell subsets has not been characterized. To study the interaction of $\gamma\delta$ T cells with MAdCAM-1, an animal model with large numbers of $\gamma\delta$ T cells in circulation (in the process of homing) is advantageous. As we have previously shown in multiple reports, $\gamma\delta$ T cells from young cattle, which constitute up to 70% of peripheral blood lymphocytes, represent an ideal population to study $\gamma\delta$ T cell/endothelial cell interactions (6, 18-23).

We recently characterized MAdCAM-1 in cattle through the use of the mAb 7G11, which was generated against recombinant human MAdCAM-1 and previously shown to cross-react in sheep (25). Anti- $\beta 7$ mAbs block bovine lymphocyte adhesion to recombinant MAdCAM-1. Using newborn animals, for which lymphocyte distribution

has been well defined, we now extend our characterization of the expression pattern of bovine MAdCAM-1. Examining principally mucosal tissues and spleen, it was investigated whether the tissue tropism of CD8⁺γδ T cells can be explained, in part, by specific interactions with this adhesion molecule. MAdCAM-1 was expressed at high levels in bovine MLN and intestinal lamina propria. Minimal expression was seen in peripheral lymph nodes (PLN), skin, chronic inflammatory lesions, and the thymus. MAdCAM-1 was expressed at high levels in bovine spleen, predominantly in the marginal zone and radiating into the red pulp. Using multi-color FACS analysis and immunohistology, a striking correlation of the localization of CD8⁺γδ T cells and MAdCAM-1 expression was noted. In *in vitro* binding assays, CD8⁺γδ T cells preferentially bound recombinant MAdCAM-1, when compared to CD8⁻γδ T cells. The *in vitro* enrichment of CD8⁺γδ T cells was specifically inhibited by the addition of function blocking anti-MAdCAM-1 mAbs. Most CD8⁺γδ T cells also expressed higher levels of β7 than CD8⁻γδ T cells. These results suggest that in some cases α4β7/MAdCAM-1 receptor ligand interactions may facilitate the tissue-specific accumulation of TCR-defined subsets of γδ T cells.

Materials and Methods

Animals

Holstein calves were purchased from local producers and housed at the Montana State University Large Animal Facilities at the Veterinary Molecular Biology Laboratory. Cattle used in this study were bull calves from 1-6 months of age. Tissue samples were collected from animals upon necropsy, cut and frozen in O.C.T. freezing medium (Tissue-Tek, Inc., Elkhart, IN). For immunohistology, frozen sections were air dried, fixed in acetone, and then stained as previously described (20).

Cells and cell lines

Peripheral blood was collected into sodium heparin anticoagulant tubes by venipuncture and PBMC were purified by Ficoll-Hypaque (Sigma Diagnostics, St. Louis, MO) gradient centrifugation. Lymphocytes were isolated from spleen, thymus and lymph nodes by first finely mincing tissues with a razor blade. Samples were then dounced in a tissue homogenizer to release lymphocytes from the stromal elements. Cell suspensions isolated from various tissues were then passed through a layer of Nytex fabric (Fairmont Fabrics, Hercules, CA) to remove cell aggregates, and the resulting cell preparation was stained for FACS analysis or used in functional assays. Intra-epithelial lymphocytes (IEL) were isolated by exteriorizing the lumen of the small intestine, washing extensively with HBSS and gently scraping the luminal surface with a razor blade. Cell scrapings were then dounced in a tissue homogenizer, and isolated as described above. MAdCAM-1 transfected CHO cells and non-transfected CHO cell

controls were kindly provided by E. Butcher and M. Briskin (Stanford School of Medicine) (9).

MAbs used in this study

The following mouse mAbs were used: GD3.8, which recognizes a pan epitope on the bovine $\gamma\delta$ TCR (26); CC58, which recognizes bovine CD8, kindly provided by Chris Howard (Institute for Animal Health, Compton, UK) (27); MHM23, which recognizes human and bovine CD18 (DAKO, Carpinteria, CA); FW4-101, which recognizes bovine CD29 (VMRD, Pullman, WA); and 7G11, which recognizes human MAdCAM-1 (25) and cross-reacts with sheep and bovine MAdCAM-1 (24, 25). The following rat mAbs were used: FIB30, a rat anti- $\beta 7$ integrin mAb that has previously been shown to specifically bind human and mouse $\beta 7$ integrin (28), and cross-reacts with bovine $\beta 7$ (24); and MECA367, which recognizes mouse MAdCAM-1 (8). Rat mAb SK208, which recognizes mouse neutrophils (M.A. Jutila unpublished observations) and mouse mAb EL81 (29) were used as isotype-matched control mAbs. PE-conjugated anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA), and avidin-conjugated CyChrome (PharMingen, San Diego, CA) were used as secondary reagents.

Three-color FACS analysis

FACS analysis was performed as follows: 100 μ l hybridoma supernatant fluid or 100 μ l of purified mAb at a final concentration of 50 μ g/ml were incubated with cells for 30 min on ice and then washed from the cells with PBS containing 5% horse serum (Sigma Diagnostics) (PBS-HS). PE-labeled second stage diluted 1:250 in PBS-HS was

then added and incubated for 30 min on ice. The samples were washed with PBS-HS, incubated in 10% mouse serum in PBS for 15 min, and washed again. Biotin-labeled GD3.8 (anti-pan $\gamma\delta$ T cell mAb) and FITC-conjugated anti-CD8 were then added and the cells incubated on ice for 30 min. The cells were washed in PBS-HS and then incubated with avidin-CyChrome diluted 1:2000 in PBS-HS. After 30 min on ice, the cells were washed in PBS-HS and analyzed using a FACSCalibur (Becton Dickinson Immunocytometry Systems, Mountain View, CA). FL1 (FITC), FL2 (PE) and FL3 (CyChrome) detectors were used and the FACSCalibur was calibrated using Calibright beads (Becton Dickinson). Compensation was set manually using single-color stains of the various fluorochromes. In assays where it was necessary to analyze small subsets of cells, live gate acquisition was utilized to acquire sufficient cell numbers for statistical analysis. Data from up to 20,000 cells was acquired and reported in two-dimensional dot plots or histograms. Negative controls included 1) single-color stains, 2) irrelevant isotype-matched antibody stains, and 3) second-stage reagent controls. For statistical analysis, markers were placed just above the upper limit of background staining of control antibodies. We found no evidence that the CyChrome reagent bound nonspecifically, as previously described for some reagents conjugated to this fluorochrome (30, 31).

MAdCAM-1 binding assay

Bovine peripheral blood was collected and PBMC purified, as described above. Mononuclear cells were then incubated for 30 min at 37 °C in T-175 flasks (Nunc, Naperville, IL) containing 1% BSA in DMEM (DMEM/BSA) (Sigma Diagnostics) to remove monocytes and other non-specifically adherent cells. The nonadherent lymphocyte population was washed in Hanks Balanced Salt Solution (HBSS) (Life

Technologies, Grand Island, NY) and resuspended at 5×10^6 cells/ml in medium consisting of 50% DMEM/BSA and 50% GPT (6) prior to use in functional analyses. The adhesion of bovine lymphocytes to mouse MAdCAM-1 cDNA-transfected CHO cells was then determined. Briefly, MAdCAM-1 binding cells were separated from whole lymphocyte preparations by incubating 5×10^7 cells in 10 ml of medium for 30 min in T-175 flasks containing confluent monolayers of MAdCAM-1 transfected CHO cells. Unbound cells were decanted and the monolayers extensively washed with DMEM/BSA. Monolayers were then washed twice in PBS and the bound lymphocytes were eluted by treatment with 2 mM EDTA/PBS. MAdCAM-1-adherent lymphocytes were then collected, washed in HBSS, and stained and analyzed by multicolor FACS analysis, as previously described (6). Specificity controls for MAdCAM-1 adhesion of lymphocytes included addition of the MAdCAM-1 blocking mAb MECA367 (50 μ g/ml) or isotype-matched negative control mAb SK208 (50 μ g/ml) to the adhesion assays. Specificity controls also included incubation of bovine PBMC on monolayers of non-transfected control CHO cells.

