

COMPREHENSIVE TRANSCRIPTIONAL PROFILING OF $\gamma\delta$ T CELLS

by

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ABSTRACT

$\gamma\delta$ T cells have been conserved since the adaptive immune system arose, yet their importance is still unclear. In an attempt to compensate for the lack of a broad knowledge-base of $\gamma\delta$ T cells across species, global analyses of $\gamma\delta$ T cell transcriptomes have been performed using serial analysis of gene expression (SAGE). Twelve new SAGE libraries were generated from the following bovine lymphocyte populations: magnetic bead-sorted blood $\gamma\delta$ T cells, spleen $\gamma\delta$ T cells and enriched $\alpha\beta$ T cells from a single calf, both rested and ConA/IL-2 stimulated, and flow cytometry-sorted blood $\gamma\delta$ and $\alpha\beta$ T cells each either rested, ConA/IL-2, or PMA/ionomycin stimulated. These databases were analyzed using new web-based bioinformatic tools, which allow the user to rapidly compare gene expression patterns within these and other SAGE and standard EST libraries generated from different cell types and different species. These analyses revealed striking differences between blood and spleen $\gamma\delta$ T cells and how these cells respond to various mitogenic stimulation. These analyses also confirm previous studies that suggest that global gene expression in $\gamma\delta$ and $\alpha\beta$ T cells is quite similar; however, a five-fold increase in $\gamma\delta$ T cell-specific transcripts could be induced by ConA/IL-2 stimulation of spleen cells. Even greater differences were seen between the two lymphocyte populations isolated from blood, regardless of activation state. These new public databases provide additional resources for the annotation/analysis of global gene expression in $\gamma\delta$ T cells, which will facilitate studies of the biology of this enigmatic lymphoid cell.

STUDY INTRODUCTION

$\gamma\delta$ T Cells

$\gamma\delta$ T cells appeared with the adaptive immune system 400-500 million years ago and have been well conserved (76), yet their specific roles in the immune system are largely undefined (rev. in 76). $\gamma\delta$ T cells were first recognized in 1986 by Brenner *et. al.* (23) and Bank *et. al.* (6) when a minor lymphocyte population lacking the T cell-receptor (TCR) α - and β -chains was identified. These cells expressed the T3 glycoprotein in a complex with other polypeptides, which have now been identified as the TCR γ - and δ -chains. Some $\gamma\delta$ T cells show monospecificity, as do $\alpha\beta$ T cells, and others show great promiscuity in the antigens they recognize (102).

A consensus concerning the importance of $\gamma\delta$ T cells to human and animal health has not developed. $\gamma\delta$ T cells seemingly can perform many of the same major functions as $\alpha\beta$ T cells, but they also display unique activities (102, Table 1.1). Four major functions of $\gamma\delta$ T cells are: 1) cytolytic destruction of stressed or transformed cells, 2) regulation of inflammation and immune responses, 3) modulation of epithelial cell growth and functional integrity, and 4) innate responses to pathogens. However, these activities are not consistently seen in all animal species. Indeed, differences in $\gamma\delta$ T cells from humans, rodents, ruminants, and birds complicate the extension of results from one species to another. For this reason, it is important to perform global gene expression

analyses to identify trends that are conserved between species that would not be identified by studying only single genes in a single species.

Table 1.1 Innate and adaptive immune cell characteristics of $\gamma\delta$ T cells.

Adaptive Immune Cell Characteristics

TCR-dependent recognition of specific antigens
 Cytokine secretion
 Regulation of Ig class switching
 Maturation into memory cells
 Regulation of adaptive immune responses
 Pro-inflammatory
 Suppressive

Bridging Functions

Immunoregulatory
 Antigen presentation

Innate Immune Cell Characteristics

Limited diversity in T cell receptor
 Located at all portals of pathogen entry
 Recognition of self-antigens
 Responses to phosphoantigens and other unconventional antigens
 Expression of myeloid-related genes
 Direct recognition of pathogen associated molecular patterns (PAMPs)
 Modulation of epithelial cell growth and functional integrity
 Intestinal lipid metabolism and cholesterol homeostasis
 Cytolytic destruction of tumor transformed or infected cells

A comprehensive discussion of our current limited understanding of $\gamma\delta$ T cells is presented in the sections that follow. Though lengthy, this review illustrates that we are only beginning to gain an understanding of $\gamma\delta$ T cells. This review incorporates information obtained from work with rodent, ruminant and human cells and ends with a summary of the limited global gene expression analyses that have been done on $\gamma\delta$ T cells to date.

The $\gamma\delta$ T Cell Receptor (TCR)

A unique feature of T and B lymphocytes is that they are the only vertebrate cell type that uses somatic DNA rearrangements to form cell surface markers, namely TCRs or B cell receptors (BCRs; ref. 76). TCRs are glycoproteins comprised of polymorphic disulfide-linked α - and β -chains or γ - and δ -chains (6) and are composed of various combinations of gene segments. α - and γ -chains are composed of single copies of different V-, J- and C-gene segments. β - and δ -chains are composed of V-, D-, J- and C-gene segments. The $\gamma\delta$ TCR structurally resembles immunoglobulin (Ig) more than the $\alpha\beta$ TCR (165,174). Specifically, the complementarity-determining region 3 (CDR3), the major antigen binding site of the $\gamma\delta$ TCR, is more similar to the Ig binding domain than the $\alpha\beta$ TCR CDR3 (102,165). The $\gamma\delta$ TCR CDR3 binds peptides 8-21 amino acids in length, whereas the $\alpha\beta$ TCR CDR3 binds peptides only 6-12 amino acids long (165).

TCRs and BCRs undergo somatic DNA rearrangements resulting in receptor diversity that enables each clone to recognize a unique antigen. The $\gamma\delta$ TCR displays less diversity than Ig and the $\alpha\beta$ TCR because fewer V-, D-, and J-gene segments are available to the $\gamma\delta$ TCR (102). Also, the majority of $\gamma\delta$ TCR rearrangements occur during embryonic development (182,29,126), prior to thymic terminal transferase activation, which limits $\gamma\delta$ TCRs to simpler V-D-J recombination events (76). However, additional diversity is added to the γ - and δ -chains via N-nucleotide additions (102). The large majority of $\alpha\beta$ TCRs recognize only a single unique antigenic peptide, whereas the structure of the $\gamma\delta$ TCR allows for individual TCRs to bind a greater diversity of antigenic ligands

(165,174). In addition, $\alpha\beta$ T cells are restricted to antigens presented by MHC molecules (122), whereas many $\gamma\delta$ T cells are capable of recognizing antigen independently of antigen-presenting molecules (165,174).

Murine studies have shown that T cells expressing the $\gamma\delta$ TCR appear during development before those expressing the $\alpha\beta$ TCR (69). $\gamma\delta$ T cells develop in and are released from the thymus in waves based on their TCR expression (13). The first set of $\gamma\delta$ T cells shows no junctional diversity (1) and migrates from the thymus to the epidermis. Consecutive waves follow, migrating from the thymus and localizing in other tissues (91,72,19). The ordered $\gamma\delta$ TCR rearrangements have been hypothesized to be controlled by programmed rearrangement of specific $V\gamma$ genes (107,83,65,209), cytokines produced by thymic epithelial cells (3,77), and regulation of transcriptional enhancers (87,113).

$\gamma\delta$ T Cell Subsets

$\gamma\delta$ T cells can be divided into multiple subsets based on various cell-surface markers, which include TCR, differentiation antigens and other molecules, such as CD40 ligand. These various subsets generally possess unique functions and/or tissue localization (as discussed below). A common division of ruminant $\gamma\delta$ T cells is by the cell surface expression of CD8, a co-receptor for MHC class I, or WC1, a scavenger receptor (207). Genomic profiling of these subsets in cattle and subsets based on $V\delta$ -chain usage in humans, has uncovered information about the unique transcriptional activities of various $\gamma\delta$ T cell subsets (128,79,108) as discussed in the final section of this chapter. Another

frequently studied murine subset is the dendritic epidermal T cell (DETC), which has unique characteristics. The large majority of studies using gene knockout mice have likely missed many interesting functions of $\gamma\delta$ T cells because every subset is eliminated. However, more studies are now being done with V γ -subset knockout mice as they are becoming available (190,147). Additional studies on isolated populations or subsets of $\gamma\delta$ T cells will help identify subset-specific characteristics that are not visible when studying the population as a whole.

Bovine CD8⁺/WC1⁻ $\gamma\delta$ T Cells and Human V δ 1⁺ $\gamma\delta$ T Cells. Bovine CD8⁺/WC1⁻ $\gamma\delta$ T cells are rare in circulation but are abundant at mucosal surfaces and in the red pulp of the spleen (128,210,120), and represent the majority of IEL (79). CD8⁺ $\gamma\delta$ T cells cannot bind E-selectin or GR antigen, and lack L-selectin expression (210). The lack of L-selectin and selectin ligands prevents these cells from accumulating at sites of inflammation but does not prevent them from trafficking to the spleen (210). CD8⁺ $\gamma\delta$ T cells colocalize with MAdCAM-1 and migrate to CCR7 ligands within mucosal tissues (211). CD8⁺ $\gamma\delta$ T cells predominate in the spleen and may produce IL-4, which drives B cell production of antibodies in the spleen (211). The human V δ 1 $\gamma\delta$ T cells appear to represent a similar $\gamma\delta$ T cell subset as the CD8⁺ $\gamma\delta$ T cells in cattle. Like bovine CD8⁺ $\gamma\delta$ T cells, V δ 1 $\gamma\delta$ T cells are found at lower levels in circulation and predominate in the gut mucosa and spleen (45,86,44,201).

Bovine CD8⁻/WC1⁺ $\gamma\delta$ T Cells and Human V δ 2⁺ $\gamma\delta$ T Cells. CD8⁻/WC1⁺ $\gamma\delta$ T cells make up the majority of $\gamma\delta$ T cells in all species (120). They are present at higher levels than CD8⁺ $\gamma\delta$ T cells in the white pulp of the spleen, peripheral lymphoid tissues, sites of inflammation and in peripheral blood (128). There are 3 known variants of WC1 in ruminants, WC1.1 and WC1.2, expressed on non-overlapping subsets, and WC1.3, which is expressed on a small population of WC1.1⁺ $\gamma\delta$ T cells (206). WC1.1⁺ and WC1.2⁺ $\gamma\delta$ T cells differ in that WC1.1⁺ $\gamma\delta$ T cells decrease with the age of the animal, whereas WC1.2⁺ $\gamma\delta$ T cells appear to increase with age (166). Like bovine CD8⁻/WC1⁺ $\gamma\delta$ T cells, human V δ 2 $\gamma\delta$ T cells predominate in blood (201,178,24). Differences in transcriptional profiles and probable functions for these subsets are described below.

Dendritic Epidermal T Cells (DETC). DETC are a specialized subset of murine $\gamma\delta$ T cells, specializing in re-growth of epithelium (18) as discussed below. This murine-specific cell type has a branched appearance, expresses Thy1 and is a derivative of the first developmental wave of $\gamma\delta$ T cells (13,18). However, DETC may not be as unique as once hypothesized since Brandes *et al.* (21) demonstrated that human V δ 2 $\gamma\delta$ T cells can present antigen and have dendritic cell-like morphology similar to DETC.

Tissue Localization of $\gamma\delta$ T Cells

Many $\gamma\delta$ T cell subsets localize to specific tissues. Tissue-specific $\gamma\delta$ T cell subsets can be identified by distinct tissue-specific $\gamma\delta$ TCR expression. Some $\gamma\delta$ T cell subsets express tissue-specific V-segments that direct localization by recognition of antigens

involving tissue-specific components (1,74). Irrespective of subsets, $\gamma\delta$ T cells also localize to certain types of tissues. $\gamma\delta$ T cells may recognize antigens without classical MHC presentation within tissues (76). Since $\gamma\delta$ T cells recognize stress antigens or common pathogen-derived moieties, such as IPP (123), it is not necessary for them to circulate to the spleen or lymph nodes. This is likely the reason for the greater abundance of $\gamma\delta$ T cells in tissues than in circulating blood of some species (75).

Epithelial Tissues. $\gamma\delta$ T cells are the major population of T cells within the epithelia of skin and the mucosa (181,73), including the uterine and vaginal epithelia, tongue, lung, trachea, bladder and mammary tissues (76,92,10,173,208). IEL within rodent skin almost exclusively express the $\gamma\delta$ TCR and represent up to 50% of all gut T cells in most species (66,47,114). Localized in epithelial barriers, $\gamma\delta$ T cells play a role in the first line of defense against invading pathogens, as described below.

Spleen. The spleen functions to filter out dead or dying red blood cells and traps blood-borne antigens for presentation to T cells that are constantly circulating through the spleen. T cells enter the spleen by migrating across vessels in the marginal zone or by entering the red pulp through blood circulation. Like most tissues, the spleen contains a unique population of $\gamma\delta$ T cells. $CD8^+$ $\gamma\delta$ T cells accumulate in large numbers in the spleen. This subset remains in the red pulp, whereas $CD8^-$ $\gamma\delta$ T cells appear to quickly re-enter circulation (210). $\gamma\delta$ T cells localize to sites of T cell traffic within the spleen (marginal zone, red pulp and the marginal sinus) and are rarely found in the conventional

T cell regions of the spleen and lymph nodes (82). They are also rarely found in B cell follicles or germinal centers (82). Many $\gamma\delta$ T cells pass through the spleen and lymph nodes rather than localizing to specific regions within the immune organs as do $\alpha\beta$ T cells (82).

Variability Between $\gamma\delta$ T Cells in Rodents, Humans and Ruminants

There are many functional and phenotypic similarities between human, rodent and ruminant $\gamma\delta$ T cells. For example, most $\gamma\delta$ T cells in all species lack expression of CD4 and CD8 (76,17,100,53,35,71). However, differences exist between species in expression of CD2, CD4 and CD8 on subsets. Specifically, all human $\gamma\delta$ T cells express CD2, whereas only a minor subset of bovine $\gamma\delta$ T cells expresses CD2 (53,35,71). The major population of circulating $\gamma\delta$ T cells in adult humans expresses the V γ 2/V δ 2 TCR (also known as V γ 9/V δ 2 in alternate nomenclature) and is responsive to various pathogens, including *M. tuberculosis* (41). Though subsets of $\gamma\delta$ T cells are present in the mouse, which respond to similar pathogens, their TCR usage and the specific antigens they recognize are different (149,148,88). Another difference between human and mice is that humans lack equivalent $\gamma\delta$ T cell subsets as those found in the epidermis and epithelium of the murine reproductive tract and tongue (DETC; ref. 20).

In most animals, $\gamma\delta$ T cells make up about 10% of total lymphocytes at birth and decrease with age (69,109). However, $\gamma\delta$ T cells from calves and other young ruminants comprise 30-70% of circulating lymphocytes (82). $\gamma\delta$ T cells can represent up to 75% of

the double-negative (CD4⁻CD8⁻) T cells in bovine PBMCs (82). Ruminants are typically hosts to many pathogens, many of which attack the mucosal surfaces, which might explain the much higher level of $\gamma\delta$ T cells in ruminants than in other species (82). The abundance of $\gamma\delta$ T cells in ruminants suggests a likely greater importance to the host's health than to those species with fewer $\gamma\delta$ T cells. Therefore, experiments performed in these systems will likely yield greater responses than in hosts with a very low level of $\gamma\delta$ T cells. The relatively high percentage of peripheral blood $\gamma\delta$ T cells in ruminants facilitates the isolation of large numbers of these cells for bulk experimental purposes.

Foreign Antigen Recognition by $\gamma\delta$ T Cells

The process of antigen recognition by $\gamma\delta$ T cells is obscure. Although some $\gamma\delta$ T cells can recognize antigens presented by both MHC class I and MHC class II molecules (106,85,124,67), many are β_2 M independent and therefore are not MHC class I-dependent (163,111,165,174). In fact, most studies demonstrate that $\gamma\delta$ T cell ligands are nonpeptidic and MHC-independent (174,191,109). There are many non-classical MHC antigen-presenting molecules that might present antigens to $\gamma\delta$ T cells. For example, CD1c, CD48 and Hsp60 appear to present non-classical antigens to $\gamma\delta$ T cells (200,84,54,134,153,159,56,121,103). CD1 is expressed on cell surfaces with β_2 M, but unlike MHC class I and MHC class II, CD1 presents antigen independently of antigen processing (102).

$\gamma\delta$ T cells recognize a greater diversity of antigen forms than do $\alpha\beta$ T cells. For example, $\gamma\delta$ T cells are stimulated by both peptide and non-peptide mycobacterial antigens (191,15,175,41,192,25) and can recognize and respond to proteins and non-proteinaceous phospholipids (156). Conventional antigen-processing pathways are not used for these non-peptide antigens (13). Human double negative ($CD4^-CD8^-$) $\gamma\delta$ T cells have been shown to recognize non-proteinaceous microbial components in the context of CD1b (8,180,160). Since $\gamma\delta$ T cells recognize a different category of antigens, they may play a complementary role, rather than a redundant role, to $\alpha\beta$ T cells.

$\gamma\delta$ T Cell Function

$\gamma\delta$ T Cells as Regulatory Cells. In many immune responses, $\gamma\delta$ T cells do not play a direct role in pathogen clearance. They instead regulate the type and intensity of the induced immune response (31). The most common effect of $\gamma\delta$ T cell deficiency during an infection is the lack of immunoregulation (17). It has been observed that pro-inflammatory and anti-inflammatory activities of $\gamma\delta$ T cells can occur at the same time (75). This is most likely due to different subsets of $\gamma\delta$ T cells (perhaps $CD8^-$ and $CD8^+$ or $V\delta 2$ and $V\delta 1$) performing their specific functions simultaneously (128,79).

$\gamma\delta$ T cells often initiate inflammation by cytokine secretion. Some pro-inflammatory chemokines expressed by $\gamma\delta$ T cells are RANTES, macrophage inflammatory protein-1 α (MIP1 α), MIP1 β and lymphotactin (79,55,179). Like NK cells, $\gamma\delta$ T cells can assist in the destruction of pathogens by secreting molecules like IFN γ , which also regulates

other cell types (76). Without $\gamma\delta$ T cell-secreted stimulatory cytokines, such as interferon- γ (IFN γ), macrophages and NK cells produce less IFN α and IFN γ , respectively (145), and thus the immune response is weakened.

A specific example of $\gamma\delta$ T cells directing inflammation is seen in the murine model of neurocysticercosis (NCC). NCC results in severe pathology of the central nervous system and intense recruitment of $\gamma\delta$ T cells and macrophages (33,34). This inflammation is caused by $\gamma\delta$ T cells secreting type 1 cytokines (34), such as MCP-1 and MIP1 α , in the brain during NCC (32). $\gamma\delta$ T cells appear to play a major role in the immunopathogenesis since $\gamma\delta$ T cell-deficient (TCR $\delta^{-/-}$) mice show less inflammation and reduced CNS pathology (32), despite the fact that they develop a systemic, disseminated infection (34).

Although $\gamma\delta$ T cells can play a pro-inflammatory role in some infection settings, it has been well documented that subsets (presumably bovine CD8 $^{+}$ and human V δ 1 $^{+}$; ref. 142,79,108) can play anti-inflammatory roles in other situations to prevent chronic disease (31,141). Consistent with an anti-inflammatory role, $\gamma\delta$ T cells secrete cytokines and other molecules to suppress and often terminate immune responses. Thymosin- β 4 (T β 4) is an immunosuppressive molecule originally identified in macrophages, but has now been identified in intraepithelial lymphocytes (IEL), murine DETC, and CD8 $^{+}$ $\gamma\delta$ T cells (79,170,93). The lymphocyte-specific isoform of T β 4 expressed by $\gamma\delta$ T cells appears to be more immunosuppressive than the other common macrophage isoform (170,93). In addition, $\gamma\delta$ T cells can down regulate $\alpha\beta$ T cell responses to environmental

antigens, irritants (75), or intracellular bacterial infections (134). $\gamma\delta$ T cells can interact with and suppress macrophages (31), as well as suppress the expansion of B cells in vivo (76). The immunosuppressive behavior is evident in $\gamma\delta$ T cell autoimmune diseases that are often manifested by a down-regulation of the extent of inflammation (31).

$\gamma\delta$ T Cells as Antigen Presenting Cells. It has been known for several years that $\gamma\delta$ T cell subsets can function as antigen presenting cells for other $\gamma\delta$ T cells (203) and $CD4^+$ $\alpha\beta$ T cells (40). These cells express CD1 on their surfaces, which might be used for presentation of unprocessed antigen (203,136). Processing and presentation of conventional peptide antigens by $\gamma\delta$ T cells to $CD4^+$ $\alpha\beta$ T cells was originally described by Collins *et al.* (40) in bovine $\gamma\delta$ T cell lines. Cultured or stimulated bovine $\gamma\delta$ T cells can endocytose, process and present soluble antigens on MHC II.

Recently, these findings from ruminants have been confirmed in the human $V\gamma2/V\delta2$ $\gamma\delta$ T cell subset (21). Brandes *et al.* (21) showed that IPP stimulated $V\delta2$ $\gamma\delta$ T cells have a phenotype of professional APCs: up-regulation of MHC II, co-stimulatory molecules and adhesion molecules. $V\delta2$ $\gamma\delta$ T cells also express the LN-homing receptor CCR7, are present in mucosal draining LNs, and have “pre-activation” expression of CD69 (22,140). If these results hold true that $V\delta2$ can process and present soluble protein antigens (21), the role of $\gamma\delta$ T cells in innate and adaptive immunity is greater than even previously believed. Now that ruminant and human $\gamma\delta$ T cells have been shown to present processed and unprocessed antigens, can recognize specific antigens or

PAMPs, and can readily circulate through the lymph and blood (see below), these cells would be even greater targets for vaccines and immunotherapy.

$\gamma\delta$ T Cells in Mucosal Immunity. $\gamma\delta$ T cells are a major population of T cells within the mucosa (181,73) and have multiple functions within the mucosal, suggesting they are part of the first line of defense against mucosal pathogens. As in all tissues where $\gamma\delta$ T cells are localized, they can play an anti-inflammatory role or pro-inflammatory role in regulating other cell types, particularly in recruiting inflammatory cells to sites of mucosal damage (179,11). For example, $\gamma\delta$ T cells play roles in regulating IgA production within the gut lumen and in regulating oral tolerance (179).

Proliferating $\gamma\delta$ T cells are found in all intestinal compartments (194) and appear to have different migratory and proliferative patterns than $\alpha\beta$ T cells and B cells (194). Gut-derived $\gamma\delta$ T cells enter the intestinal lymph and re-enter intestinal tissue more often than other cells (194). $\gamma\delta$ T cells originating from the intestine return to the gut rapidly via the intestinal lymph (194). Intestinal $\gamma\delta$ T cells secrete cytokines distinct from those within peripheral lymphoid organs (14). Whether intestinal $\gamma\delta$ T cells have a thymic or intestinal origin is still unclear (61,89). It is thought that the CD8 $\alpha\alpha$ marker is an indicator of extra-thymic origin of CD8 $\alpha\alpha^+$ $\gamma\delta$ T cells (68). There is a $\gamma\delta$ T cell subset that can enter the cell cycle in the intestine, re-circulate quickly, preferentially through the gut, and is comparable to the residual cells in thymectomized pigs, suggesting this subset is extra-thymically derived (194).

$\gamma\delta$ T cells localized in the large intestine are different from those within the small intestine. Those in the large intestine express CD2 and L-selectin and are only weakly cytolytic, whereas those in the small intestine do not express CD2 nor L-selectin and are much more cytolytic (7,28). The majority of proliferating $\gamma\delta$ T cells in blood are actually proliferating $\gamma\delta$ T cells that were recently in the intestine (194); however, some clonally expanded $\gamma\delta$ T cells have been identified in the intestine that were not found in blood nor lymph (194).

$\gamma\delta$ T Cells as Innate Immune Cells. Though $\gamma\delta$ T cells display many conventional T cell characteristics, they also serve many innate immune functions as well. These characteristics include the ability to recognize self-antigens on transformed cells as do NK cells, respond to self- or pathogen-derived prenyl pyrophosphates, or directly recognize and respond to pathogen associated microbial patterns (PAMPs) through the expression of a variety of myeloid-associated genes. $\gamma\delta$ T cells are located at all portals of entry and can immediately respond to phosphate antigens or PAMPs, not needing clonal expansion first.

$\gamma\delta$ T Cells Recognize Self-Antigens. $\gamma\delta$ T cells function in innate immunity by responding to stressed cells and non-microbial tissue damage (142) by recognition of heat shock proteins (hsps; ref. 102) or other host cell surface molecules. Hsps, known to bind small peptides (13), and non-classical MHC molecules have been suggested as alternative antigen presenting molecules for $\gamma\delta$ T cells (69,189,57,187). Some $\gamma\delta$ T cell subsets

likely survey for cell damage or distressed cells, rather than specific pathogens (13) and can initiate a polyclonal response to these self-proteins up-regulated by infected, transformed or stressed self cells (36). Because $\gamma\delta$ T cells recognize self-antigens, the same $\gamma\delta$ T cell population can respond to cells infected with herpes simplex virus-1 or the unrelated vaccinia virus, for example (25). This recognition causes expansion and activation of $\gamma\delta$ T cells regardless of their TCR specificities.

This role in innate immunity is highlighted by the similarities of $\gamma\delta$ T cells to NK cells. Virtually all $\gamma\delta$ T cells express NK receptors, whereas $\alpha\beta$ T cells rarely express any NK receptors (60,96,188). $\gamma\delta$ T cells can detect target cells with altered MHC I expression due to tumor transformation or viral infection, therefore functioning in immunosurveillance like NK cells (169). NKG2D, a common NK receptor found on $\gamma\delta$ T cells, recognizes self-molecules on transformed or infected cells (144,154). $V\gamma 6/V\delta 1$ T cells express NKG2D in response to kidney damage and therefore may recognize endogenous tissue ligands up-regulated by inflammation (213). Because of innate receptor expression, $\gamma\delta$ T cells may recognize virus and tumor cells in non-antigen-specific or TCR-independent ways (51).

Killer cell lectin-like receptor G1 (KLRG1) is expressed on NK, effector memory $\alpha\beta$ T cells and the majority of human peripheral $V\gamma 2/V\delta 2$ T cells (49). $KLRG1^+$ $V\gamma 2/V\delta 2$ T cells have an effector memory phenotype of $CD27^-$, $CD45RA^-$, $CD62L^-$, and $CCR7^-$. Although effector memory $\alpha\beta$ T cells can express KLRG1, $KLRG1^+$ $\gamma\delta$ and $\alpha\beta$ T cells

respond differently to stimulation. KLRG1 expression on $\alpha\beta$ T cells decreases proliferation, whereas KLRG1 expression on $\gamma\delta$ T cells induces proliferation (49).

$\gamma\delta$ T Cells Respond to Prenyl Pyrophosphates. A common stimulant of human $\gamma\delta$ T cells is isopentenyl pyrophosphate (IPP; ref. 191), a nucleotide derivative from two separate metabolic pathways: the mevalonate pathway (eukaryotes and Gram-positive bacteria; ref. 64) and the non-mevalonate pathway (eubacteria; ref. 41,168). $\gamma\delta$ T cells respond to phospho-antigens likely produced from stressed cells over-expressing IPP, living bacteria at the site of infection (146), or nitrogen-containing bisphosphonates and alkylamines that block the mevalonate pathway and induce an intracellular accumulation of IPP (195). HMB-PP is an intermediate in the non-mevalonate pathway of IPP biosynthesis and is present in all V γ 2/V δ 2 $\gamma\delta$ T cell stimulating microbial pathogens (*E.coli*, mycobacterium and malaria) and is not present in non-stimulating microorganisms (streptococci, staphylococci and fungi) or the human host (50). There is no evidence that HMB-PP binds the $\gamma\delta$ TCR (136,26,25,2). Likewise, IPP is recognized by $\gamma\delta$ T cells independently of MHC, MHC-like and CD1 molecules (136), though the effect of IPP appears to be specific to human V γ 2/V δ 2 T cells since that population can be expanded from cultures of PBMCs with HMB-PP (49) or IPP (108).

In response to phosphate antigens, activated $\gamma\delta$ T cells play an immunoregulatory role by stimulating other innate cells, including dendritic cells in both a paracrine and cell-contact mediated fashion (123). For example, when $\gamma\delta$ T cells encounter IPP, the

transcription factors NF- κ B and AP-1 are activated and TNF α secretion is induced (38). Blocking of NF- κ B blocks IPP-induced production of TNF α , MIP1 β and RANTES (38).

$\gamma\delta$ T Cells Express Myeloid-Related Genes. Recent genomic analyses of $\gamma\delta$ T cells have identified the expression of many transcripts thought to be specific to myeloid cells. These genes encode for secreted factors, transcription factors, cell surface proteins and PAMP receptors. SAGE analysis identified cytokines that support myeloid cell differentiation and activation, such as GM-CSF and G-CSF, to be produced by $\gamma\delta$ T cells. Likewise, IgE-dependent histamine-releasing factor, a B cell growth factor and activator of basophils was also identified (128). Interestingly, message for B lymphocyte-induced maturation protein-1 (Blimp-1), described as a master regulator of B cell and monocyte differentiation (157,197), is expressed and regulated in bovine and human $\gamma\delta$ T cells (128, J.C. Graff and M.A. Jutila manuscript in preparation). One of the most abundant transcripts identified by SAGE in $\gamma\delta$ T cells was connexin43, which is expressed in activated macrophages (95) and may allow $\gamma\delta$ T cells to form gap junctions with epithelial and/or endothelial cells (128).

$\gamma\delta$ T cells can respond to microbial compounds such as glycoproteins, peptides, carbohydrates, and lipids (pathogen associated molecular patterns (PAMPS); ref. 81,146) through the expression of myeloid-related intracellular and cell surface receptors. These PAMP recognition receptors include Toll-like receptors (TLRs) and scavenger receptors. Scavenger receptors identified on $\gamma\delta$ T cells include scavenger receptor 1 (128), SRBI, CD36 (81,118), CD68 (128), mannose-binding lectin (MBL) (79,81) and the ruminant-

specific WC1 (139,205). Transcripts for many TLRs and related genes have been identified in $\gamma\delta$ T cells, including TLRs 1-5, 8 and 9 (81,132) and MyD88, TRAM and SARM (81). CD14, PKR (PRKR), CD38 and CD11b are among other innate receptors identified on $\gamma\delta$ T cells (128,81). Nucleotide-binding oligomerization domain 1 (NOD1) and NOD2 are intracellular receptors thought to be of myeloid origin that are also expressed by $\gamma\delta$ T cells (81).

Cells of the innate immune system can often recognize dsRNA through cell-surface receptors (TLR3) or through intracellular targets such as the IFN-inducible dsRNA-activated protein kinase R (PKR or PRKR; ref. 115,130). To date, it is not clear whether $\gamma\delta$ T cells are able to respond directly to dsRNA. Kunzmann *et al.* (110) demonstrated that $\gamma\delta$ T cells do not respond to poly(I:C) in PBMCs without dendritic cells (CD11c⁺) that are TLR3 positive. However, microarrays have recently indicated that $\gamma\delta$ T cells do express PKR transcripts (78), and TLR3 transcripts have been identified in $\gamma\delta$ T cells by quantitative real-time RT-PCR (81). Either way, $\gamma\delta$ T cells can become partially activated (increased CD69 expression, but no change in CD25 or HLA-DR) in response to type I IFNs produced by dsRNA-stimulated NK cells or dendritic cells (110). $\gamma\delta$ T cells may participate in rapid innate immune responses through PAMP receptors or other myeloid proteins. However, the expression and function of these innate genes on $\gamma\delta$ T cells is just beginning to be characterized.

$\gamma\delta$ T Cells in Epithelial Cell Health and Wound Maintenance. In addition to recognizing and responding to altered-self antigens and PAMPS, $\gamma\delta$ T cells also function in innate immunity by helping to maintain the body's protective barriers. $\gamma\delta$ T cells are the major population of T cells within the epithelia of skin and the mucosa (181,73). They support epithelial cell growth and maintenance by expressing cytokines, specifically keratinocyte growth factor (KGF; ref. 179,18). KGF is a member of the fibroblast growth factor family that shows strict specificity for epithelial cells in various tissues. It is produced by activated $\gamma\delta$ IEL and DETC (12). DETC can recognize host-derived ligands, possibly heat-stress ligands (18). Intestinal $\gamma\delta$ IEL also express KGF, which regulates turnover and maturation of enterocytes (105). Unlike $\alpha\beta$ IEL that do not express KGF, activated, but not resting, $\gamma\delta$ IEL from intestine, skin and other tissues express KGF (12). Therefore, $\gamma\delta$ T cells may regulate tissue growth and differentiation in response to injury (11).

$\gamma\delta$ T Cells and Infectious Disease. $\gamma\delta$ T cells play varying roles to different degrees during various infections. This is likely due to the differences in subsets localized to different infected tissues, immunoregulatory effects, or expression of various innate receptors by $\gamma\delta$ T cells. Some studies even show that some specific anti-pathogen functions of $\gamma\delta$ T cells are irrelevant if functional $\alpha\beta$ T cells are present (rev. in 76). For example, there is no difference in susceptibility to larval *Taenia crassiceps* infection between δ -gene knockout mice and wild type mice (196). However, $\gamma\delta$ T cell responses

to many pathogens have been identified. Early or late $\gamma\delta$ T cell responses may depend on the type and/or dose of bacteria, as well as the site of infection (52). Though it was previously thought that $\gamma\delta$ and $\alpha\beta$ T cells had similar functions, it is becoming clear that the roles of $\gamma\delta$ T cells are distinct and complementary to that of $\alpha\beta$ T cells. It has been hypothesized that $\gamma\delta$ T cells function during the interval between the innate response and induction of the adaptive response (48).