Statistical analysis

Results were analyzed using paired Student's *t* test. Significant *P* values are indicated.

Results

Bovine distribution of MAdCAM-1

The distribution of bovine MAdCAM-1 closely resembled the MAdCAM-1 distribution described in the mouse (8, 13), with high expression at mucosal sites and minimal expression in peripheral tissues. MAdCAM-1 was expressed at high levels in the MLN (Fig 1a) as well as throughout the ileal mucosa. Immunoperoxidase staining of calf ileum showed high expression of MAdCAM-1 in the lamina propria (Fig 1b), as well as in the connective tissue between the mucosa muscularis and Peyer's patches (Fig 1c). Most PLN examined had undetectable levels of MAdCAM-1; however, in some PLNs, minimal expression of MAdCAM-1 was occasionally seen in the paracortical area (Fig 1d). This observation is similar to the PLN expression of MAdCAM-1 described in the sheep (25). MAdCAM-1 expression was not seen in skin, thymus, or chronic inflammatory lesions established under the skin (data not shown).

The major difference observed between bovine and murine MAdCAM-1 expression was seen in the spleen. Mouse splenic MAdCAM-1 was found predominantly in areas of intense expression along the marginal zone and generally ended abruptly at the outside edge of the white pulp (Fig 2A), as previously reported (13). Conversely, the bovine distribution of MAdCAM-1 was less restricted. Expression of bovine MAdCAM-1 predominated in the marginal zone of the spleen; however, unlike mouse, bovine MAdCAM-1 extended from the edge of the marginal zone, outward into the red pulp of the spleen (Fig. 2B).

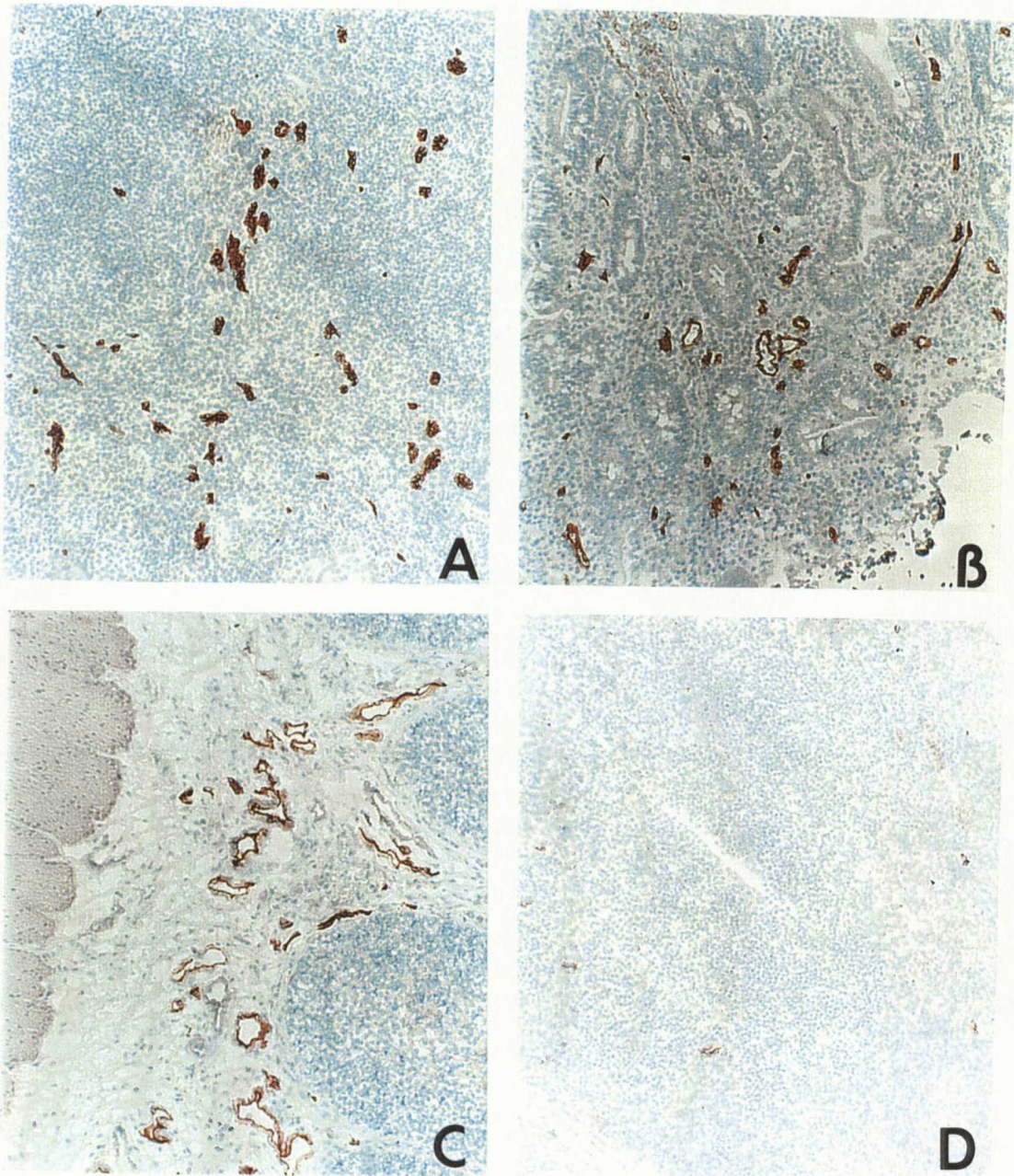


Figure 10

Immunohistologic staining of bovine MAdCAM-1. Tissues from calves were prepared and stained with mAb 7G11 to determine the distribution of bovine MAdCAM-1 as described in Materials and Methods. Panel A, paracortex of MLN. Panel B, ileal lamina propria. Panel C, connective tissue between the mucosa muscularis and the Peyer's patch. Panel D, Paracortex of PLN. Original magnification 200X.

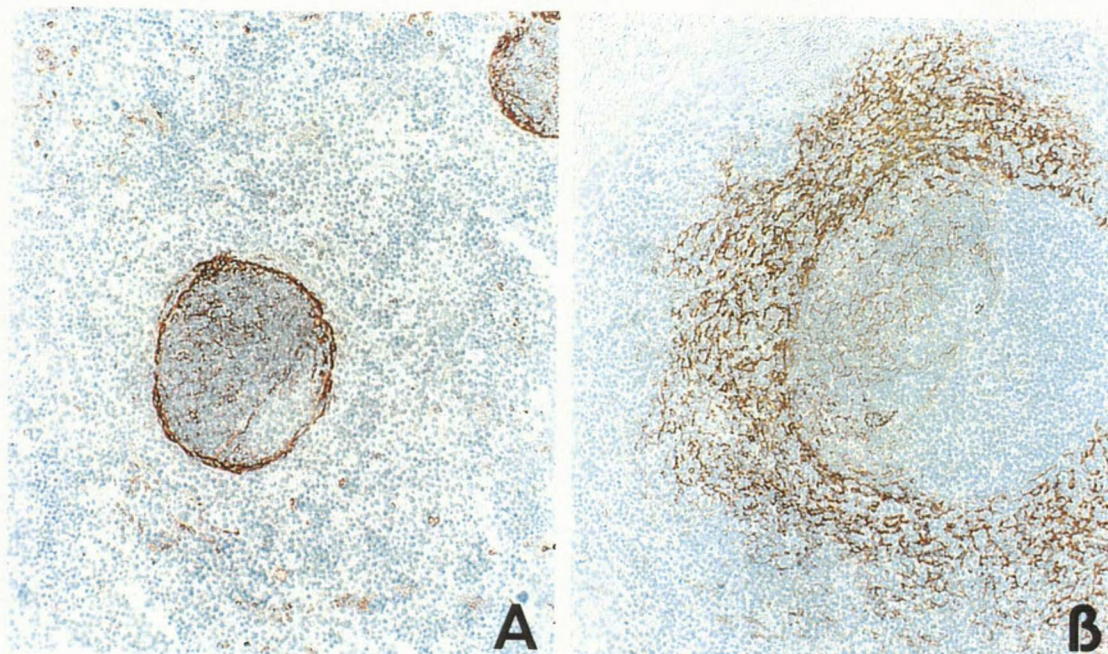


Figure 11

Immunohistologic staining of mouse and bovine splenic MAdCAM-1. Mouse and bovine spleen sections were prepared and stained as described in Materials and Methods. Panel A, mouse spleen stained with MAdCAM-1 specific mAb MECA367. Panel B, bovine spleen stained with MAdCAM-1 specific mAb 7G11. Original magnification 200X.