Murine models of microbial infection demonstrate the necessity of $\gamma\delta$ T cells by depletion using $\gamma\delta$ T cell-specific antibodies (102) or gene knockout mutants (135). It has been demonstrated that $\gamma\delta$ T cells are required for host survival against *Nocardia asteroides* infections (104) and early protection against *Staphylococcus aureus* (151) and *Listeria monocytogenes* (215) infections. A critical role of $\gamma\delta$ T cells during acute, extracellular bacterial infections has been demonstrated through *Klebsiella pneumoniae* infection in $\gamma\delta$ T cell knock-out mice (135). Through cytotoxic responses, $\gamma\delta$ T cells can also control herpes simplex virus (HSV) replication and spread (177). $\gamma\delta$ T cells can lyse human herpes virus-6 (HHV-6) infected cells (13) until they themselves become infected (119). $\gamma\delta$ T cell cytotoxicity may depend on the perforin pathway and Fas ligation to target cells (116,214). $\gamma\delta$ T cells play an important role in fighting other viral infections, as demonstrated by their ability to initiate an inflammatory response during influenza infection (31,30).

$\gamma\delta$ T cells may act as part of the first line of defense against many infections initiated within the epithelium. As $\gamma\delta$ T cells localize in epithelial layers, they survey for

microbial infections (102). For example, $\gamma\delta$ T cells limit the ability of *Staphylococcus aureus* to replicate and spread (133). $\gamma\delta$ T cells also recognize self-stress antigens, which are quickly identified, allowing $\gamma\delta$ T cells to respond rapidly to infected cells (94).

$\gamma\delta$ T cells accumulate at sites of inflammation associated with certain microbial infections, such as reactive lesions of leprosy and cutaneous leishmaniasis (102,131), as well as viral and parasitic infections such as malaria and toxoplasmosis (52). Human IBD patients and murine IBD models show an increased level of $\gamma\delta$ T cells (134,62,127,184). An increase in intestinal $\gamma\delta$ T cells circulating in the peripheral blood and near intestinal lesions in IBD models may be due to activated $\gamma\delta$ T cells migrating from the intestine or expansion of the few intestinal $\gamma\delta$ T cells normally present in blood (11). Circulating $\gamma\delta$ T cell numbers increase in several infectious states (69), such as tuberculosis, malaria, toxoplasmosis, salmonellosis and HIV infection (34,78). An increase in $\gamma\delta$ T cells is also seen in some non-infectious diseases. For example, asthma patients have much higher levels of $\gamma\delta$ T cells in their bronchoalveolar lavage (BAL) fluid (20%) compared to non-asthma patients (0.3%; ref. 186,185).

$\gamma\delta$ T cells can also be directly stimulated by many pathogens, such as mycobacteria (102), and can recognize both intact pathogens as well as pathogen components. *Plasmodium falciparum* infection activates $\gamma\delta$ T cells (171,97,176) but interestingly requires MHC class I. Lipoteichoic acid (component of *Staphylococcus aureus* cell wall) provides an activating signal to $\gamma\delta$ T cells through CD36 expression (118), which helps to eliminate the bacteria (133).

$\gamma\delta$ T cells may have immunoregulatory roles during many infections (76). For example, during influenza infection, the $\gamma\delta$ T cell response follows the $\alpha\beta$ T cell response, suggesting $\gamma\delta$ T cells function to repress the $\alpha\beta$ T cell response (76) and/or function in repair of tissue damage. Similarly, the $\gamma\delta$ T cell response is at its highest about 2-3 days after listeria infection has been defeated by macrophages, NK cells, and $\alpha\beta$ T cells (134,5). This response remains elevated for another 6-7 weeks (76). During a *Nocardia* infection, $\gamma\delta$ T cells recruit polymorphonuclear cells (PMNs) and macrophages to the lungs (76). Similarly, $\gamma\delta$ T cells produce Th1 cytokines (TNF α and IFN γ) and may therefore mediate protective immunity, playing a protective role against *Plasmodium falciparum* (171). During viral infection, $\gamma\delta$ T cells stimulate NK cells to secrete perforin and IFN γ , as well as stimulating CD8⁺ $\alpha\beta$ T cells (76). Splenic CD8⁺ $\gamma\delta$ T cells suppress IgE production and are powerful anti-inflammatory cells (112). For example, $\gamma\delta$ T cells may help regulate *Listeria monocytogenes* infections by suppressing the formation of liver lesions (134). On the other hand, $\gamma\delta$ T cells can be pro-inflammatory and recruit eosinophils resulting in allergic inflammation (112).

Measles virus (MV) glycoproteins cause negative regulation of T cell expansion, although this inhibition can be overcome by $\gamma\delta$ T cell-macrophage interactions (9). In vitro, human $\gamma\delta$ T cells can be expanded in culture by stimulation with phosphorylated nucleotide-containing microbial compounds or IPP in the presence of IL-2 (204). Expansion was inhibited by co-culturing $\gamma\delta$ T cells with UV-inactivated MV and persistently MV-infected T or monocytic cell lines (9). On the other hand, MV-infected

dendritic cells (DCs) promote the expansion of $\gamma\delta$ T cells (9). $\gamma\delta$ T cell resistance to MV-inhibition requires cell contact with macrophages, rather than soluble mediators (9).

$\gamma\delta$ T Cells in Mycobacterium Infection. Perhaps the best-defined $\gamma\delta$ T cell infectious agent response is that against mycobacterium. Human $\gamma\delta$ T cells represent the largest peripheral blood T cell population that is reactive towards *Mycobacterium tuberculosis* (Mtb; ref. 200,99) and the large majority of these $\gamma\delta$ T cells express the V γ 2V δ 2 TCR (98,152,70). In vitro, $\gamma\delta$ T cells proliferate vigorously and release cytokines in response to live Mtb or Mtb cell-associated proteins (99,16,143). They also respond similarly to non-protein, phosphorylated compounds, such as IPP (137). By depleting V γ 2V δ 2 $\gamma\delta$ T cells from peripheral blood, no $\gamma\delta$ T cells proliferate in response to Mtb (98). Without these Mtb-responsive $\gamma\delta$ T cells, persistent infection and/or chronic disease often occurs (31).

$\gamma\delta$ T cells peak about one week after *Mycobacterium bovis* bacillus Calmette-Guerin (BCG) infection, whereas the $\alpha\beta$ T cell response does not reach its peak until four weeks post-infection (48). The $\gamma\delta$ T cell response is characterized by the expansion of V γ 2 T cells and an influx of T cells expressing V γ 1 and four V δ genes (48). After stimulation with phorbol ester and ionomycin in vitro, $\gamma\delta$ T cells secrete IFN γ and are cytotoxic to BCG-infected macrophages or uninfected J774 macrophage cell line (48). This cytotoxicity is blocked by anti- $\gamma\delta$ TCR antibodies (48). Depletion of $\gamma\delta$ T cells has no

effect on the CD4⁺ $\alpha\beta$ T cell response but results in decreased CD8⁺ $\alpha\beta$ T cell cytotoxicity and IFN γ production (48).

There appear to be three pathways in which $\gamma\delta$ T cells are involved during a Mtb infection: 1) secretion of IFN γ , 2) lysis of infected target cells, and 3) induction of CD4⁺ and/or CD8⁺ $\alpha\beta$ T cell responses (48). $\gamma\delta$ T cells in BCG-infected lungs secrete IFN γ and TNF α upon antigen stimulation (48). IFN γ and TNF α induce NO synthase and production of NO by macrophages, which then causes a cytotoxic effect on intracellular bacteria (58,42,59). $\gamma\delta$ T cells may play a regulatory role following inoculation with low levels of Mtb by decreasing inflammation but play an important protective role if there is a high level of Mtb inocula (183). Similar to *Listeria monocytogenes* infection, $\gamma\delta$ T cells could be stimulated by cytokine production rather than direct antigen contact in Mtb infection (125).

$\gamma\delta$ T Cells in Salmonella Infection. A very recent study comparing the responses of mucosal-derived $\gamma\delta$ and $\alpha\beta$ T cells in an experimental model of *Salmonella enterica* serovar *typhimurium* enterocolitis demonstrated the dramatic differences in the early responses of the two major T cell subsets (78). Unlike $\alpha\beta$ T cells or B cells, the frequency of $\gamma\delta$ T cells in lymphatic fluid draining the gut mucosa increased in *Salmonella typhimurium*-infected calves. Included in the expanded $\gamma\delta$ T cell population were inflammatory WC1⁺ $\gamma\delta$ T cells that are normally found in circulation. Further microarray studies of the lymphatic $\gamma\delta$ T cells identified dramatic gene expression

changes at 48 hours p.i., the same time that the animals became clinically ill. The majority of differentially expressed genes in $\alpha\beta$ T cells decreased during infection. However, the majority of regulated genes in $\gamma\delta$ T cells increased during infection, including two PAMP receptors, TLR2 and PKR. Activation markers also increased on $\gamma\delta$ T cells, including CD25 and CD69. This study concluded that $\gamma\delta$ T cells respond rapidly to *S. typhimurium* infection by proliferating or migrating to the gut mucosa and by up-regulating the expression of many genes, including PAMP receptors, thus contributing to the innate response (78).

Use of Functional Genomics in the Analysis of $\gamma\delta$ T Cells

A method that can be used to overcome many of the obstacles faced when comparing $\gamma\delta$ T cells between species is to identify conserved trends, rather than comparing expression of single genes. Two widely used genomic analysis techniques have recently been developed to compare transcriptional profiles of various cell populations in multiple biological settings (128,79,55,179,78,81,108,154). The serial analysis of gene expression (SAGE) and microarrays have previously been used to compare transcriptional profiles of activated tissue or blood $\gamma\delta$ T cells (rev. in 80). These studies identified general trends of $\gamma\delta$ T cells that may not have been identified had the transcript expression of only a few genes been investigated.

Genomic Analysis Tools

The mRNA transcripts expressed by a cell in various situations is critical to determining cellular function. There are several techniques for identifying such transcripts. Northern blots, RNase protection assays and reverse transcriptase-polymerase chain reaction (RT-PCR) are commonly used to quantify relative amounts of specific mRNAs within a cell population. Although these techniques can yield large quantities of information, they are limited to transcripts with known sequences and logistically only a limited number of genes can be tested. Because these techniques are limited to specific genes, the data can be easily biased, and unexpected trends may never be identified. Recently, two high-throughput techniques, microarrays and SAGE, have become widely used to analyze global transcript expression within cell populations.

Briefly, microarray technology involves a microchip spotted with either cDNAs or oligonucleotides representing thousands of selected genes (117,80; Fig 1.1a.). Originally, total mRNA from two different cell populations were differentially fluorescently labeled and then hybridized to the microchip. Based on the abundance of specific transcripts within the two cell populations, the individual transcripts competed for hybridization to their complementary sequence on the microchip. By analysis of fluorescence intensity, relative levels of transcripts could be determined, thus identifying transcript abundance in each population. A recent advance in microarray technology allows a single sample to be probed on a single chip and then directly compared to other samples probed on equivalent chips. This allows for the direct comparison of multiple samples, rather than the original two-way comparison.

Although SAGE results in similar information as microarrays, the principles and techniques behind SAGE are quite different. The theory behind SAGE is that 10 base pairs (bp) of sequence, isolated from a specific region from each transcript (for a total of 14 bp), contains enough information to uniquely identify the gene from which it was derived (199,80; Fig 1.1b). Through a series of ligations, digestions and PCR amplifications, a specific 14 bp sequence (tag) from each transcript is isolated, based upon the natural location of NlaIII restriction sites within the transcripts. The tags are then concatenated to form a long strand, which can be easily sequenced. Transcripts are identified by comparing the isolated tags to sequence databases. Based upon the frequency of specific tags sequenced, the relative level of the corresponding gene transcript can be determined (199).

Though both techniques can provide large quantities of information about transcript expression for specific cell types, they both have their advantages and disadvantages. Microarrays are highly sensitive and comparatively less technically complicated than SAGE. Analysis of microarrays is simplified due to the prior annotation of the genes spotted onto the array, the ability to design arrays containing only genes of interest and the increased availability of bioinformatics tools. Because of these advantages, microarrays are the most widely used genomic analysis tool used in immunology (4). For example, microarrays have been used to study CD4⁺ and CD8⁺ type 1 and type 2 $\alpha\beta$ T cells (37), activation of $\alpha\beta$ T cells (193), and to characterize $\alpha\beta$ helper T cell development (167). Although there can be thousands of genes represented on a single

microarray, the genes represented are limited to ones with known sequence, and the genes selected can be biased, thus limiting the identification of unexpected transcripts.

SAGE also has advantages and disadvantages. Since genes that are identified are not predetermined, SAGE is unbiased. In addition, SAGE is comprehensive because even the very low abundance transcripts can be identified if adequate sequencing is performed. To gain confidence in the regulation of low abundance genes, additional sequencing from the same SAGE library can be performed. Another advantage of SAGE is that after the data is adjusted to the number of tags sequenced, it can be directly compared to any other SAGE library, regardless of the time or location of its construction. If a gene is not identified from the SAGE tag sequence, the tags may be used as primers for new gene discovery. A slight modification of the SAGE procedure, termed long SAGE (172), yields tags of 21 bp, rather than 14 bp, providing additional sequence helpful for gene identification. However, SAGE is much more technically challenging than are microarrays and the cost for concatemer sequencing can be very high. Since SAGE only relies on a short segment isolated from the 3' ends of sequences, alternatively spliced mRNAs cannot be identified in most cases. Both microarrays and SAGE frequently reveal genes that display unexpected expression patterns and warrant further study.

Global Gene Expression Analysis of $\gamma\delta$ T Cells.

Recently, global analyses of $\gamma\delta$ T cell transcriptomes from various species have been performed using both microarrays and SAGE, which have provided unique insights into the function of these cells (80). In one study (55), oligonucleotide microarrays were used

to examine murine $\gamma\delta$ intraepithelial lymphocytes ($\gamma\delta$ IELs) in a *Yersinia* infection model. Though a role for $\gamma\delta$ T cells in the *Yersinia* infection was not identified, some unique gene expression patterns were revealed. For example, $\gamma\delta$ T cells were implicated in intestinal lipid metabolism and cholesterol homeostasis. Another group compared $\gamma\delta$ and $\alpha\beta$ T cells isolated from the same tissue (murine IELs) using SAGE (179). The analysis showed that these two T cell populations have highly similar gene expression profiles, though some $\gamma\delta$ T cell-specific transcripts were detected. A similar study compared murine $\gamma\delta$ T cells to unconventional T cells using SAGE (154). It was concluded that $\gamma\delta$ T cell profiles are similar to that of unconventional $\text{CD8}\alpha\alpha^+ \alpha\beta$ T cells.

We have previously compared specific subsets of $\gamma\delta$ T cells isolated from bovine peripheral blood (128,79). In these studies, CD8^- and $\text{CD8}^+ \gamma\delta$ T cells were isolated and treated with two different global stimulants. SAGE was used to compare the subsets stimulated with PMA/ionomycin and cDNA microarrays were used to study the same subsets following ConA/IL-2 stimulation. Results revealed that bovine $\text{CD8}^+ \gamma\delta$ T cells appear to be anti-inflammatory (128,79,210) and express genes promoting quiescence and trafficking to the mucosa, as well as interferon-inducible genes (79). Supporting this finding, BAP37, a prohibitin-related molecule that inhibits RNA translation efficiency and cell proliferation, is expressed at higher levels in these cells than their $\text{CD8}^- \gamma\delta$ T cell counterparts (128,43). Unlike $\text{CD8}^+ \gamma\delta$ T cells, bovine $\text{CD8}^- \gamma\delta$ T cells appear to be pro-inflammatory (128,79). $\text{CD8}^- \gamma\delta$ T cells show a higher level of mRNA for transcription and translation regulatory genes, as well as proliferation-inducing and apoptosis-

inhibiting genes, suggesting they are likely transcriptionally and translationally more active than CD8⁺ $\gamma\delta$ T cells. For example, galectin-1, an immune-regulatory and apoptosis-inducing molecule, is more abundant in bovine CD8⁻ $\gamma\delta$ T cells (128). These data suggest that circulating CD8⁻ $\gamma\delta$ T cells may be pre-activated or in an activated/resting state so they are capable of responding quickly to external stimuli (128,79).