CD8⁺γδ T cells accumulate in tissues expressing MAdCAM-1

Bovine γδ T cell numbers, as a percent of total lymphocytes, vary dramatically between different tissues and organs. Large numbers of γδ T cells are found in the blood, spleen, and gut, whereas minimal numbers accumulate in lymph nodes.

Previous reports by our lab and others have shown high numbers of CD8⁺γδ T cells in the spleen and gut, but not in the blood or inflamed tissues (5-7, 32). In an effort to determine whether CD8⁺γδ T cells, or any other defined subset of γδ T cells, accumulated in sites expressing MAdCAM-1, γδ T cell subset analysis of various mucosal and non-mucosal organs was performed. The percent γδ T cells as a function of total lymphocytes varied greatly from tissue to tissue as previously reported (33, 34). However, the percent of CD8⁺γδ T cells as a function of total γδ T cells correlated directly with the amount of MAdCAM-1 expressed in the tissue. For example, at sites expressing high levels of MAdCAM-1, such as intestinal lamina propria and MLN, over 60% of γδ T cells co-expressed CD8. This is in contrast to PLN which contained minimal levels of MAdCAM-1, and the percentage of CD8⁺γδ T cells was essentially the same as in blood (30%). These data demonstrate that the percentage of CD8⁺γδ T cells, as a proportion of total γδ T cells in a tissue, correlated with the expression of MAdCAM-1 (Table 1).

Peripheral blood CD8⁺γδ T cells express high levels of the β7 integrin

To determine if CD8⁺γδ T cells in circulation express the MAdCAM-1 counter receptor α4β7, FACS analysis of various γδ T cell subsets was performed. Although α4β7 has not been molecularly characterized in the bovine system, a mAb, FIB30, which binds the murine β7 integrin, has been shown to cross-react in the cow (26). Multi-color FACS analysis of bovine peripheral blood γδ T cells revealed that the fluorescence of the β7 integrin on CD8⁺γδ T cells (mode 554) was approximately 1.5 fold higher than that of CD8⁻γδ T cells (mode 322) (difference significant at $P < 0.01$) (Fig 3). Analysis of other β-subunit integrins showed that the level of β1 (CD29) (CD8⁺γδ T cell mode fluorescence 223, CD8⁻γδ T cell mode fluorescence 254) and β2

(CD18) (CD8 $^+$ $\gamma\delta$ T cell mode fluorescence 245, CD8 $^+$ $\gamma\delta$ T cell mode fluorescence 271) integrins on CD8 $^+$ and CD8 $^+$ $\gamma\delta$ T cells did not differ statistically.

Table 1

MAdCAM-1 expression correlates with the CD8 $^+$ $\gamma\delta$ T cell/ $\gamma\delta$ T cell ratio in a tissue.

Tissue	MAdCAM-1	$\gamma\delta$ T cells/ lymphocytes	CD8 $^+$ $\gamma\delta$ T cells/ $\gamma\delta$ T cells
Blood		17.8% $\pm$ 3.6	20.9% $\pm$ 6.35
Thymus	.*	9.6% $\pm$ 0.4 [#]	28.2% $\pm$ 4.3
Peripheral node	+/-	3.7% $\pm$ 1.7 [#]	30.8% $\pm$ 5.9
Mesenteric node	++++	2.4% $\pm$ 0.7 [#]	64.8% $\pm$ 9.8 [#]
Intestinal Lamina Propria	++++	20.2% $\pm$ 7.8	63.2% $\pm$ 5.1 [#]
Spleen	++++	23.7% $\pm$ 3.5	43.1% $\pm$ 14.6 [§]

MAdCAM-1 expression was determined by immunoperoxidase staining. Lymphocyte data was generated through multi-color FACS analysis of tissue homogenates, as described in Materials and Methods. * MAdCAM-1 expression was judged on a scale of no detectable MAdCAM-1 (-) to high expression of MAdCAM-1 (++++). [#] Indicates statistical significance compared to peripheral blood ($P < 0.01$). [§] Although statistical significance ($P > 0.05$) was not seen in the CD8 $^+$ $\gamma\delta$ T cell/ $\gamma\delta$ T cell ratio in the spleen in this experiment, previous research by our lab and others has shown that the percentage of CD8 $^+$ $\gamma\delta$ T cells is significantly higher in the spleen than in peripheral blood (5-7). Data was generated from three animals tested.

Peripheral blood CD8⁺γδ T cells preferentially bind recombinant mouse MAdCAM-1

Mode fluorescence values of β7 expression suggested that CD8⁺γδ T cells expressed higher levels of α4β7, the lymphocyte ligand for MAdCAM-1, than CD8⁻γδ T cells, potentially explaining, in part, the accumulation of CD8⁺γδ T cells in MAdCAM-1 expressing sites. Although the mode fluorescence of CD8⁺ was significantly higher than that of CD8⁻γδ T cells, some CD8⁻γδ T cells did express high levels of the β7 integrin. Lymphocyte expressed integrins have previously been shown to exist in low affinity as well as high affinity/functional states (35). To determine if CD8⁺ and CD8⁻γδ T cells expressed different functional levels of α4β7, we examined the ability of γδ T cells to bind MAdCAM-1 transfected CHO cells. In this *in vitro* adhesion assay, peripheral blood lymphocytes, expressing functional levels of α4β7, were selected by their adhesion to monolayers of MAdCAM-1 cDNA transfected CHO cells. As expected a variety of cells, including αβ T cells, B cells and γδ T cells, bound MAdCAM-1 and were recovered in these assays. Importantly, CD8⁺γδ T cells bound to recombinant MAdCAM-1 and were recovered at a significantly higher frequency than CD8⁻γδ T cells ($P < 0.05$) (Figure 4). Thus, in this *in vitro* adhesion assay using a single molecule, MAdCAM-1, the frequency of CD8⁺γδ T cells increased dramatically over the percentage found in peripheral blood. This result mimics the selective accumulation of CD8⁺γδ T cells seen in MAdCAM-1 expressing tissues *in vivo*.

Splenic γδ T cells express heterogeneous patterns of β7 expression

Since peripheral blood CD8⁺γδ T cells expressed high levels of functional α4β7, we next analyzed expression patterns on cells isolated from different tissues to determine

if high $\beta 7$ levels are maintained once the cells leave the blood, or in the case of the thymus, before the cells enter the blood. Lymphocytes were isolated from PLN, MLN, thymus, intestinal lamina propria, spleen, and blood, and $\beta 7$ levels compared. $CD8^{+}\gamma\delta$ T cells isolated from PLN, MLN, and thymus essentially had the same profile of $\beta 7$ expression as cells in the blood: mode fluorescence was higher on $CD8^{+}\gamma\delta$ T cells than on $CD8^{-}\gamma\delta$ T cells (data not shown and Fig. 5). The results in the thymus suggested that $\beta 7$ expression may be developmentally regulated on some $CD8^{+}\gamma\delta$ T cells, and is expressed at high levels prior to antigen stimulation. All $\gamma\delta$ T cells isolated from intestinal lamina propria expressed high levels of $\beta 7$ (Fig. 5), correlating with the dependence on MAdCAM-1 for recruitment of lymphocytes to this site.

$\beta 7$ expression on $\gamma\delta$ T cells isolated from the spleen exhibited an unexpected level of complexity. Two distinct populations of $\gamma\delta$ T cells were identified based on $\beta 7$ expression: one which expressed low levels of the antigen and another which expressed greater than ten-fold higher levels (average mode fluorescence of 7 in the $\beta 7$ low cells and an average of 115 in the $\beta 7$ high cells). The $\beta 7$ high and low expressing $\gamma\delta$ T cells could be further divided by their expression of CD8. As shown in Figure 5, four distinct subsets of $\gamma\delta$ T cells could be identified by analysis of $\beta 7$ and CD8: $\beta 7^{low}CD8^{-}$; $\beta 7^{high}CD8^{-}$; $\beta 7^{low}CD8^{+}$; and $\beta 7^{high}CD8^{+}$. Thus, the spleen is unique among the lymphoid organs examined with respect to the $\beta 7$ expression profile of its resident $\gamma\delta$ T cell populations.

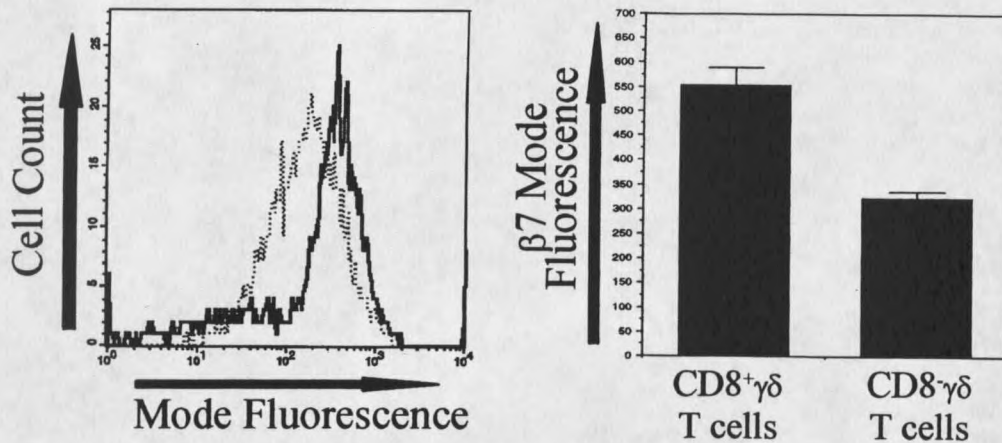


Figure 12

Peripheral blood CD8⁺γδ T cells express higher levels β7 integrin than CD8⁻γδ T cells. Three-color FACS analysis was performed, as described in Materials and Methods. Panel A, representative histogram of β7 expression on CD8⁻γδ T cells (dotted line) and CD8⁺γδ T cells (solid line). Panel B, mode fluorescence of β7 expression levels on CD8⁺ and CD8⁻γδ T cells from five pooled experiments, indicating that CD8⁺γδ T cells express higher levels of the β7 integrin than CD8⁻γδ T cells $P < 0.01$. y-axis of graph shows mean fluorescence intensity. Error bars represent standard error of the mean.

CD8⁺γδ T cells express low levels of IL-2R

The results described above suggest that MAdCAM-1 contributes to selective accumulation of CD8⁺γδ T cells in some tissues. Another potential mechanism of lymphocyte accumulation in tissues is the selective proliferation and expansion of cell subsets. Therefore, we examined the potential contribution of proliferation on CD8⁺γδ T cell tissue-specific accumulation, by staining cells for expression of the IL-2 receptor (IL-2R). As shown in Figure 6, peripheral blood and splenic CD8⁺γδ T cells exhibited a lower level of IL-2R expression than CD8⁻γδ T cells from these tissues. The minimal

expression of IL-2R on CD8 $^{+}$ $\gamma\delta$ T cells suggested that these cells were not proliferating in response to IL-2. These results also showed that CD8 $^{+}$ and CD8 $^{-}$ $\gamma\delta$ T cells exhibited distinct profiles with respect to the levels of IL-2R expressed on their cell surface. This differential cytokine receptor expression was seen on peripheral blood and spleen $\gamma\delta$ T cells (Fig. 6), as well as on cells from MLN and PLN (data not shown). In contrast, in the thymus, CD8 $^{-}$ $\gamma\delta$ T cells expressed only slightly more IL-2R than CD8 $^{+}$ $\gamma\delta$ T cells (Fig. 6).

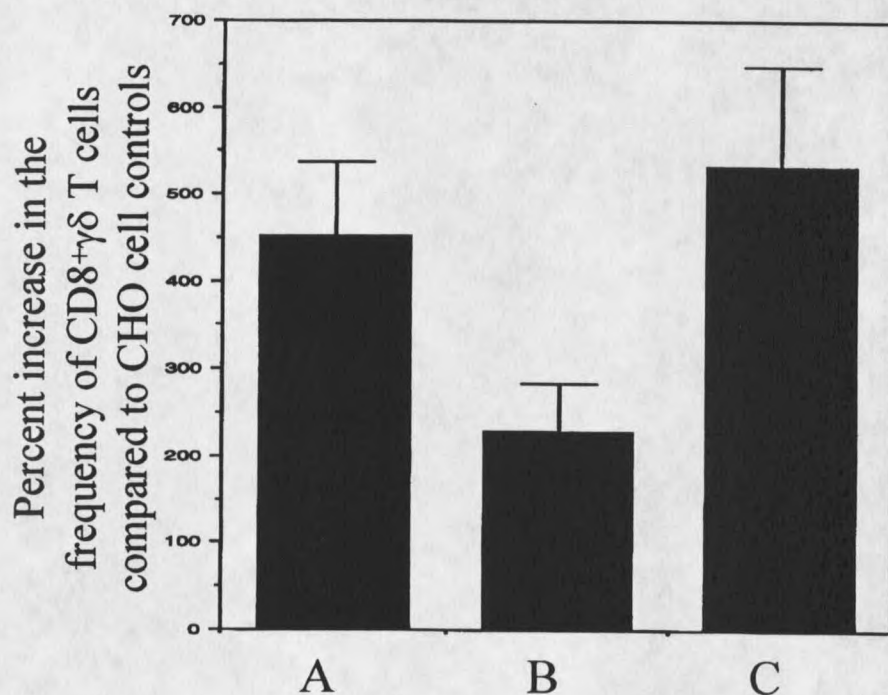


Figure 13

CD8 $^{+}$ $\gamma\delta$ T cells bind MAdCAM-1 more efficiently than CD8 $^{-}$ $\gamma\delta$ T cells. Lymphocytes capable of binding MAdCAM-1 were separated from total peripheral blood mononuclear cells by adhesion to monolayers of MAdCAM-1 transfected CHO cells, as described in Materials and Methods. Incubation of PBMC on non-transfected CHO cells was used to establish the background binding of CD8 $^{+}$ and CD8 $^{-}$ $\gamma\delta$ T cell subsets in adhesion assays. The change in frequency of CD8 $^{+}$ $\gamma\delta$ /CD8 $^{-}$ $\gamma\delta$ T cells in each experimental group, when compared to binding of cells on CHO cell controls, is indicated in the y-axis. A, change in CD8 $^{+}$ $\gamma\delta$ /CD8 $^{-}$ $\gamma\delta$ T cell frequency after binding to MAdCAM-1 transfected CHO cells. B, change in CD8 $^{+}$ $\gamma\delta$ /CD8 $^{-}$ $\gamma\delta$ T cell frequency after binding to MAdCAM-1 transfected CHO cells with 50 μ g/ml MAdCAM-1 function blocking mAb MECA367. C, change in CD8 $^{+}$ $\gamma\delta$ /CD8 $^{-}$ $\gamma\delta$ T cell frequency after binding to MAdCAM-1 transfected CHO cells with 50 μ g/ml isotype control mAb SK208. Difference between groups A and B, as well as, B and C were statistically different at $P < 0.05$. Error bars represent standard error of the mean. Data generated from six experiments.