A complementary study was just completed that compared human V δ 1 and V δ 2 $\gamma\delta$ T cell subsets under various activation conditions (108). These subsets were found to have similar profiles as the bovine CD8⁺ and CD8⁻ $\gamma\delta$ T cell subsets, respectively. V δ 1 $\gamma\delta$ T cells have a non-inflamed tissue homing phenotype (CCR7) and appear to be immunoregulatory due to their expression of cytokines like IL-10 and IL-11 (108). V δ 2 $\gamma\delta$ T cells respond to inflammation/infection by secretion of inflammatory cytokines (201,178,24). They also appear to be more responsive to inflammatory cytokines (CCR1, CCR2, CCR5 and CXCR6 ligands and IL-12; ref. 108,63) and may produce more inflammatory mediators than V δ 1 $\gamma\delta$ T cells (108). Unlike the more quiescent V δ 1 $\gamma\delta$ T cells, V δ 2 $\gamma\delta$ T cells can be activated by prenyl pyrophosphates and alkylamines produced by plants and microbes (201,178,24,136,138). These studies of human and bovine subsets demonstrate the utility of genomic analysis and comparison across species.

Two other studies have recently examined the response of $\gamma\delta$ T cells to PAMPs or directly to salmonella infection. The first study examined human $\gamma\delta$ T cell responses to

LPS or peptidoglycan by microarray (81). The second study, also using microarrays, compared the in vivo responses of $\gamma\delta$ and $\alpha\beta$ T cells to an experimental bovine model of *Salmonella enterica* serovar *typhimurium* enterocolitis (78), as discussed above. These two studies underscored the role of $\gamma\delta$ T cells in innate immunity.

Although these studies have provided insight into the differences and similarities of $\gamma\delta$ and $\alpha\beta$ T cells, as well as provided specific genes to target in future studies, additional, properly-designed genomic studies could dramatically increase the current understanding of these still elusive cells. Genomic analyses on $\gamma\delta$ T cells have not been performed that compare resting and activated profiles or that compare populations isolated from different microenvironments. Also, direct comparison of $\alpha\beta$ and $\gamma\delta$ T cells isolated from the same anatomical location would likely yield information as to the unique functions of both cell types. To gain a better understanding of unique $\gamma\delta$ T cell responses to mitogenic stimuli in different microenvironments and in comparison to $\alpha\beta$ T cells, we have built 12 new SAGE libraries. These new SAGE libraries have identified unique gene expression profiles of multiple $\gamma\delta$ T cell populations, as well as general transcript expression trends that will likely apply not only to ruminants, but to rodents and humans as well.

TRANSCRIPTIONAL PROFILING OF $\gamma\delta$ T CELLSIntroduction

$\gamma\delta$ T cells represent only a minor population of circulating T lymphocytes in most animals, but are evolutionarily conserved. $\gamma\delta$ T cells have been the focus of many studies over the last two decades, yet their specific functions within the tissues to which they localize are still unclear (76). Unique biological and immunological functions of $\gamma\delta$ T cells have possibly been overlooked because many of the earlier studies of these cells were designed based on known $\alpha\beta$ T cell biology. The unique functions of $\gamma\delta$ T cells also remain unclear due to differences identified between the various species studied (27,53,173). For the most part, sequential analysis of single functional attributes has been used to study these cells in various animals, including humans. This approach is effective in studying $\gamma\delta$ T cells in a single species but does not efficiently reveal clusters or patterns of activities of $\gamma\delta$ T cells, which may be conserved across species.

Recently, global analyses of $\gamma\delta$ T cell transcriptomes have been performed using both microarrays and serial analysis of gene expression (SAGE; ref. 199), which have provided unique insights into the function of these cells (80). In one study (55), oligonucleotide microarrays were used to examine murine $\gamma\delta$ intraepithelial lymphocytes ($\gamma\delta$ IELs) in a *Yersinia* infection model. Though a role for $\gamma\delta$ T cells in the *Yersinia* infection was not identified, some unique gene expression patterns were revealed. For example, $\gamma\delta$ T cells were implicated in intestinal lipid metabolism and cholesterol

homeostasis. Another group compared $\gamma\delta$ and $\alpha\beta$ T cells isolated from the same tissue (murine IELs) using SAGE (179). The analysis showed that these two T cell populations have highly similar gene expression profiles, though some $\gamma\delta$ T cell-specific transcripts were detected. A similar study compared murine $\gamma\delta$ T cells to unconventional T cells using SAGE (154). It was concluded that $\gamma\delta$ T cell gene expression profiles are similar to those of unconventional $CD8\alpha\alpha^+ \alpha\beta$ T cells.

We have previously compared specific subsets of $\gamma\delta$ T cells isolated from bovine peripheral blood (79,128). SAGE was used to compare $CD8^-$ and $CD8^+$ $\gamma\delta$ T cell subsets stimulated with PMA/ionomycin, and cDNA microarrays were used to study the same subsets following ConA/IL-2 stimulation. Results revealed that $CD8^-$ $\gamma\delta$ T cells expressed genes that correlated with inflammatory and proliferative profiles, while $CD8^+$ $\gamma\delta$ T cells expressed genes of more quiescent and apoptotic profiles. A complementary study was just completed that compared human $V\delta 1^+$ and $V\delta 2^+$ $\gamma\delta$ T cell subsets under various activation conditions (108). These subsets were found to have similar profiles as the bovine $CD8^+$ and $CD8^-$ $\gamma\delta$ T cell subsets, respectively. These findings show the utility of global gene expression analyses in identifying trends between species.

Though the global analysis of gene expression in $\gamma\delta$ T cells has provided new insights into the biology of these cells, transcriptomes of only a few select populations of $\gamma\delta$ T cells in limited activation states and that identify few unique gene expression patterns are available to date (80). To increase the potential of identifying unique $\gamma\delta$ T cell transcripts and to provide a more comprehensive dataset, we employed SAGE to construct twelve

additional SAGE libraries. SAGE was chosen for these analyses because data from any system, including data from different species and laboratories, is directly comparable due to the unique advantage of the SAGE technique in that the data is internally normalized (199). These new libraries represent the following bovine lymphocyte populations: magnetic bead-sorted blood $\gamma\delta$ T cells (>90% pure), spleen $\gamma\delta$ T cells (>90% pure) and enriched $\alpha\beta$ T cells (>80% pure) from a single calf, both resting and ConA/IL-2 stimulated for 6 hours and flow cytometry-sorted blood $\gamma\delta$ and $\alpha\beta$ T cells (pooled from 3 calves; >95% pure) each either resting, ConA/IL-2, or PMA/ionomycin stimulated as above. This study complements our original $CD8^+$ and $CD8^-$ $\gamma\delta$ T cell SAGE data sets.

We hypothesized that many $\gamma\delta$ T cell-specific/selective transcripts and gene expression patterns would be identified by comparing these libraries against each other and with other data sets. Results indicate that these libraries do exhibit several gene expression differences between resting and mitogen-stimulated blood $\gamma\delta$ T cells, between blood and spleen $\gamma\delta$ T cells, as well as differences between spleen and blood $\gamma\delta$ and $\alpha\beta$ T cells. A cross comparison of these libraries revealed unique $\gamma\delta$ T cell-specific transcripts, as well as tissue-specific gene expression patterns. In addition, a web-based resource for the use of these libraries is described.

Materials and Methods

Cell Extraction, Magnetic Bead Cell Sorting (MACS) and Culturing of Calf Spleen and Peripheral Blood Lymphocytes

A bovine calf spleen was surgically removed and kept on ice in Hanks balanced salt solution (HBSS; Mediatech, Inc., Herndon, VA). Tissue was homogenized using tissue grinders. Single cell suspensions of mononuclear cells were isolated using Histopaque 1077 (Sigma Aldrich, St. Louis, MO), as per the manufacturer's instructions and filtered through a 70 μm filter. Peripheral blood mononuclear cells (PBMCs) were also isolated using Histopaque 1077. PBMCs were monocyte-depleted by incubation in plastic flasks and collecting all non-adherent peripheral blood lymphocytes (PBLs). $\gamma\delta$ T cells were isolated from both PBLs and single-cell suspensions of spleen lymphocytes using MACS LS Separation Columns (Miltenyi Biotec, Auburn, CA) as per the manufacturer's instructions and GD3.8 (anti-bovine $\gamma\delta$ T cells), as previously described (46). Briefly, PBLs were first depleted of B cells using the magnetic bead separation and CC21 antibody (specific for bovine B cells). Purified $\gamma\delta$ T cells and $\alpha\beta$ T cell-enriched populations were obtained (purity of: >90% spleen and >95% blood $\gamma\delta$ T cells and >80% spleen $\alpha\beta$ T cells; data not shown). Cells were rested overnight in complete RPMI (10% fetal bovine serum in RPMI supplemented with 1% each of essential amino acids, penicillin/streptomycin, L-glutamine, and 10 mM HEPES; Mediatech, Inc.) at 37° C, 10% CO₂ at a concentration of 1×10^7 cells/ml. After approximately 16 hours of rest, lymphocytes were either stimulated for 6 hrs with 5 $\mu\text{g/ml}$ Concanavalin A (ConA;

Sigma Aldrich) and 1 ng/ml IL-2 (Pepro Tech, Inc., Rocky Hill, NJ) or rested for an additional 6 hours.

Flow Cytometric Analysis

A small fraction of cells were stimulated or remained resting for 24 hours for use in flow cytometric analysis. Previously described techniques were used to perform single and two-color flow cytometric analyses using a FACSCalibur (BD Biosciences; 79,128,212). Staining with the following antibodies was used to determine the extent of stimulation: GD3.8 ($\gamma\delta$ TCR) directly labeled with FITC, and GD3.5 (nearly all CD8⁻ $\gamma\delta$ T cells, but no CD8⁺ $\gamma\delta$ T cells), DREG56 (anti-bovine L-selectin), H58A (anti-bovine MHC class I; VMRD, Inc., Pullman, WA), CACT1165A (anti-bovine IL-2 receptor; VMRD, Inc.), CAT82A (MHC class II; VMRD, Inc.), and MSA3 (MHC class II; VMRD, Inc.). Second-stage reagents included PE-conjugated anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA). The indirect stain was done first, followed by incubation in 10% mouse serum to block all reactive sites of the immobilized second stage antibody and then the FITC-labeled GD3.8 mAb stain. A successful activation profile resulted in decreased expression of L-selectin and/or GD3.5 and/or increased expression of MHC class I and class II and/or IL-2R.

Flow Cytometric Cell Sorting (FACS) and Culturing of Peripheral Blood Lymphocytes

High-speed cell sorting on a Vantage SE cell sorter (BD Biosciences, San Jose, CA) was used to obtain >95% pure $\gamma\delta$ and $\alpha\beta$ T cell populations from three calves, as

described previously (79,128,212). Briefly, PBMCs were isolated as described above, and stained using CC42 mAb (anti-bovine CD2) and a FITC-labeled GD3.8 mAb. All GD3.8⁺ cells were included in the $\gamma\delta$ T cell sorted population, and only GD3.8⁻/CD2⁺ cells were included in the $\alpha\beta$ T cell population. Cells were rested overnight and then stimulated as above with ConA/IL-2 or 20ng/ml PMA (phorbol 12-myristate 13-acetate; Sigma Aldrich) and 0.5 μ g/ml ionomycin (Sigma Aldrich) or remained resting for 6 hours prior to RNA extraction.

SAGE Library Construction

RNA was extracted from cells using TRIzol reagent (Invitrogen, Carlsbad, CA) per the manufacturer's protocol. SAGE libraries were constructed using the SAGE-lite method, utilizing template switching and PCR amplification, as previously described (155). Briefly, a biotinylated oligo(dT) primer (biotin-5'-AAGCAGTCCTAACAACGCAGAGTAC(T)₃₀VN-3', where V = A,C or G, and N = T, C, G or A) and Superscript II reverse transcriptase (Invitrogen) were used to make first-strand cDNA from 100 ng of total RNA. Second-strand synthesis and cDNA amplification was completed by PCR using Advantage2 Polymerase Mix (Clontech, Palo Alto, CA) and a switching primer (5'-AAGCAGTGGTAACAACGCAGAGTACGCGGG-3') in combination with the original biotinylated oligo(dT) primer. SAGE library construction was completed using 5 μ g of the ds-cDNA following standard protocols (199,161,162). FACS sorted PBL libraries were built with equal amounts of cDNA from cell preparations from 3 different calves.

Large-scale sequencing of concatemerized ditags was completed by Agencourt Bioscience Corporation (Beverly, MA).

SAGE Data Analysis

Ditags were pulled from sequences and duplicate ditags that arise during PCR amplification were eliminated (39). Ditag sequence was processed using software previously developed to extract individual SAGE tag information, record tag frequency and correct sequence error in the raw dataset by nearest neighbor analysis (39; <http://195.70.0.115/bioinf/sage/>). Tag frequencies for each library were adjusted for slight differences in sequencing depth by multiplying the tag count by the adjusted library size, divided by the actual size—where the adjusted size was equal to 25,000 tags. This corrected data set provides the highest level of confidence and therefore, was used in all of the analyses presented. Tags were compared to the TIGR bovine gene index release 11.0 sequence (<http://www.tigr.org/>). Annotation of SAGE/EST matches was performed by BLAST comparisons to the Human 15.0 and Mouse 14.0 TIGR databases using $1e^{-6}$ cutoff. Uncorrected and adjusted tag frequencies and annotations can be viewed and analyzed on the public Bovine SAGE database (<http://vmbmod10.msu.montana.edu/vmb/jutila-lab/sagebov.htm>).

SAGE tags with adjusted frequencies of 10 or greater within all 12 libraries were imported into GeneSpring 7.2 (Agilent Technologies Inc., Palo Alto, CA) to build visual representations of the 12 libraries. Data was analyzed in GeneSpring 7.2 using the log of ratios mode. These datasets were compared by cluster analysis using the Pearson

correlation as a similarity measurement to generate gene trees. Condition trees were also generated based on the Pearson correlation. Data sets united by the shortest branches were the most similar. Correlation coefficients can be determined by subtracting the length of the branches (represented on the condition trees) from 1. Gene lists were generated by comparing fold-changes of the adjusted tag frequencies.

Reference Long SAGE Library Construction

A long SAGE library was constructed, as previously described (172). Briefly, RNA was collected as described above from lymphocytes isolated from calf spleen, lymphatic fluid, lymph nodes, and peripheral blood and pooled together. The long SAGE library was built as described for the SAGE libraries, with the following modifications. Linkers containing restriction sites for MmeI were used rather than linkers containing BsmFI restriction sites. Cleavage of cDNA with MmeI resulted in tags of 21 bp in length rather than only 14 bp. Elimination of linkers from the ditags was performed with 4 washes over strept-avidin magnetic beads. Concatemerization and sequencing was done as described above for the SAGE libraries. Data analysis was done using in-house programs designed to isolate 17-22 bp tags. Annotation of tags was done as described above. In-house programs were also used to compare short SAGE tags with the corresponding long SAGE tags.

Web Database Platform

The web site (<http://vmbmod10.msu.montana.edu/vmb/jutila-lab/sagebov.htm>) is served from a Dell 4400 running RedHat Linux (<http://www.redhat.com/>) and Apache

web server (<http://apache.org/>). All scripts created to implement and serve the web site were written in Perl. The Perl DBI and CGI modules are used respectively to connect to the MySQL (<http://dev.mysql.com/>) database and serve the site to web browsers.

cDNA Construction and Real-Time RT-PCR

RNA was extracted from equivalent cell populations as described above, DNase treated (Promega), and re-extracted with phenol/chloroform and ethanol precipitated. Reverse transcription was performed with random primers and SuperScript II or III (Invitrogen), per the manufacturer's instruction. Specific gene transcripts were measured by SYBR green incorporation during real-time RT-PCR using the relative standard curve method. Gene specific primers were designed using Primer Express software (Applied Biosystems) and the bovine gene sequences. Primers to bovine 18S were used as endogenous controls, and standard curves were constructed using serially diluted, similarly extracted PBL RNA in RT reactions. One μ l of RT reaction was used in each 25 μ l PCR reaction. Reactions were done in triplicate and run on an ABI PRISM 7500 Sequence Detection System. Calculations were performed as described in the User Bulletin 2 for the ABI PRISM 7700 Sequence Detection System.

Results

SAGE Libraries/Annotation of SAGE Tags/Bioinformatics tools

Twelve new SAGE libraries were generated from the following bovine lymphocyte populations: MACS sorted blood $\gamma\delta$ T cells, spleen $\gamma\delta$ T cells and enriched $\alpha\beta$ T cells

from a single calf, both rested and ConA/IL-2 stimulated for 6 hours and FACS sorted blood $\gamma\delta$ and $\alpha\beta$ T cells (pooled from 3 calves) each either resting, ConA/IL-2, or PMA/ionomycin stimulated as above. Activation of the blood and spleen populations was confirmed by increased staining by IL-2 receptor- and/or MHC class I and II-specific antibodies, and/or decreased staining by an L-selectin-specific antibody in flow cytometric assays (Fig 2.1) after 24 hours of stimulation. These 12 libraries were separated for analysis based on the sorting procedures.