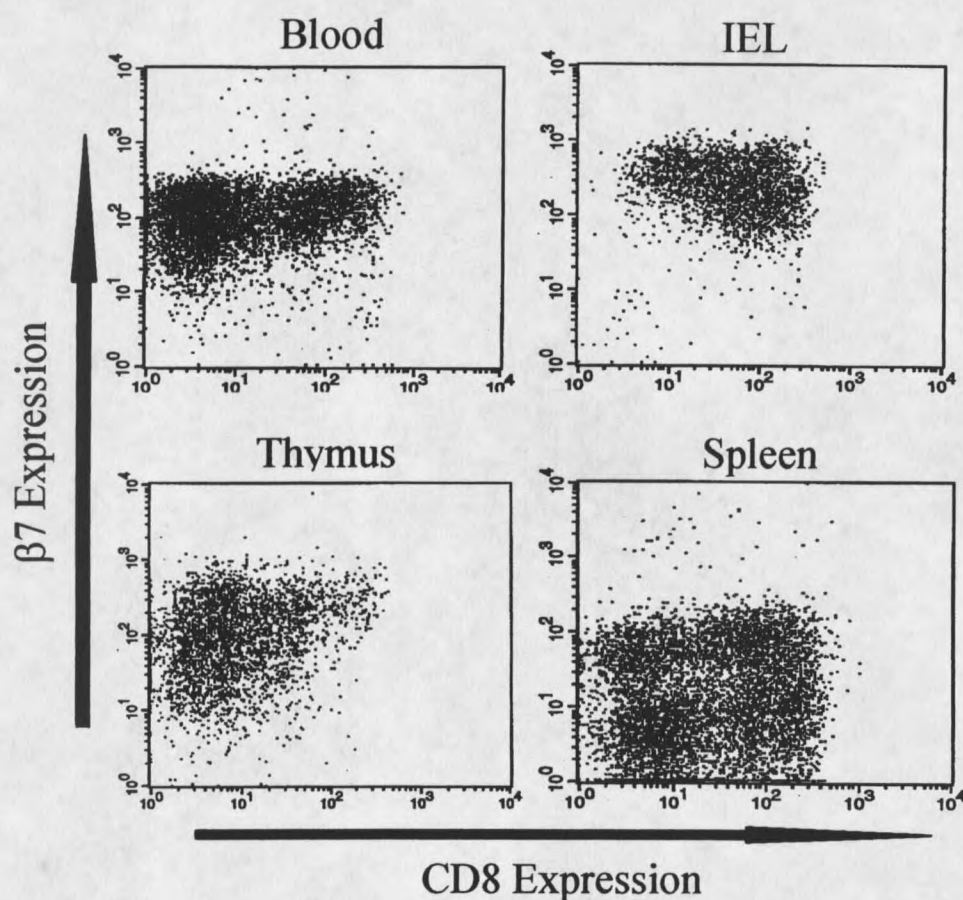


Figure 14

Expression of the $\beta 7$ integrin on $\gamma\delta$ T cells varies between tissues and organs. Single cell suspensions were generated from blood, intestinal lamina propria, thymus, and spleen, and stained for FACS analysis, as described in Materials and Methods. Dot plots were generated by gating on total $\gamma\delta$ T cells (FL 3). CD8 expression is shown in the x-axis (FL 1), while $\beta 7$ expression is shown in the y-axis (FL 2). Each panel represents the $\beta 7$ expression on CD8⁻ and CD8⁺ $\gamma\delta$ T cells from the tissue indicated above each plot. Data representative of three calves tested.

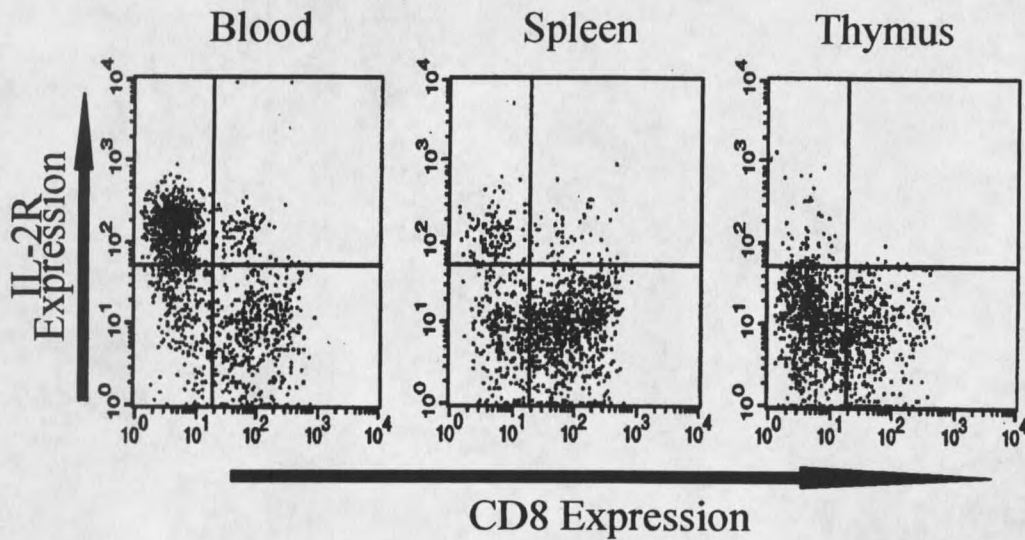


Figure 15

Constitutive levels of IL-2R are higher on CD8⁻ than CD8⁺γδ T cells. Single cell suspensions were generated from various organs and stained for FACs analysis, as described in Materials and Methods. Dot plots were generated by gating on total γδ T cells (FL 3). CD8 expression is shown in the x-axis (FL-1), IL-2R expression is shown in the y-axis (FL-2). Cells were obtained from the tissue indicated above each plot. Data representative of three calves tested.

Discussion

The accumulation of CD8⁺γδ T cells within a tissue may be the result of site-specific retention, proliferation/expansion, or homing. In this study a strong correlation was found between the accumulation of CD8⁺γδ T cells and the expression of the mucosal addressin MAdCAM-1 in tissues, suggesting that these cells may accumulate via α4β7 integrin mediated homing. In order for an integrin to mediate leukocyte homing, it must be expressed on the cell surface as well as be in a functional/active state (35). CD8⁺γδ T cells were shown to express higher levels of the β7 integrin than CD8⁻γδ T cells. CD8⁺γδ T cells also preferentially bound MAdCAM-1 in *in vitro* binding assays when compared to CD8⁻γδ T cells, indicating that the β7 integrin expressed on CD8⁺γδ T cells was functional. These results suggest that the unique tissue-tropism of bovine CD8⁺γδ T cells is controlled, at least in part, by MAdCAM-1/α4β7 interactions. Furthermore, high expression of the β7 integrin on CD8⁺γδ T cells was found on cells isolated from the thymus, suggesting that β7 integrin expression may be developmentally regulated before thymic emigration. This is the first study to show that quantifiable differences in the adhesion molecules expressed on γδ T cell subsets potentially exist as early as during lymphocyte ontogeny in the thymus.