Over 10,000 tags were generated from each library (except for the PMA/ionomycin stimulated blood $\gamma\delta$ T cells, which had only 7638 tags sequenced) for a total of over 210,000 tags to provide an initial “corrected” data set for comparison (Table 2.1, Bovine SAGE database: <http://vmbmod10.msu.montana.edu/vmb/jutila-lab/sagebov.htm>). Confirmation of SAGE as a predictor of gene expression in bovine blood T cells was previously performed in our laboratory (128). Here, differences in MACS-sorted resting and ConA/IL-2 stimulated blood $\gamma\delta$ T cells were confirmed using real time RT-PCR in 9 out of 13 candidate genes, with only one gene showing an opposite regulation (Fig. 2.2). This level of confirmation was similar to that shown in our previous study (128), further validating SAGE in this system and also illustrating the consistency of gene expression profiles in blood $\gamma\delta$ T cells from different animals. Spleen cell preparations were not as consistent in follow-up analyses of specific genes, which is discussed below.

Between all 12 SAGE libraries there were over 88,000 unique tags sequenced; however, 71.2% were represented only once in a single library (Table 2.2). In theory,

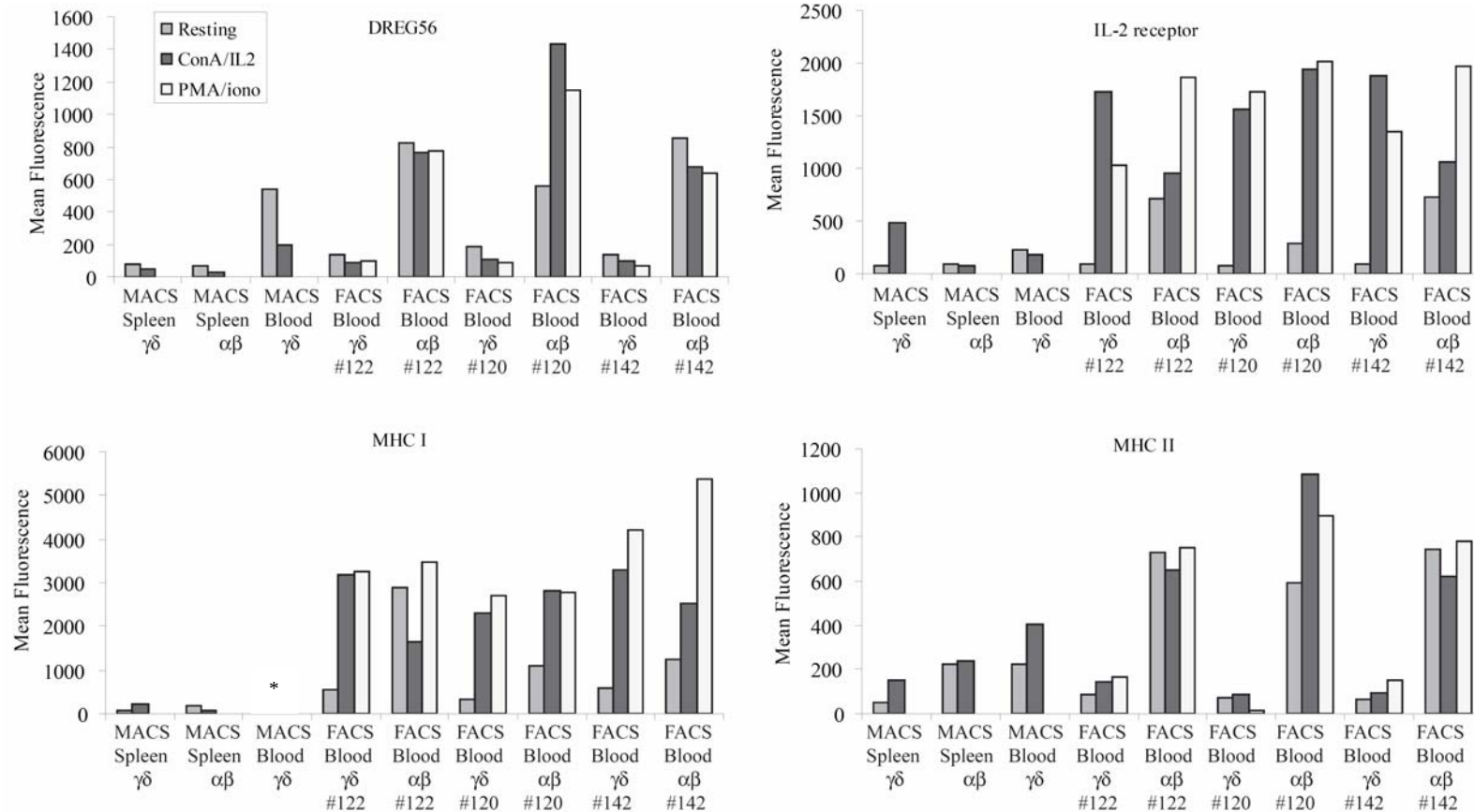


Figure. 2.1 Activation of spleen and blood $\gamma\delta$ and $\alpha\beta$ T cells was confirmed by flow cytometry. A small aliquot of cells were cultured for 24 hours after the majority were collected at 6 hours for RNA extraction. The cultured cells were then subjected to FACS analysis using antibodies against 4 activation markers. A successful activation profile consisted of decreased staining of DREG56 (anti-L-selectin), and/or increased staining of the IL-2R, MHC I and/or MHC II. Data from FACS analysis representing blood T cells from each of the three calves (#122, #120, and #142) are included. *Data not available.

Table 2.1 Twelve new SAGE libraries are represented in the bovine SAGE database.

		Total Ditags Sequenced	Total Ditags Kept	Total Ditags Thrown ^a	% Ditags Thrown	Total Tags Sequenced ^b	Total Unique Tags	Total Unique Tags Hit Sequence ^c	% Total Unique Tags Hit Sequence
SAGE Library									
MACS-Separated	Blood $\gamma\delta$ T cells (Resting)	11,275	7,556	3,719	33.0	14,542	5,261	3,220	61.2
	Blood $\gamma\delta$ T cells (ConA/IL-2)	13,710	11,251	2,459	17.9	21,492	7,951	3,912	49.2
	Spleen $\gamma\delta$ T cells (Resting)	17,665	14,793	2,872	16.3	27,462	18,170	7,304	40.2
	Spleen $\gamma\delta$ T cells (ConA/IL-2)	18,198	11,381	6,817	37.5	21,134	14,056	6,003	42.7
	Enriched Spleen $\alpha\beta$ T cells (Resting)	21,330	16,092	5,238	24.6	29,956	16,476	7,031	42.7
	Enriched Spleen $\alpha\beta$ T cells (ConA/IL-2)	14,590	10,302	4,288	29.4	19,170	12,348	5,345	43.3
FACS-Separated	Pooled Blood $\gamma\delta$ T cells (Resting)	12,012	7,402	4,610	38.4	13,936	6,484	3,863	59.6
	Pooled Blood $\gamma\delta$ T cells (ConA/IL-2)	9,036	6,562	2,474	27.4	12,503	5,326	3,444	64.7
	Pooled Blood $\gamma\delta$ T cells (PMA/iono)	11,861	3,702	8,159	68.8	7,047	3,366	2,283	67.8
	Pooled Blood $\alpha\beta$ T cells (Resting)	13,070	9,236	3,834	29.3	17,483	6,574	4,116	62.6
	Pooled Blood $\alpha\beta$ T cells (ConA/IL-2)	6,249	5,330	919	14.7	10,196	4,736	2,991	63.2
	Pooled Blood $\alpha\beta$ T cells (PMA/iono)	13,056	5,554	7,502	57.5	10,644	4,533	3,011	66.4
Total		162,052	109,161	52,891	32.6	205,565	88,462^d	36,348^d	41.1^d

^aDuplicated ditags were eliminated from analysis due to the probability of PCR artifact

^bThe number of tags sequenced after duplicated ditags and poor sequence were eliminated from analysis

^cTags were BLASTed against current bovine ESTs

^dData based on all 12 libraries combined, therefore the numbers are not the sum or average of the libraries analyzed separately

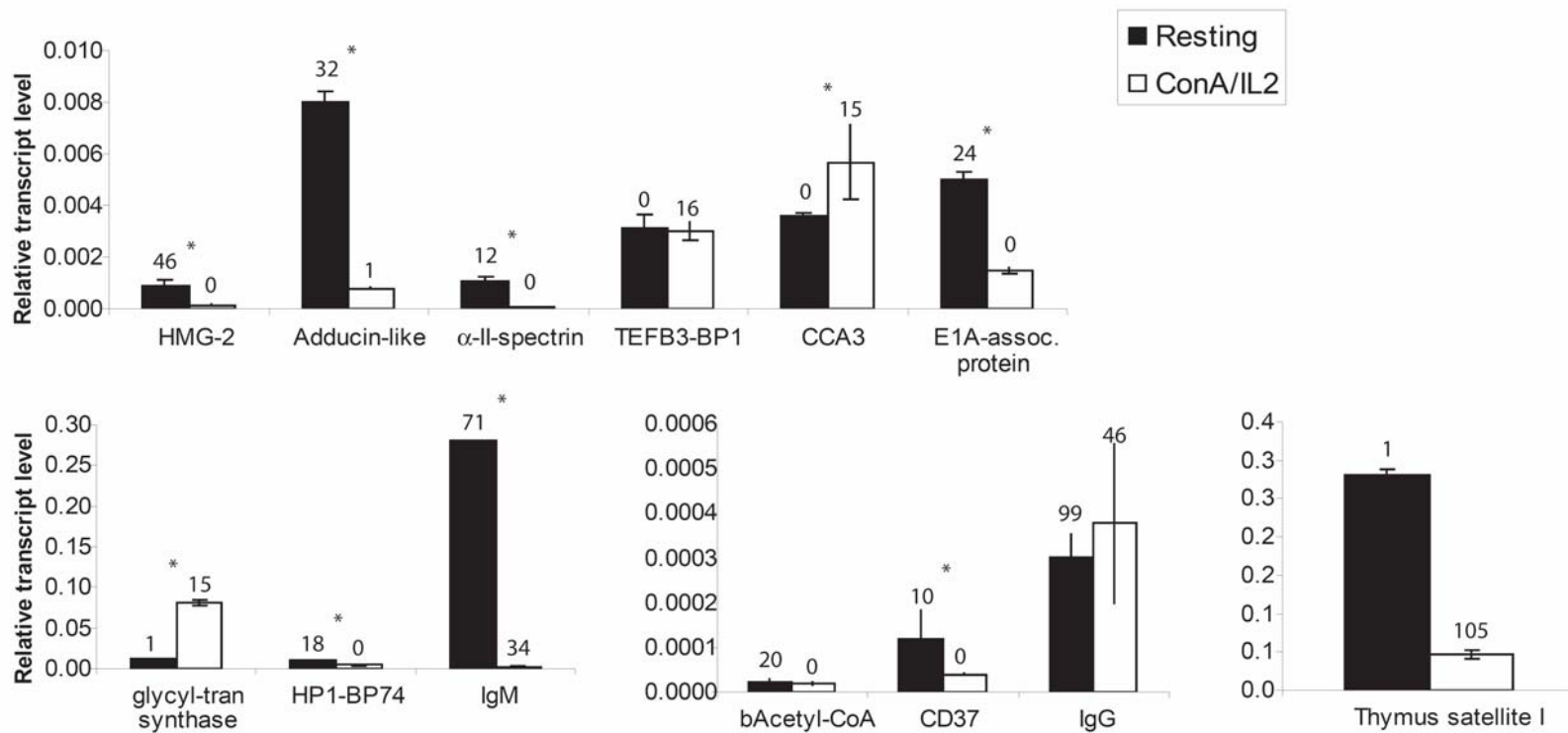


Figure 2.2 Quantitative real-time RT-PCR was performed in triplicate using RNA isolated from calf peripheral blood $\gamma\delta$ T cells to confirm the blood SAGE libraries. $\gamma\delta$ T cells were sorted to greater than 97% purity using a Vantage-SE cell sorter (BD Biosciences). Cells were rested overnight before being stimulated with ConA/IL-2 or rested for an additional 6 hours. 18S was used as an endogenous control. Numbers above each column represent the tag frequency for that gene in the corrected data set. *Genes that show the same regulation in both the SAGE libraries and the real time RT-PCR analysis.

each tag represents a unique gene; however, some unique tags are created by PCR or sequencing error due to a single base pair change (39). Highly-abundant tags were likely to be the source of several singletons (tags represented only once) that differ by only single base pair from the abundant tag. Therefore, singletons were not used in the analyses reported here, but are made available in the bovine SAGE database. Tag frequencies were adjusted to the total number of tags sequenced from each library. Classification of tags by increasing frequency cut-offs greatly decreased the number of tags in each category (Table 2.2). For example, tags with an adjusted frequency of 5 or greater between all 12 libraries represented only 5.2% of the unique tags, correlating with approximately 4,600 genes.

Table 2.2 Statistical information on data from all 12 libraries combined

Tag Frequency Classifications	Unique Tags	% Total Tags	Total Unique Tags Hit Sequence	% Tags Hit Sequence
Total unique tags	88,462	100.0	36,348	41.1
Freq. =1	62,973	71.2	24,322	38.6
Freq. >=2	21,489	24.3	12,026	56.0
Freq. >=3	9,735	11.0	6,377	65.5
Freq. >=4	6,170	7.9	4,411	71.5
Freq. >=5	4,609	5.2	3,436	74.5

The SAGE tags were annotated using bovine ESTs that were in turn annotated by comparison to the human, mouse and bovine genomes. Approximately 41% of the unique tags from each library matched a known sequence for annotation. As expected, a greater percentage of the medium to high abundance tags matched a sequence, increasing

confidence that they represent “real” genes (Table 2.2). For this reason, our global comparisons use only tags with a total frequency of 10 or greater between all twelve libraries.

GeneSpring 7.2 software was used to build visual representations of each SAGE library, facilitating comparison to the others. Using the high confidence cut-off of tags represented by an adjusted frequency of 10 or more, all 12 libraries were compared (Fig. 2.3). Many tags were expressed at similar levels in all libraries, most likely representing housekeeping genes. However, as predicted, similarities and differences became apparent between the various activation states and cell types.

Effect of Mitogen Stimulation on Gene Expression in MACS Separated Blood and Spleen $\gamma\delta$ T Cells

We hypothesized that several genes would be identified as differentially expressed between resting and mitogen-stimulated blood $\gamma\delta$ T cells. For an initial comparison, SAGE libraries were constructed from MACS-sorted resting and ConA/IL-2 stimulated blood $\gamma\delta$ T cells. Magnetic bead separation was used because others have previously used the same technique to study gene expression in $\gamma\delta$ T cells (46), and we found that the separation procedure did not “activate” the cells as measured by proliferation and IL-2 receptor up-regulation (data not shown). Several tags were identified that were differentially expressed between the two libraries. Specifically, 43 tags were expressed at a 10-fold or greater level in resting cells, and 60 tags were expressed at a 10-fold higher level in ConA/IL-2 stimulated cells (Tables 2.3 and 2.4). Notably, some tags were differentially expressed at levels over 100-fold.