If selective proliferation of CD8⁺γδ T cells in response to antigen stimulation were responsible for the increased proportion of CD8⁺γδ T cells found in some tissues, it would be expected that a significant portion of these cells would be actively proliferating. The IL-2Rα chain is expressed on lymphocytes which proliferate in response to the cytokine IL-2 (36, 37). IL-2Rα has also been shown to be expressed at low levels on some memory T cells (38). Although IL-2Rα expression is not a definitive marker of activated or memory cells, the presence of this cytokine receptor on

lymphocytes indicates that they are capable of proliferation in response to IL-2. Strikingly, IL-2R α , though expressed at high levels on CD8 $\gamma\delta$ T cells, was absent on nearly all CD8 $\gamma\delta$ T cells in all tissues examined. This suggests that IL-2 driven expansion was not actively contributing to the accumulation of CD8 $\gamma\delta$ T cells at the time of tissue collection. Interestingly, even though CD8 $\gamma\delta$ T cells isolated directly from the animal seldom expressed detectable levels of the IL-2R, CD8 $\gamma\delta$ T cells have been shown to express the IL-2R α chain and proliferate in response to IL-2 when stimulated under appropriate conditions (39).

Increased retention of lymphocytes through specific interactions with stromal elements could contribute to the accumulation of CD8 $\gamma\delta$ T cells at some sites. A potential mechanism of specific retention of CD8 $\gamma\delta$ T cells in the intestinal lamina propria is the interaction of the α E β 7 integrin with E-cadherin. Others have shown that the α E β 7/E-cadherin interaction is important in the retention of lymphocytes in the gut mucosa following extravasation from the blood (40). Reagents specific for bovine α E β 7 are not currently available; however, based on information from other animal systems, and the high expression of β 7 on intestinal IELs shown in this chapter, it is likely that E-cadherin contributes to the accumulation of bovine CD8 $\gamma\delta$ T cells in intestinal lamina propria. When appropriate reagents become available, the issue of E-cadherin mediated retention will be able to be examined in detail.

The results of this study, as well as previous work (6), have shown that the adhesion molecule profile expressed by $\gamma\delta$ T cells is useful in predicting whether a cell enters and/or remains in a tissue. However, this correlation is not always direct. For example, most circulating $\gamma\delta$ T cells (CD8 $\gamma\delta$ T cells) express L-selectin and avidly bind vessels in PLN using L-selectin; however, few of these $\gamma\delta$ T cells accumulate in underlying tissue (23). In this study, we found that tissues, such as MLN, express high

levels of MAdCAM-1, and as a function of total $\gamma\delta$ T cells, CD8⁺ $\gamma\delta$ T cells are greatly enriched in these tissues. However, as seen in both MLN and PLN, the total number of $\gamma\delta$ T cells remains low (less than four percent of total lymphocytes) suggesting that factors, in addition to the expression of adhesion molecules, are involved in the migration of lymphocytes to some sites.

The migration of lymphocytes into tissues is thought to occur by a multi-step process, involving leukocyte recognition of the vascular endothelium, which leads to a reversible rolling interaction of the leukocyte along the vessel wall. If an appropriate secondary signal is detected by the rolling leukocyte, it tightly adheres to the vessel wall through an integrin-mediated adhesion event. If the adhered leukocyte then responds to factors released by the underlying tissue, it may migrate through the vessel. Importantly, if at any time the leukocyte does not detect the appropriate signal, the cell may detach and re-enter the circulation (41-45).

Some factors inducing the rapid arrest and transendothelial migration of lymphocytes have been identified as chemokines. 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 possess exquisite leukocyte subset specificity, some acting on myeloid and others on lymphoid cells (reviewed in ref. 46). In some cases, lymphocyte subsets have been shown to differentially express chemokine receptors. For example, the chemokine receptor CCR6, which binds the chemokine MIP-3 α , has been shown to be expressed almost exclusively on memory T cells expressing $\alpha 4\beta 7$ integrins or the CLA antigen (47). Conversely, the CCR4 chemokine receptor, which binds the chemokine TARC, has been shown to mediate chemokine responses on CLA memory cells, but not on $\alpha 4\beta 7$ memory cells (48). Hence, differential sensitivity to chemokines

enables elegant selectivity in the recruitment of lymphocytes.

Although $\beta 7$ mode fluorescence on $CD8^+\gamma\delta$ T cells was significantly higher than $CD8^-\gamma\delta$ T cells, many $CD8^-\gamma\delta$ T cells did express high levels of the $\beta 7$ integrin and likely traffic via this molecule. However, the *in vivo* accumulation of $CD8^-\gamma\delta$ T cells to MAdCAM-1 expressing sites is apparently not as efficient as $CD8^+\gamma\delta$ T cell accumulation. Likewise, in *in vitro* MAdCAM-1 adhesion assays, $CD8^-\gamma\delta$ T cells are not enriched as are $CD8^+\gamma\delta$ T cells. One result of chemokine activation of T cells is the rapid transition of integrins from their low affinity to high affinity/functional forms (48-50). The accumulation patterns of $CD8^+$ and $CD8^-\gamma\delta$ T cells in MAdCAM-1 expressing sites is likely a function of both the level of $\alpha 4\beta 7$ expression and the functional state of the molecule. Chemokines, which upregulate integrin function and possibly lead to selective lymphocyte migration, have been shown to be expressed by (51, 52) and presented on (53) vascular endothelial cells. Therefore, qualitative or quantitative differences in the expression of chemokine receptors on $CD8^+$ and $CD8^-\gamma\delta$ T cells may ultimately regulate their accumulation in MAdCAM-1 expressing tissues. Experiments are currently underway to compare the response of $CD8^+$ and $CD8^-\gamma\delta$ T cells to known chemokines in an effort to better elucidate factors influencing the accumulation of these cells in certain tissues.

As already mentioned, $CD8^+\gamma\delta$ T cells accumulate in large numbers in the spleen. In fact, more lymphocytes recirculate through the spleen than to all lymph nodes combined (54, 55). Lymphocytes generally exit the blood flow and enter the spleen in the marginal zone (56, 57). From the marginal zone, lymphocytes migrate into distinct regions of the spleen. The molecular mechanisms that control the entry of lymphocytes into the spleen are unclear. As in other tissues, specific homing in the spleen may be a complex process depending both on the adhesion molecule profile, as well as the chemokine sensitivity of a cell. High levels of MAdCAM-1 are expressed in the

marginal zone; however, homing assays have not shown a functional role for splenic MAdCAM-1 in the mouse (13). Importantly, these studies focused only on total lymphocyte numbers entering the spleen and did not address the possibility that the homing of some subsets of lymphocytes may have been inhibited. Evidence for MAdCAM-1 mediated splenic lymphocyte accumulation has come from the study of human gastric lymphomas (56). In this study the authors suggest that the localization of mucosal associated lymphoma cells to the splenic marginal zone may be due to $\alpha 4\beta 7$ /MAdCAM-1 mediated adhesion. Sensitivity to soluble chemokines may act as a second level of discrimination, regulating the accumulation of CD8 $^{+}$ $\gamma\delta$ T cells, and other cell types, to distinct areas of the spleen, once they have exited the blood via MAdCAM-1. Indeed, the migration of B cells to the B cell zone of the follicle has been shown to be mediated by a specific chemotactic response to the soluble chemokine BLC (58).

If MAdCAM-1 is important in lymphocyte trafficking to the spleen, the question arises: why are there large numbers of $\gamma\delta$ T cells in the spleen with minimal or no expression of the $\beta 7$ integrin? The accumulation of $\beta 7$ -low cells in the spleen may be accomplished through several different modes. $\gamma\delta$ T cells may home to the spleen using MAdCAM-1/ $\alpha 4\beta 7$ interactions and subsequently undergo a down regulation of the $\beta 7$ integrin in response to cytokines or other factors within the spleen. Conversely, $\beta 7$ -low $\gamma\delta$ T cells, which are not found in appreciable numbers in other tissues, may represent a non-circulating population of cells, possibly unique to the spleen or produced in the spleen, and may never express high levels of the $\beta 7$ integrin. Finally, $\beta 7$ -low cells may accumulate in the spleen by unidentified homing and/or retention mechanisms. Although the role of $\gamma\delta$ T cells in the spleen is largely unknown, the observation that a large proportion of the $\gamma\delta$ T cells in the spleen express low levels of the $\beta 7$ integrin

demonstrates that these cells represent a distinct population of cells compared to $\gamma\delta$ T cells from all other organs and tissues examined.