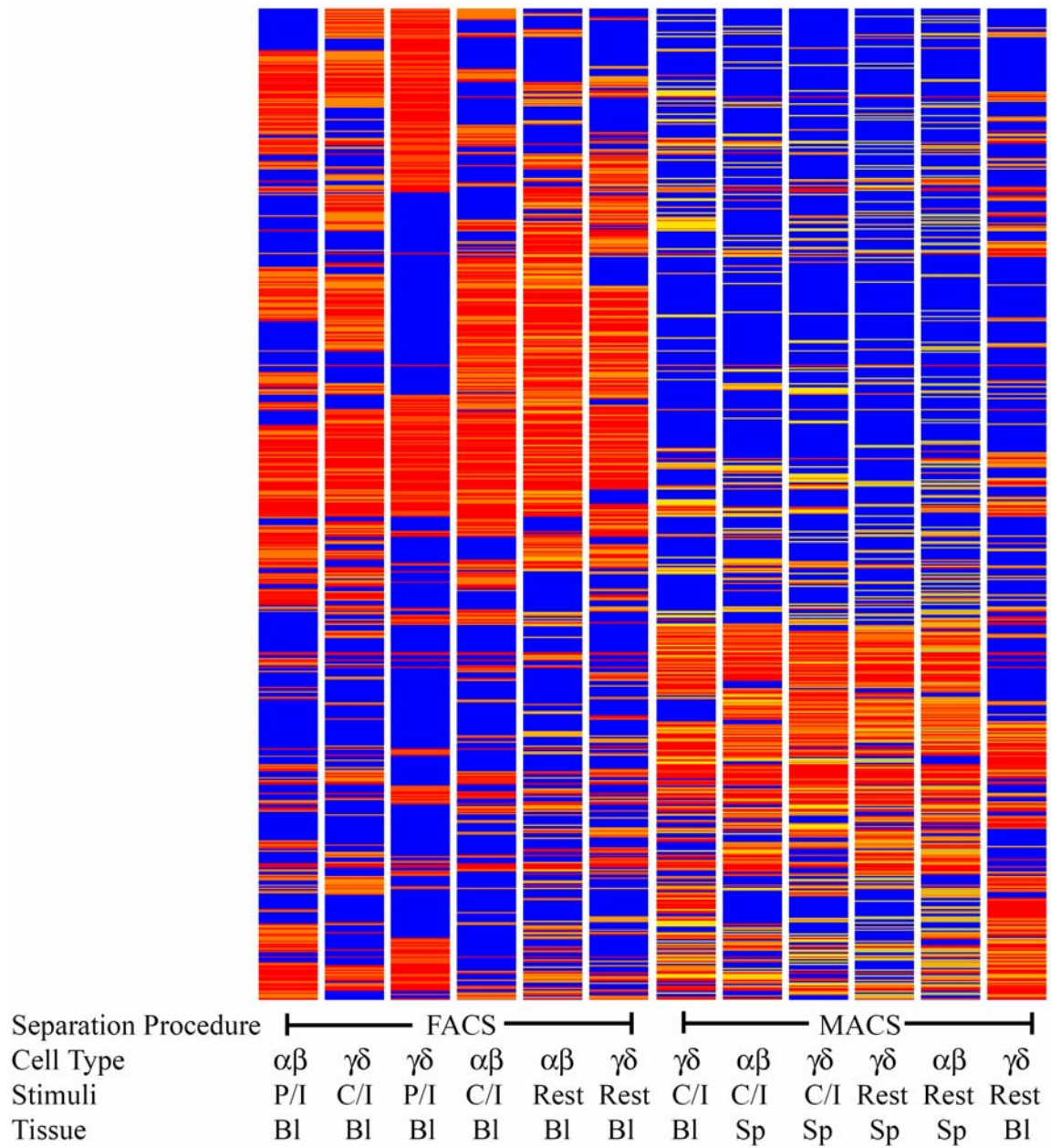


Fig. 2.3 All 12 SAGE libraries display different gene expression profiles. Each column represents a unique library and each row represents a specific expressed tag. Tags expressed at high levels are shaded dark and tags expressed at low levels are light. $\gamma\delta$: $\gamma\delta$ T cells, $\alpha\beta$: $\alpha\beta$ T cells, Bl: blood, Sp: spleen, P/I: PMA/ionomycin, C/I: ConA/IL-2.

Many tags could not be uniquely annotated as a specific gene. Some tags did not match any known bovine ESTs or matched a non-annotated bovine EST, and thus may represent novel genes. Likewise, those tags may also represent novel genes. Other tags matched several known bovine ESTs, and thus could not be uniquely identified as a specific gene. Curiously, tags that were represented at a greater level in the stimulated population were much more difficult to uniquely annotate because on average those tags matched 24 different ESTs (data not shown). However, tags expressed at higher levels in resting cells matched fewer than 3 sequences on average and could be annotated with a greater level of confidence.

An experiment was done to examine how $\gamma\delta$ T cell subsets localized to different microenvironments responded to mitogenic stimulation. $\gamma\delta$ T cells were isolated for SAGE from bovine spleen using the single-step MACS bead separation protocol and stimulated with ConA/IL-2 or remained resting. Spleen $\gamma\delta$ T cells had a much less robust change in gene expression after ConA/IL-2 stimulation when compared to the changes seen in the corresponding blood populations (Fig. 2.4). Specifically, only 2 tags were expressed at a 10-fold higher level in activated spleen cells than in the resting population, and only 1 tag was expressed at a 10-fold higher level in resting spleen $\gamma\delta$ T cells (data not shown).

Table 2.3. Tags expressed at a 10-fold or greater level in MACS-sorted resting blood $\gamma\delta$ T cells than in ConA/IL-2 stimulated blood $\gamma\delta$ T cells.

Tag	Blood $\gamma\delta$ T cells		Fold difference	Spleen $\gamma\delta$ T cells		Spleen $\alpha\beta$ T cells		Annotation
	Resting	ConA/IL2		Resting	ConA/IL2	Resting	ConA/IL2	
CATGGAGGAGGAAG	101 ^a	1	95.7	4	1	2	9	CAMPATH-1 antigen precursor (CD52)
CATGGCAAAAAGTG	42	0.99 ^b	42.6	5	4	5	6	Nonhistone chromosomal protein HMG-2
CATGATTATTGACT	30	1	28.0	1	1	1	0	Adducin-like protein
CATGAGTGCAGAAT	23	0.99	23.7	0	0	0	0	No matches
CATGCGATTAAAAT	22	0.99	22.1	0	0	1	2	E1A-associated protein p300
CATGAGTGCAGATT	20	0.99	20.5	0	0	0	0	No matches
CATGGGAGCCGCCG	19	0.99	18.9	0	1	2	1	Acetyl-CoA acyltransferase
CATGGTCACCGGCT	17	0.99	17.3	0	0	0	0	IgG2a heavy chain C region
CATGCCGATTTCGTC	17	0.99	17.3	2	1	0	2	HP1-BP74 protein
CATGCACAAACAGT	17	0.99	17.3	0	0	0	0	40S ribosomal protein S27
CATGGAGCCTGTAA	17	1	16.2	2	1	2	0	Nef associated protein 1
CATGTGGCAACAGT	16	0.99	15.8	2	0	0	0	H2A histone family, member V, isoform 1
CATGGAGTCAGGAT	16	0.99	15.8	1	0	0	1	Multiple matches
CATGAGCGCAGACT	16	0.99	15.8	0	0	0	0	No matches
CATGTCTCCCTTCT	16	1	14.7	2	0	2	2	Glutathione peroxidase
CATGGCGCCCCTGC	16	1	14.7	2	3	4	5	No annotation
CATGTTGTAATTGT	14	0.99	14.2	0	1	3	2	Multiple matches
CATGTGGCTACTTA	14	0.99	14.2	2	1	0	0	No matches
CATGGCGGCGGTCT	14	1	13.3	0	1	0	5	Histone H2a.2
CATGAAGATCAAGT	14	1	13.3	0	1	0	0	Multiple matches
CATGTTTTCTAATC	12	0.99	12.6	2	0	3	2	Multiple matches
CATGTGGCTTAATG	12	0.99	12.6	0	0	0	1	Multiple matches
CATGGTACATAGAT	12	0.99	12.6	0	1	1	0	No matches
CATGGGCTGGCTGG	12	0.99	12.6	0	0	0	0	Alpha-galactosidase B

(Table 2.3 Continued)

CATGCTACCCAACG	12	0.99	12.6	0	1	8	5	Chloride intracellular channel protein 1 (Nuclear chloride ion channel 27)
CATGAGGGCCATCC	12	0.99	12.6	1	0	1	2	Hydroxyacylglutathione hydrolase
CATGAGAGATGAGA	76	6	12.1	4	2	4	7	Immune associated nucleotide protein
CATGTGATCACCTG	12	1	11.8	1	1	1	0	Multiple matches
CATGGCAAAGGCGG	12	1	11.8	1	0	0	1	High mobility group 1 protein
CATGGAGCCCGCAG	12	1	11.8	1	2	1	11	IgM heavy chain C region
CATGTCCTTCTTAC	11	0.99	11.0	0	0	0	0	No annotation
CATGTAAAAGACAA	11	0.99	11.0	0	0	0	0	Alpha II spectrin
CATGGGATCATTTTC	11	0.99	11.0	1	0	1	0	No matches
CATGGAGAAGGTCA	11	0.99	11.0	0	2	0	1	Immunity-associated nucleotide 4-like 1 protein
CATGAGTGCATACT	11	0.99	11.0	0	0	0	0	No matches
CATGGACGACACGA	58	5	10.9	2	5	7	5	40S ribosomal protein S28
CATGTGGACTTGTTG	22	2	10.4	1	0	0	2	CD3 protein
CATGGGGGACGCCG	11	1	10.3	0	1	0	0	No matches
CATGAGTTCTGAAG	11	1	10.3	0	2	0	1	No annotation
CATGAGCAAGGCAA	11	1	10.3	0	1	0	0	No matches
CATGAAGGGCGTGG	11	1	10.3	1	1	0	1	Leukocyte common antigen precursor (CD45 antigen)
CATGTTGGTGAAGG	53	5	10.1	1	3	2	1	Thymosin beta-4

^aAdjusted tag frequencies^bTags with a frequency of 0 were given a frequency of 0.99 to determine an approximate fold-difference

Table 2.4. Tags expressed at a 10-fold or greater level in ConA/IL-2 stimulated blood $\gamma\delta$ T cells than in resting blood $\gamma\delta$ T cells.

Tag	Blood $\gamma\delta$ T cells		Fold difference	Spleen $\gamma\delta$ T cells		Spleen $\alpha\beta$ T cells		Annotation
	Resting	ConA/IL2		Resting	ConA/IL2	Resting	ConA/IL2	
CATGGCCTAGAGAC	0.99 ^{a,b}	127	128.0	12	27	65	32	No annotation
CATGGAAGTCCGCC	0.99	110	110.9	8	11	50	13	No annotation
CATGGAGACCCGAC	2	103	66.3	10	26	61	18	No matches
CATGGAAGTCCGCT	8	481	61.6	31	78	204	104	No matches
CATGAGAAGCCCCC	2	96	61.6	2	4	38	18	No annotation
CATGCAAAGGACAA	5	210	44.9	207	229	196	178	No annotation
CATGAGATTTTCCA	0.99	39	39.5	8	11	26	15	EP3B receptor
CATGGCCTAGAGGC	2	55	35.2	2	11	20	13	No matches
CATGCACTGATCAC	0.99	35	35.2	26	47	8	8	No annotation
CATGTTTCAATGCC	0.99	33	33.1	7	13	26	25	Multiple matches
CATGTATGAAACGA	0.99	33	33.1	29	25	38	30	Organic anion transporting polypeptide 1 (OATP1)
CATGGCTCGGGAGC	2	51	32.5	6	10	33	12	U4/U6 small nuclear ribonucleoprotein Prp3
CATGTGTTCCAAGA	0.99	32	32.0	0	3	3	1	No annotation
CATGGGGTCACACA	0.99	32	32.0	7	2	5	7	Calpastatin
CATGCCACATACCT	2	46	29.8	21	31	20	13	No annotation
CATGGGGTCGCTCA	0.99	27	27.7	1	1	3	0	No annotation
CATGCATTGGAGGA	0.99	27	27.7	2	5	14	7	No annotation
CATGGCTGCAATCA	0.99	26	26.7	18	25	25	14	Multiple matches
CATGAGATAGAGAA	2	41	26.4	0	0	0	0	No matches
CATGACCACTGGAA	0.99	24	24.5	12	13	17	7	No annotation
CATGTCCAGTTCTA	2	34	21.7	12	21	17	20	Multiple matches
CATGAATTGCAGCA	0.99	20	20.3	2	5	8	0	HMG-box containing protein 1
CATGGGATTTTCTA	0.99	20	20.3	6	19	10	12	Ubiquitin activating enzyme
CATGTATGTGCCAG	2	31	19.6	12	11	17	12	No matches
CATGTATGGATGTG	5	92	19.6	56	49	68	50	Multiple matches

(Table 2.4 Continued)

CATGGTGGCTCTGA	11	211	19.3	40	78	71	38	No annotation
CATGTACTGCCTCA	0.99	19	19.2	2	1	7	2	Multiple matches
CATGACCACAGGAA	0.99	19	19.2	7	13	9	9	Type-1 angiotensin II receptor (AT1) (AT1AR)
CATGATGTACTCTG	0.99	18	18.1	7	6	8	8	Aromatase cytochrome P450 pseudogene
CATGGATGTTCTTT	0.99	18	18.1	15	15	17	9	Multiple matches
CATGCAGGAGACGA	11	185	16.9	75	209	108	52	No annotation
CATGATCAGTCACG	0.99	16	16.0	0	0	2	0	No matches
CATGGGCTGGTTCC	0.99	16	16.0	2	0	17	4	No annotation
CATGGCAAATAGAT	0.99	16	16.0	16	12	13	14	Multiple matches
CATGTATACCTGTG	17	269	15.7	475	445	459	369	Multiple matches
CATGCTGCTCAGCT	0.99	15	14.9	2	2	2	0	Transcription elongation factor B polypeptide 3 binding protein 1
CATGCTCAGTCACG	0.99	15	14.9	2	4	8	12	No annotation
CATGTGCCCCCTCC	0.99	15	14.9	47	50	20	26	Acylphosphatase
CATGACTACTGGAA	2	23	14.9	12	22	15	12	Multiple matches
CATGTCCATTGAGT	3	43	13.9	32	32	38	41	Multiple matches
CATGGTGAGACCAA	0.99	14	13.9	0	3	9	5	No matches
CATGGGATTTTCCG	0.99	14	13.9	3	4	6	9	No annotation
CATGATCTGAGCCA	0.99	14	13.9	12	4	5	2	No annotation
CATGATCTGAGCCA	0.99	14	13.9	12	4	5	2	No annotation
CATGAAAAGATGCT	0.99	14	13.9	10	5	7	5	Multiple matches
CATGTATGCCTGTG	0.99	14	13.9	17	17	20	15	CCA3
CATGTGTTCCCCAT	6	86	13.7	25	21	50	39	Succinate dehydrogenase [ubiquinone] flavoprotein subunit
CATGAAATTTAAAAG	2	21	13.5	21	27	14	17	Multiple matches
CATGGCCTAGCGAC	9	127	13.5	4	35	80	38	No matches

(Table 2.4 Continued)

CATGTGTTTGTTAG	0.99	13	12.8	3	0	4	4	No annotation
CATGAGATTCTCCA	0.99	13	12.8	3	4	5	4	Ubiquitin-specific protease
CATGAAAGTGAAAA	2	19	12.2	7	8	14	17	Multiple matches
CATGAGGAAGGAGG	5	55	11.7	13	10	31	35	Seryl-tRNA synthetase
CATGGACATCACCA	0.99	12	11.7	16	16	21	8	NADH-ubiquinone oxidoreductase chain 6
CATGGTGGCTCGGA	0.99	11	10.7	0	3	3	0	No annotation
CATGGGCAGAGGAG	0.99	11	10.7	2	4	8	2	Multiple matches
CATGTGAAGAGTTG	0.99	11	10.7	6	4	8	2	No annotation
CATGCAAAGATGGG	0.99	11	10.7	15	14	9	11	Multiple matches
CATGGACTATAGCC	2	16	10.2	7	10	11	8	Multiple matches
CATGAAGTGATGGG	2	16	10.2	9	12	19	8	Multiple matches

^aAdjusted tag frequencies^bTags with a frequency of 0 were given a frequency of 0.99 to determine an approximate fold-difference

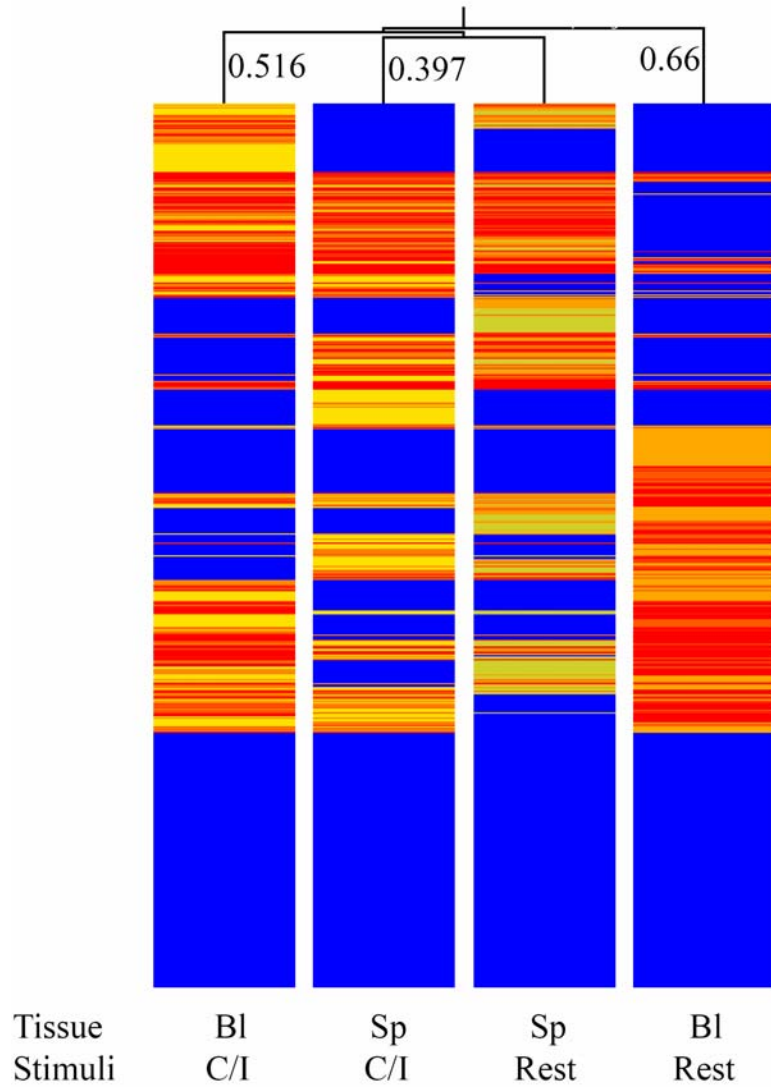


Fig. 2.4 MACS-sorted $\gamma\delta$ T cells from blood and spleen display unique gene expression profiles in both resting and ConA/IL-2-activated states. The condition tree at top displays the relatedness of the libraries based on the Pearson correlation of the gene expression profiles including only tags with an adjusted frequency of 10 or greater between all 12 libraries. The numerical values represent the length of the condition tree branches; shorter branches represent more similar datasets. Bl: blood, Sp: spleen, C/I: ConA/IL-2.