In summary, we previously showed that bovine $CD8^+\gamma\delta$ T cells, which constitute a TCR-defined subset of $\gamma\delta$ T cells, lack the adhesion molecules necessary to efficiently home to sites of inflammation. Although $CD8^+\gamma\delta$ T cells lack several homing-associated molecules they accumulate in large numbers in some organs and tissues. This study now extends the original findings by demonstrating $CD8^+\gamma\delta$ T cells predominantly accumulate in tissues expressing the homing molecule MAdCAM-1. $CD8^+\gamma\delta$ T cells express high levels of the $\beta 7$ integrin, a component of the lymphocyte ligand for MAdCAM-1. The $\beta 7$ integrin detected on $\gamma\delta$ T cells appears to be functional $\alpha 4\beta 7$, based on the observation that the percentage of $CD8^+\gamma\delta$ T cells is greatly enriched through adhesion to MAdCAM-1 transfected CHO cells. These results demonstrate a direct correlation between the accumulation of a TCR-defined subset of $\gamma\delta$ T cells with the expression of an adhesive phenotype, and suggest that in some cases site specific homing is, in part, responsible for the accumulation of $CD8^+\gamma\delta$ T cells in tissues.

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CHAPTER 5

SUMMARY AND CONCLUSION

Almost a decade ago it was discovered that in the mouse, $\gamma\delta$ T cells, defined by TCR usage, accumulated in distinct tissues and organs. This TCR-defined subset accumulation is especially striking, in that, within a certain tissue virtually every $\gamma\delta$ T cell may use the same $V\gamma$ region. A similar phenomenon has since been described in the human system. Although TCR-defined subset accumulation has been well described, many basic facts of this phenomenon remain unanswered. Specifically, how do TCR-defined subsets of cells accumulate in distinct locations, and does the function of one subset of $\gamma\delta$ T cells differ from that of another?

It was with these basic questions in mind that I formulated my hypothesis, that selective homing contributed to the accumulation of TCR-defined subsets of $\gamma\delta$ T cells, and began this research project. The first project attempted was to characterize antibodies against the bovine $\gamma\delta$ TCR. These antibodies were needed to confirm that TCR-defined subsets of $\gamma\delta$ T cells existed in cattle, as had been shown in the mouse and human. This work is described in Chapter 2 of this dissertation. Using these antibodies, four distinct TCR-defined subsets of bovine $\gamma\delta$ T cells were defined. The analysis of TCR-defined subsets of $\gamma\delta$ T cells revealed a preferential accumulation of GD3.1 positive cells in artificially inflamed lymph nodes. This increase in GD3.1 positive cells was either due to the selective accumulation of subset 1 cells, or the selective accumulation of subset 3 cells in concert with the selective exodus of subset 2 cells from the node. Although either of these scenarios is possible, the selective exodus of cells from a site has not been

previously reported. This data indicates that the increase of GD3.1 positive cells in inflamed lymph nodes is most likely due to the accumulation of subset 1 cells. Subset 4 cells were found preferentially in the spleen. These results indicate that, as previously shown in mice and humans, TCR-defined subsets of $\gamma\delta$ T cells exist in cattle and these subsets specifically accumulate in distinct tissues.

With the tools in hand to identify TCR-defined subsets of $\gamma\delta$ T cells in the bovine system and evidence that differences exist both in the homing and accumulation patterns of these cells, characterization of bovine $\gamma\delta$ T cell subsets in context of the leukocyte adhesion molecule profile was performed. Subset 4 cells were of particular interest because large numbers of them were found preferentially in the spleen and gut, but not in peripheral tissues, suggesting these cells utilize distinct homing mechanisms.

In chapter 3, it was demonstrated that many subset 4 cells co-express the CD8 and CD2 molecules. Furthermore, these cells express a unique adhesion molecule profile, distinctly different from that which was previously described for circulating bovine $\gamma\delta$ T cells. CD8⁺ $\gamma\delta$ T cells were shown to express low levels of a variety of large molecular weight proteins that are expressed by the majority of $\gamma\delta$ T cells, including the adhesion molecules L-selectin, E-selectin ligands, GR antigen ligands, and the lineage specific molecules WC1 and GD3.5. In a novel *in vivo* homing assay it was demonstrated that CD8⁺ $\gamma\delta$ T cells, lacking these adhesion molecules, accumulated inefficiently at sites of inflammation, whereas, $\gamma\delta$ T cells expressing these adhesion molecules accumulated in inflammatory sites. At this point in my research I had successfully demonstrated that CD8⁺ $\gamma\delta$ T cells did not express a wide array of leukocyte adhesion molecules. Furthermore, the lack of adhesion molecules on CD8⁺ $\gamma\delta$ T cells correlated with the inability of these cells to accumulate in sites of inflammation. However, potential modes of accumulation of these cells in mucosal sites and the spleen remained enigmatic due to the lack of a positive correlation between homing molecule

expression and the tissue specific accumulation observed *in vivo*.

In chapter 4, the described studies show that CD8⁺γδ T cells were found to express functional α4β7 integrin, a component of the lymphocyte ligand for the mucosal addressin MAdCAM-1. Furthermore, as a function of total γδ T cells, the accumulation of CD8⁺γδ T cells correlated directly with the tissue expression of MAdCAM-1. In support of the hypothesis that MAdCAM-1/α4β7 interactions contributed to lymphocyte accumulation in these sites, it was demonstrated that the β7 integrin expressed on CD8⁺γδ T cells was functional. Using an *in vitro* adhesion assay utilizing MAdCAM-1 transfected cells, peripheral blood CD8⁺γδ T cells were greatly enriched, essentially mimicking the accumulation of CD8⁺γδ T cells observed in MAdCAM-1 expressing tissues *in vivo*. Thus, these results provide a positive correlation of the expression of the adhesion molecule β7, and the accumulation of CD8⁺γδ T cells in MAdCAM-1-expressing tissues.

In conclusion, I have tested the hypothesis that tissue specific accumulation of γδ T cells is controlled, in part, by selective homing. Differential expression of various lymphocyte expressed adhesion molecules on γδ T cells was demonstrated and the expression of lymphocyte adhesion molecules on γδ T cell subsets was found to correlate with the accumulation of these subsets in some tissues. These results demonstrate for the first time that the tissue specific localization of TCR-defined subsets of γδ T cells can be predicted, in part, by the expression of defined homing molecules on these cells. These results are significant in that they elucidate some of the mechanisms used by γδ T cell subsets to home to specific tissues. Ultimately, understanding how cells efficiently migrate to defined tissues may facilitate novel approaches to the treatment of inflammatory diseases, as well as more efficient targeting and better design of

therapeutic agents. In addition, if unique functions of $\gamma\delta$ T cell subsets are found, the function of individual subsets could potentially be modulated through the disruption, or enhancement, of lymphocyte homing.

CHAPTER 6

COLLABORATIVE STUDIES

The preceding chapters reflect the highly focused experiments of my dissertation work. However, I have also been involved in several collaborative studies not detailed in this dissertation. Some of these collaborative studies as well as some of my current research is outlined below.

Identification and characterization of the GR antigen

Working in conjunction with Dr. Mark A. Jutila and Sandy Kurk, we have described the GR antigen, a novel homing molecule involved in the trafficking of bovine leukocytes to sites of chronic inflammation. Using functional analysis of leukocyte rolling on endothelial cells, we showed that the rolling of $\gamma\delta$ T cells and neutrophils on LPS or cytokine activated endothelial cells can be greatly reduced or abrogated through the addition of an anti-GR antigen mAb. The GR antigen was also expressed on bovine platelets and functional GR antigen was secreted from activated platelets. Using platelet-derived soluble antigen we studied the distribution of GR antigen ligands on leukocytes. We found that bovine neutrophils, monocytes, as well as, most $\gamma\delta$ T cells expressed functional ligands for the GR antigen. Using bovine platelet derived GR antigen we were also able to demonstrate ligands for the GR antigen on human lymphocytes. Human GR ligands were predominantly expressed on memory $\alpha\beta$ T cells, with expression correlating

to high expression of the CLA antigen. This work is detailed in the manuscript:

Mark A. Jutila, **Eric Wilson**, and Sandy Kurk. 1997. Characterization of an Adhesion Molecule that Mediates Leukocyte Rolling on 24 hr Cytokine- or Lipopolysaccharide-stimulated Bovine Endothelial cells under Flow Conditions. *Journal of Experimental Medicine* 186:1701.

CHAPTER 7

CURRENT RESULTS AND FUTURE STUDIES

Differential expression of chemokine receptors on $\gamma\delta$ T cell subsets

The adhesion molecule profile of a lymphocyte is useful in predicting if a leukocyte can tether and roll on endothelial cells. However, as discussed in chapter 4, it is not always possible to predict which cells will transmigrate by the adhesion molecule profile expressed on the cell surface. The ability of a cell to firmly adhere and transmigrate is believed to be partly due to the chemokine sensitivity of the cell. The ability to detect and respond to chemokines is in turn dependent on the array of chemokine receptors expressed on the cell surface.

Recently I have found that CD8⁺ $\gamma\delta$ T cells express up to 15-fold higher levels of the chemokine receptor CXCR4, when compared to $\alpha\beta$ T cells and CD8⁻ $\gamma\delta$ T cells (data not shown and Fig. 1). CXCR4, which binds the chemokine SDF-1 α , was recently shown to induce rapid firm adhesion of rolling bovine $\gamma\delta$ T cells. These results demonstrate that distinct differences exist between $\alpha\beta$ T cells as well as CD8⁻ and CD8⁺ $\gamma\delta$ T cells with respect to the expression of chemokine receptors. Varying expression levels of CXCR4 potentially influence the differential migration of CD8⁺ $\gamma\delta$ cells into tissues such as gut and spleen. Studies are ongoing to determine if high levels of CXCR4 expressed on the surface of CD8⁺ $\gamma\delta$ T cells correlate with increased sensitivity to the chemokine SDF-1 α .

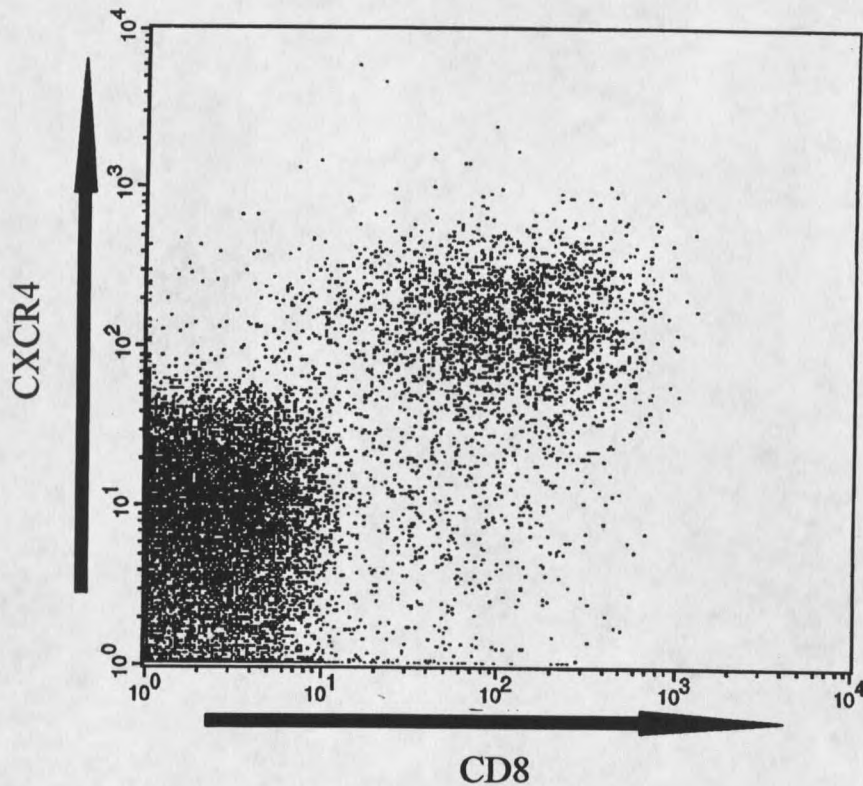


Figure 16

CD8⁺γδ T cells express high levels of the chemokine receptor CXCR4. Three-color FACS analysis was performed on peripheral blood lymphocytes using mAbs recognizing γδ T cells, CD8, and the CXCR4 chemokine receptor. Plot was generated by gating on all γδ T cells. CD8 and CXCR4 expression are shown in the x and y axis respectively. Mode CXCR4 fluorescence of CD8⁻γδ T cells, 14. Mode CXCR4 fluorescence of CD8⁺γδ T cells, 156.

γδ T cell subset accumulation in milk

The accumulation of lymphocytes in the milk is believed to be primarily a result of specific homing, rather than selective retention or expansion of cells. Milk and milk

lymphocytes are seldom retained in the cistern of the mammary gland (udder) for the time required for even a single doubling of cells to occur, suggesting that selective proliferation/expansion or selective retention of cells do not contribute to the accumulation of any subset of cells in the milk. Therefore, if a subset of lymphocytes is found in a greater proportion in the milk than in the blood, these cells most likely accumulated there through specific homing. Homing receptors in the mammary gland are not well characterized. However, MAdCAM-1 has been reported to be expressed in lactating mouse mammary gland. In an attempt to definitively prove that the accumulation of CD8 $\gamma\delta$ T cells to sites expressing MAdCAM-1 is due to the specific homing of these cells, the lymphocyte populations in bovine milk were analyzed. Working in conjunction with Dr. Jindrich Soltys, we have found that in the milk of some dairy cows, the percentage of CD8 $\gamma\delta$ T cells, as a function of total $\gamma\delta$ T cells, is greatly increased when compared to circulating blood lymphocytes. However, this has not been the case in all cows tested. We are currently exploring the possibility that in lactating cattle the co-expression of the CD2 molecule on $\gamma\delta$ T cells may be a more reliable marker of MAdCAM-1 homing cells than the CD8 molecule.

Cytokine expression of $\gamma\delta$ T cell subsets

One of the original questions I was interested in when beginning my work with $\gamma\delta$ T cells was: are TCR-defined subsets of $\gamma\delta$ T cells responsible for different immunological functions? Recently our laboratory has acquired the ability to efficiently sort large numbers of $\gamma\delta$ T cell subsets using a high speed cell sorter. Working in conjunction with Dr. Nicole Meissner we are now sorting pure populations of $\gamma\delta$ T cells subsets, and through the use of RT-PCR we are beginning to analyze these cells for the production of various cytokines. Thus far, Dr. Meissner has preliminary evidence

showing that CD8⁺γδ T cells differ from other γδ T cells in the production of IL-4. Ultimately, we plan to use cDNA collected from sorted populations to do SAGE analysis in hopes of better understanding the differences between γδ T cell subsets at the molecular level.

In the preceding dissertation I have characterized the adhesion profile of TCR-defined subsets of γδ T cells. In work peripheral to this dissertation, I have assisted in the initial characterization of the GR antigen, a novel adhesion molecule used in the trafficking of γδ T cells. Currently, I am working on further characterization of the homing and accumulation profiles of distinct subsets of γδ T cells. The potential role of differential chemokine sensitivity of γδ T cell subsets is also being investigated to determine the mechanism of lymphocyte subset accumulation. This current research extends the work described in detail, in chapters 2 through 4, and may increase our understanding of the mechanisms by which γδ T cell subsets accumulate, with such striking efficiency, in some tissues and organs.

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