Table 2.5 Blood- and spleen $\gamma\delta$ T cell-specific tags.

Tag	Blood $\gamma\delta$ T cells			Spleen $\gamma\delta$ T cells			Annotation
	Resting	ConA/IL-2	Total	Resting	ConA/IL-2	Total	
CATGATTAAAGTAA	101 ^a	43	145	0	0	0	No annotation
CATGTCAGAGGTGG	119	16	134	0	0	0	Multiple matches
CATGCAGAAAGTCCA	91	42	133	0	0	0	IgG2a heavy chain C region
CATGGTCACCAGCT	70	24	95	0	0	0	Multiple matches
CATGGCTTTTACAA	28	18	46	0	0	0	Potential phospholipid-transporting ATPase IA
CATGAGATAGAGAA	2	41	43	0	0	0	No matches
CATGAGTGCAGAGT	25	10	34	0	0	0	No annotation
CATGAAGTGTTGCC	28	5	33	0	0	0	No annotation
CATGAGTGCACACT	25	7	32	0	0	0	No matches
CATGGAAGTGGAAG	17	15	32	0	0	0	MGC2477 protein
CATGCTGCTGGCCA	19	12	30	0	0	0	Multiple matches
CATGAGTGCAAACCT	22	4	26	0	0	0	No matches
CATGTCTATCCCTG	22	4	26	0	0	0	MHC class I molecule precursor
CATGACAACAATGG	20	5	26	0	0	0	Testosterone regulated apoptosis inducer and tumor suppressor (EAF2)
CATGTGCAGAGAAA	16	10	25	0	0	0	No annotation
CATGGAGGTAATAT	12	12	24	0	0	0	Cytochrome c oxidase subunit I
CATGGGTGCAGACT	17	6	24	0	0	0	No matches
CATGGCAGTTATAA	20	3	23	0	0	0	Thymine-DNA glycosylase
CATGAGTGCAGAAT	23	0	23	0	0	0	No matches
CATGAGATCTATAC	17	5	22	0	0	0	DEC-205/CD205 protein
CATGTATGTGACTA	17	5	22	0	0	0	Multiple matches
CATGAATTGGAGGA	19	3	22	0	0	0	Cytochrome b

(Table 2.5 Continued)

CATGCGATTAAAAT	22	0	22	0	0	0	E1A-associated protein p300
CATGAGTGCAGATT	20	0	20	0	0	0	No matches
CATGCAGGTGAGGC	16	4	20	0	0	0	No matches
CATGCTGTGGATGG	17	2	19	0	0	0	Spectrin beta chain
CATGCAGGTGACGC	12	6	19	0	0	0	No annotation
CATGACACTGTCAC	5	14	18	0	0	0	No annotation
CATGAGTGCGGACT	14	4	18	0	0	0	No matches
CATGGACAGGTCCC	9	8	18	0	0	0	No matches
CATGCTCTGAATAC	11	6	17	0	0	0	Protein disulfide isomerase related protein
CATGGTCACCGGCT	17	0	17	0	0	0	IgG2a heavy chain C region
CATGCACAAACAGT	17	0	17	0	0	0	40S ribosomal protein S27
CATGTTTGAAACCA	12	4	17	0	0	0	No matches
CATGCTGCCAAGCA	14	2	16	0	0	0	Multiple matches
CATGATCAGTCACG	0	16	16	0	0	0	No matches
CATGAGCGCAGACT	16	0	16	0	0	0	No matches
CATGTGGGCGGATG	2	14	15	0	0	0	Glycyl-trans synthetase
CATGGACTGTGCCA	8	7	15	0	0	0	Cytoplasmic dynein light polypeptide 1
CATGGGATTTCCAG	3	12	15	0	0	0	No annotation
CATGCACAGGGCTA	5	10	14	0	0	0	No annotation
CATGTCTCGGTAGC	8	6	14	0	0	0	No matches
CATGAAATAAAACA	8	6	14	0	0	0	Multiple matches
CATGATGGGAAATT	11	3	14	0	0	0	No annotation
CATGGCGGCCAAGA	8	5	13	0	0	0	No annotation
CATGGGAAGAAGCT	11	2	13	0	0	0	No matches
CATGCAGCTCCGCG	11	2	13	0	0	0	Cytochrome c oxidase subunit IV isoform 1

(Table 2.5 Continued)

CATGAGTAATAAAT	3	10	13	0	0	0	Cell cycle progression 2 protein, isoform 1
CATGGAAGAGCCCC	6	6	13	0	0	0	Phospholipid hydroperoxide glutathione peroxidase
CATGTCTCCTGATA	6	6	13	0	0	0	Multiple matches
CATGGGCTGGCTGG	12	0	12	0	0	0	Alpha-galactosidase B
CATGTGGCTTAATG	12	0	12	0	0	0	Multiple matches
CATGGAGCTCCGCC	2	11	12	0	0	0	No matches
CATGGACGCATATG	8	4	12	0	0	0	Microsomal signal peptidase 25 kDa subunit
CATGCTCAAAAATG	6	5	12	0	0	0	Multiple matches
CATGTGGGAAATTC	6	5	12	0	0	0	Glutamine-asparagine rich protein
CATGTAACTGCCCCA	9	2	11	0	0	0	No annotation
CATGGTGCCATCCT	9	2	11	0	0	0	Ab2-402
CATGCTGGAGATCA	9	2	11	0	0	0	No annotation
CATGGTCATCGGCT	8	3	11	0	0	0	No matches
CATGGGAGCTAATT	8	3	11	0	0	0	No matches
CATGAGGAGGAGGA	8	3	11	0	0	0	Calreticulin (Calregulin)
CATGAGTGCATACT	11	0	11	0	0	0	No matches
CATGTCCTTCTTAC	11	0	11	0	0	0	No annotation
CATGTAAAAGACAA	11	0	11	0	0	0	Alpha II spectrin
CATGGGGTCGTGAA	3	7	11	0	0	0	Multiple matches
CATGGAATCAAAGA	6	4	10	0	0	0	No annotation
CATGTGATGTTTGA	9	1	10	0	0	0	No annotation
CATGGAGGAGGGTG	9	1	10	0	0	0	Nuclear inhibitor of protein phosphatase-1 (NIPP-1)

(Table 2.5 Continued)

CATGAAGGAGAAAT	9	1	10	0	0	0	Multiple matches
CATGGGAAAAGGTT	0	0	0	21	12	33	No matches
CATGACTTAGCAAC	0	0	0	13	13	26	Multiple matches
CATGCTGCAGATTA	0	0	0	8	13	21	No matches
CATGCCACAGCTAA	0	0	0	4	13	17	Multiple matches
CATGTGTAGAGTCT	0	0	0	7	9	16	No annotation
CATGCTTCCACTGC	0	0	0	6	10	15	Multiple matches
CATGAGCTCCTTCC	0	0	0	8	6	15	No matches
CATGCTGTGTGGTA	0	0	0	9	3	12	No annotation
CATGGGAGAAAAAA	0	0	0	6	6	12	Nuclear receptor corepressor 1 (N-CoR1)
CATGGGATCGCAGA	0	0	0	2	10	12	No annotation
CATGGGTAAAAAAA	0	0	0	7	4	12	cAMP-dependent protein kinase type II-beta regulatory chain
CATGGGGCAAAAAA	0	0	0	3	8	11	No annotation
CATGACATTGTATA	0	0	0	5	5	10	No matches
CATGACAGTAGTCA	0	0	0	9	1	10	No matches

^aAdjusted tag frequencies

All four populations of blood and spleen $\gamma\delta$ T cells (resting and ConA/IL-2 stimulated) shared expression of many of the same genes (Fig. 2.4). However, cells from both locations expressed tissue-specific genes. This study identified 70 different genes that had an adjusted frequency of at least 10 when both blood libraries were combined and were not present in the spleen libraries, including CD205, a dendritic and myeloid cell antigen-recognition molecule (101; Table 2.5). There were 14 genes identified as specific to $\gamma\delta$ T cells in this one spleen (Table 2.5), including nuclear receptor co-repressor 1 (N-CoR1), which has been implicated in regulation of T cell development and macrophage activation (90,150).

On a global level of comparison, $\gamma\delta$ T cells localized to the spleen were in a more transcriptionally active state than were circulating $\gamma\delta$ T cells. Blood $\gamma\delta$ T cells following activation with ConA/IL-2 had a gene expression pattern more similar to both the resting and activated spleen cells than to the resting blood cell population (Fig 2.4). Thus, resting blood cells clearly had the lowest levels of overall gene transcription. The 5-fold greater number of blood-specific genes than spleen-specific genes was partially due to the resting population of blood $\gamma\delta$ T cells, which displayed very different gene expression profiles than activated blood or either population of spleen $\gamma\delta$ T cells. Tables 2.3 and 2.4 illustrate that genes expressed at high levels in stimulated blood $\gamma\delta$ T cells were also expressed at high levels in both spleen populations. Likewise, genes that were expressed at higher levels in resting blood $\gamma\delta$ T cells were expressed, on average, at low levels in the spleen. These results suggest that $\gamma\delta$ T cells isolated from various microenvironments

can have dramatically different gene expression profiles, thus warranting future studies on $\gamma\delta$ T cells from different tissues.

As an initial experiment to compare $\gamma\delta$ T cells to the predominant $\alpha\beta$ T cell population, SAGE libraries were constructed from resting and ConA/IL-2 activated $\alpha\beta$ T cell enriched preparations from the same spleen used in the analyses described above. A shortcoming in the bovine model is that there are no reagents available to efficiently purify $\alpha\beta$ T cells using a single mAb and the MACS bead separation protocol. At best, we achieved in the non- $\gamma\delta$ T cell fraction, $\alpha\beta$ T cell preparations which were 80% pure versus >90% pure $\gamma\delta$ T cells (data not shown). The contaminating cells in the $\alpha\beta$ T cell preparation included B cells (5%), residual $\gamma\delta$ T cells (10%), and a few monocytes and/or NK cells (<5%).

As expected, both spleen T cell populations expressed many of the same genes, though at slightly different levels (Fig. 2.5a). However, several genes unique to $\gamma\delta$ T cells were observed, such as N-CoR1 (also spleen-specific, Table 2.5). As another means of illustrating this data, the percentage of expressed genes (adjusted frequency of 3 or more in a single library) that were similar or different in 2-way comparisons of the four libraries are shown in pie graph form (Fig. 2.5b). When resting $\gamma\delta$ T cells were compared to resting $\alpha\beta$ T cells, only 1% of the unique tags were specific to $\gamma\delta$ T cells, suggesting a minimal impact of the MACS bead isolation protocol on generating artificial differences between the cell populations. However, following ConA/IL-2 stimulation, which

provided a robust signal for both populations, the percentage of tags specific to $\gamma\delta$ T cells increased to 5%.

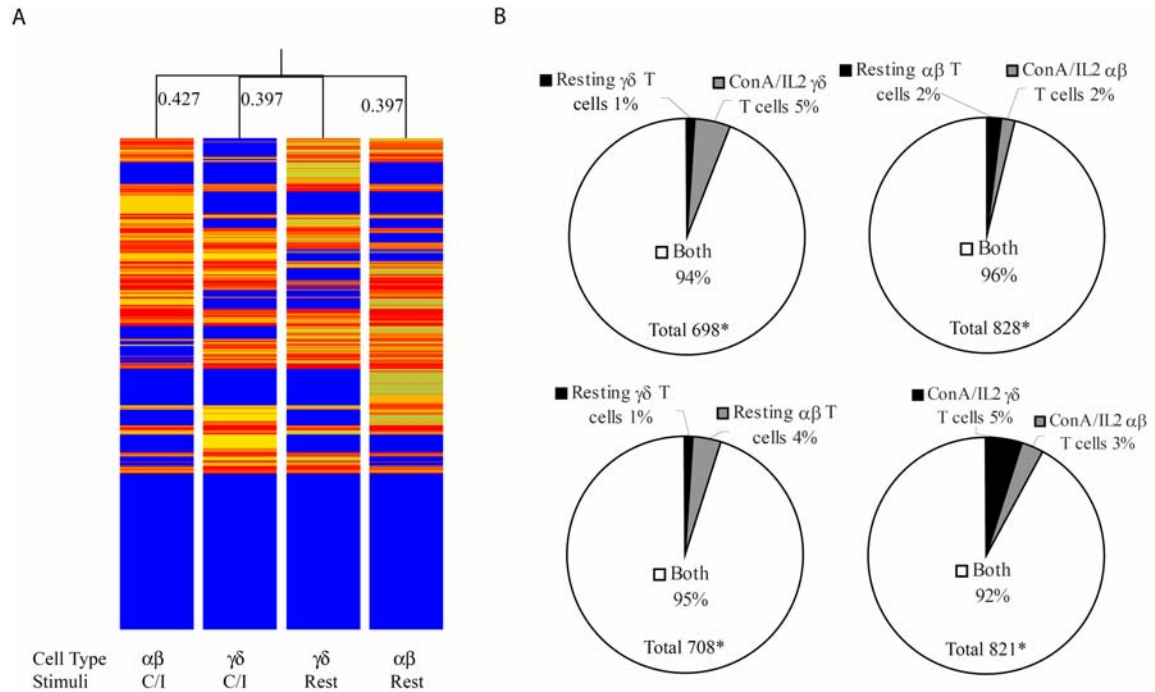


Fig. 2.5 MACS-sorted spleen $\gamma\delta$ and $\alpha\beta$ T cells share the majority of their expressed genes, however $\gamma\delta$ T cells up-regulate a greater number of genes upon stimulation than do $\alpha\beta$ T cells. A. Condition tree demonstrating the relatedness of all four libraries based on the Pearson correlation of the gene expression profiles including only tags with an adjusted frequency of 10 or greater between all 12 libraries. B. Comparison of libraries using unique tags (3 adjusted tags or more in a single library and not present in the compared library). $\alpha\beta$: enriched $\alpha\beta$ T cells, $\gamma\delta$: pure $\gamma\delta$ T cells, C/I: ConA/IL-2. *Total number of tags used in each comparison.

Comparisons of total blood $\gamma\delta$ T cells, total spleen $\gamma\delta$ T cells and total spleen $\alpha\beta$ T cells indicated that the gene expression profiles of spleen $\gamma\delta$ and $\alpha\beta$ T cells were more similar to each other than are $\gamma\delta$ T cells isolated from different microenvironments (Fig. 2.6). These data suggested that microenvironments, or the T cell subsets that localize to

them, play a greater role in determining gene expression profiles in T cells than does specific TCR chain expression. It should be noted that the cell isolation procedure needed for isolating tissue cells is much more extensive than that used to isolate blood cells. The difference in cell health (due to sorting procedures) of the isolated populations may affect the quality of RNA isolated for the library construction, which would affect the resulting SAGE data. However, these data also suggested that more insight into unique functions of $\gamma\delta$ T cells may be gained when these cells are analyzed following their response to specific agonists in vivo or in vitro.

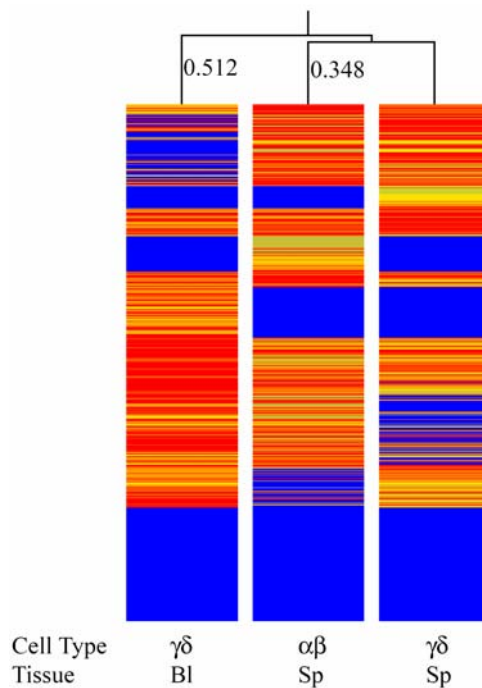


Fig. 2.6 The microenvironment in which a T cell is found plays a greater role in determining the gene expression profile than does the TCR lineage of the cell. Data from the resting and ConA/IL-2 stimulated libraries were combined from each cell preparation. The condition tree at top displays the relatedness of the libraries based on the Pearson correlation of the gene expression profiles including only tags with an adjusted frequency of 10 or greater between all 12 libraries.

Analysis of FACS Sorted PBLs

Another series of SAGE experiments were done using FACS in the absence of TCR crosslinking, as done in the MACS bead protocol, which isolated purer preparations of $\gamma\delta$ and $\alpha\beta$ T cells (>95%), to test the conclusions drawn from the SAGE libraries built from MACS-separated lymphocytes. To compare the impact on gene expression by different activating procedures, ConA/IL-2 and PMA/ionomycin stimulation were tested. Also, to minimize the effect of gene expression variability between animals, these SAGE libraries were generated from equivalent, pooled cDNA preparations from three animals.

Comparison of the data from ConA/IL-2 and PMA/ionomycin stimulated total $\gamma\delta$ T cells suggested that $\gamma\delta$ T cells responded to the different mitogenic stimuli by up- and down-regulating different sets of genes. The gene expression profiles of ConA/IL-2 stimulated $\gamma\delta$ T cells were more closely related to those of resting blood $\gamma\delta$ T cells, whereas PMA/ionomycin stimulated cells expressed a greater number of unique genes (Fig. 2.7, Tables 2.6 and 2.7). Specifically, there were 57 genes that were 15-fold down-regulated in PMA/ionomycin stimulated $\gamma\delta$ T cells and only 14 genes down-regulated in ConA/IL-2 stimulated $\gamma\delta$ T cells. Ten of these genes were down-regulated over 15-fold under both conditions. Likewise, PMA/ionomycin induced up-regulation of 58 genes, whereas ConA/IL-2 induced only 18 genes 15-fold over resting $\gamma\delta$ T cells. This difference is readily seen in figure 2.7b, in that resting and ConA/IL-2 stimulated $\gamma\delta$ T cells share 70% of their expressed genes, whereas resting and PMA/ionomycin stimulated $\gamma\delta$ T cells share only 43% of their expressed genes. Interestingly, of the genes expressed

by ConA/IL-2 or PMA/ionomycin stimulated $\gamma\delta$ T cells, nearly one-third of them were unique to each activation state.

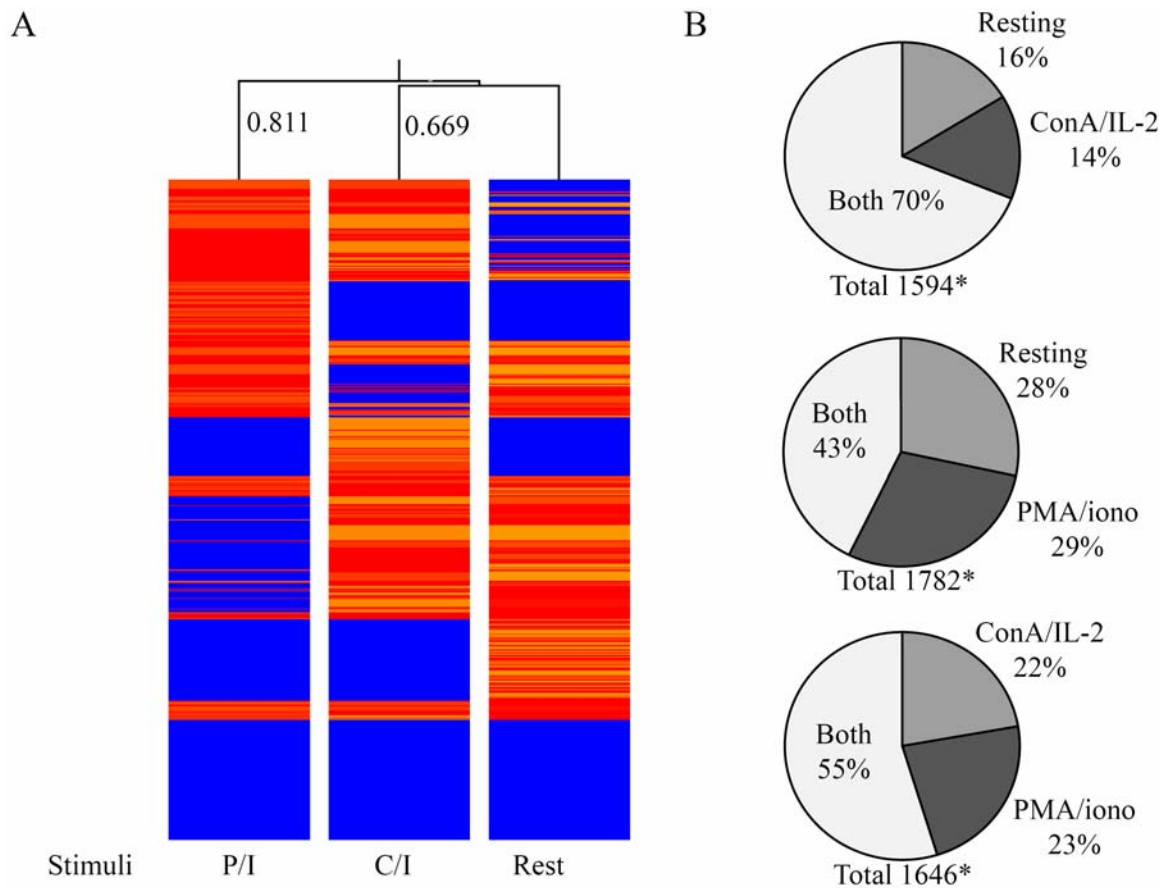


Fig. 2.7 FACS-sorted $\gamma\delta$ T cells respond by up- and down-regulating different sets of genes in response to ConA/IL-2 and PMA/ionomycin stimulation. A. Condition tree demonstrating the relatedness of the three blood $\gamma\delta$ T cell libraries based on the Pearson correlation of the gene expression profiles including only tags with an adjusted frequency of 10 or greater between all 12 libraries. B. Comparison of the libraries using unique tags (3 adjusted tags or more in a single library and not present in the compared library). *Total number of tags used in the comparisons.

Table 2.6 Tags expressed at a 10-fold or greater level in FACS-sorted resting blood $\gamma\delta$ T cells than in ConA/IL-2 and/or PMA/ionomycin stimulated blood $\gamma\delta$ T cells.

Tag	Blood $\gamma\delta$ T cells					Blood $\alpha\beta$ T cells					Annotation
	Resting	ConA/ IL2	Fold diff.	PMA/ iono	Fold diff.	Resting	ConA/ IL2	Fold diff.	PMA/ iono	Fold diff.	
CATGTGCCCCTGCA	45 ^a	0.99 ^b	45.3	0.99	45.3	9	0.99	8.7	0.99	8.7	Growth factor regulated calcium channel
CATGCCTCCCGAAG	31	0.99	30.8	0.99	30.8	36	15	2.4	7	5.1	No matches
CATGTATTAGGAAT	25	0.99	25.4	0.99	25.4	7	10	0.7	0.99	7.2	No annotation
CATGTGAACACTGT	48	2	24.2	0.99	48.9	10	12	0.8	12	0.9	Thrombospondin 3
CATGCCCCGAACTA	23	0.99	23.6	0.99	23.6	4	0.99	4.3	0.99	4.3	No matches
CATGAAGGTGGAAG	23	0.99	23.6	0.99	23.6	21	0.99	21.7	0.99	21.7	Hepatocyte growth factor-regulated tyrosine kinase substrate
CATGTACACGGATG	22	0.99	21.7	0.99	21.7	7	5	1.5	5	1.5	No matches
CATGCCAAATGTTT	16	0.99	16.3	0.99	16.3	1	0.99	1.4	0.99	1.4	No matches
CATGGAGGAGGAAG	188	12	15.7	7	26.5	412	56	7.3	23	17.5	CAMPATH-1 antigen precursor (CD52)
CATGAACTCTTTCT	31	2	15.3	0.99	30.8	41	7	5.6	0.99	41.9	No matches
CATGAAATTCATAT	18	0.99	18.1	7	2.5	4	5	0.9	14	0.3	No matches
CATGACTAAAGACG	18	0.99	18.1	4	5.1	13	2	5.3	2	5.5	No annotation
CATGCTTCATTTCC	65	4	16.1	7	9.1	29	17	1.7	0.99	28.9	No matches
CATGTTGGTGAAGG	32	2	16.1	4	9.1	60	10	6.1	19	3.2	Thymosin beta-4
CATGCTTGCTTGGG	29	2	14.4	0.99	29.0	61	12	5.0	0.99	62.1	Tumor necrosis factor, alpha-induced protein 4
CATGGATAAAAATG	50	4	12.6	0.99	50.7	11	27	0.4	0.99	11.6	No matches
CATGTCAATCTGTG	22	2	10.8	0.99	21.7	27	34	0.8	0.99	27.4	Microtubule-associated protein 4 isoform 1
CATGCTGGCCCGGA	20	2	9.9	0.99	19.9	14	4.9	2.9	5	3.0	Multiple matches
CATGAACTTGAATA	20	2	9.9	0.99	19.9	19	37	0.5	7	2.6	No matches
CATGGGCTAGAGGG	20	2	9.9	0.99	19.9	16	20	0.8	16	1.0	No matches

(Table 2.6 Continued)

CATGTAACCCATTA	18	2	9.0	0.99	18.1	1	0.99	1.4	0.99	1.4	No annotation
CATGATTAGTCCAT	18	2	9.0	0.99	18.1	6	2	2.3	0.99	5.8	No matches
CATGAGACAGGAAT	18	2	9.0	0.99	18.1	14	7	1.9	0.99	14.4	No matches
CATGTGCAAATAGT	106	12	8.8	0.99	106.9	66	34	1.9	0.99	66.4	No matches
CATGATAACTCCAT	52	6	8.7	0.99	52.5	14	20	0.7	0.99	14.4	No matches
CATGAGTGTTGTGT	16	2	8.1	0.99	16.3	17	12.26	1.4	0.99	17.3	No matches
CATGGAGACCACCG	31	4	7.6	0.99	30.8	34	2.45	14.0	7	4.9	No matches
CATGTTCCCTCACGA	25	4	6.3	0.99	25.4	0.99	0.99	1.0	0.99	1.0	No matches
CATGCTGGTGCTTT	31	6	5.1	0.99	30.8	11	5	2.3	2	4.9	No matches
CATGATTTTTGAAG	20	4	4.9	0.99	19.9	7	5	1.5	0.99	7.2	No matches
CATGCTTGTGGGTG	29	6	4.8	0.99	29.0	4	7	0.6	2	1.8	No matches
CATGCTGTACATTT	160	34	4.7	7	22.5	197	27	7.3	14	14.0	ATP-dependent RNA helicase DeaD
CATGAATCCTTCCC	74	16	4.6	4	20.7	63	49	1.3	5	13.4	No matches
CATGAATTACTGAG	18	4	4.5	0.99	18.1	0.99	0.99	1.0	0.99	1.0	Isocitrate dehydrogenase [NAD] subunit alpha
CATGCCACCGTCTC	18	4	4.5	0.99	18.1	0.99	0.99	1.0	0.99	1.0	No matches
CATGTGCCTTGTGC	36	8	4.5	0.99	36.2	113	54	2.1	0.99	114.1	Calpactin I heavy chain (p36)
CATGGGAAGCGGCA	36	8	4.5	0.99	36.2	36	27	1.3	2	15.2	Galectin-9
CATGAGATAACTAT	16	4	4.0	0.99	16.3	4	2	1.8	2	1.8	No matches
CATGGGATGATTTT	23	6	3.9	0.99	23.6	20	15	1.4	0.99	20.2	Multiple matches
CATGGAGCCAAAGG	22	6	3.6	0.99	21.7	17	37	0.5	2	7.3	No matches
CATGTCAACATCTA	27	8	3.4	0.99	27.2	33	15	2.2	5	7.0	No matches
CATGAAACACCAGT	25	8	3.1	0.99	25.4	20	32	0.6	16	1.2	No matches
CATGCGTACTCCTG	18	6	3.0	0.99	18.1	21	15	1.5	7	3.0	No annotation
CATGGACTGCAGCC	16	6	2.7	0.99	16.3	11	7	1.6	0.99	11.6	Multiple matches
CATGCACCAAATAA	16	6	2.7	0.99	16.3	10	5	2.0	2	4.3	No matches

(Table 2.6 Continued)

CATGGCTTTTTATT	27	10	2.7	0.99	27.2	4	7	0.6	2	1.8	HT014
CATGGAATTCTCCA	25	10	2.5	0.99	25.4	11	15	0.8	7	1.6	Multiple matches
CATGCAGCTAGCAA	20	8	2.5	0.99	19.9	0.99	2	0.4	0.99	1.0	No matches
CATGGTGTTTAGCA	18	8	2.2	0.99	18.1	57	7	7.8	0.99	57.8	No matches
CATGGCTCGGGCGG	18	8	2.2	0.99	18.1	17	12	1.4	0.99	17.3	Multiple matches
CATGTCCGACTCTG	27	12	2.2	0.99	27.2	20	2	10.6	14	1.4	Multiple matches
CATGTGCTGCAACT	22	10	2.2	0.99	21.7	10	7	1.4	12	0.9	Triggering receptor expressed on myeloid cells 1 (TREM-1)
CATGACTGAGCACA	22	12	1.8	0.99	21.7	9	17	0.5	12	0.7	Multiple matches
CATGAAGTCCTTAG	18	10	1.8	0.99	18.1	23	5	4.7	2	9.7	No matches
CATGGCAGCAGCGT	18	10	1.8	0.99	18.1	14	7	1.9	2	6.1	No matches
CATGGATGAACCTA	41	32	1.3	0.99	41.7	46	93	0.5	7	6.5	No annotation
CATGTGGACTTGTG	32	30	1.1	0.99	32.6	30	12	2.4	9	3.2	CD3 protein
CATGCTTACCTGCC	22	20	1.1	0.99	21.7	17	27	0.6	0.99	17.3	No annotation
CATGTAATTTAAAA	16	16	1.0	0.99	16.3	20	7	2.7	4.7	4.3	No annotation
CATGCACAAACGTG	16	18	0.9	0.99	16.3	20	17	1.2	7	2.8	No matches
CATGGTCATAATGG	22	30	0.7	0.99	21.7	0.99	12	0.1	21	0.0	No matches

^aAdjusted tag frequencies^bTags with a frequency of 0 were given a frequency of 0.99 to determine an approximate fold-difference

Table 2.7 Tags expressed at a 10-fold or greater level in FACS-sorted ConA/IL-2 and/or PMA/ionomycin stimulated blood $\gamma\delta$ T cells than in resting blood $\gamma\delta$ T cells.

Tag	Resting	Blood $\gamma\delta$ T cells				Resting	Blood $\alpha\beta$ T cells				Annotation
		ConA/ IL2	Fold diff.	PMA/ iono	Fold diff.		ConA/ IL2	Fold diff.	PMA/ iono	Fold diff.	
CATGATCTCCCCAG	0.99 ^{a,b}	50	50.5	32	32.3	19	22	1.2	45	2.4	No matches
CATGCTTTCCATTT	4	104	29.0	103	28.7	3	12	4.3	26	9.0	No annotation
CATGCTGTCCATTT	0.99	28	28.3	28	28.7	0.99	4.9	4.9	14	14.2	No annotation
CATGAGATAGAGAA	0.99	26	26.3	64	64.5	0.99	5	4.9	63	64.1	No matches
CATGCAGCCGACTA	0.99	26	26.3	43	43.0	0.99	2	2.5	9	9.5	No matches
CATGGTGCCTGCCT	2	40	22.3	114	63.4	14	0.99	0.1	33	2.3	No annotation
CATGGCTAGGATCC	2	38	21.2	57	31.7	4	10	2.3	70	16.4	Nuclear factor erythroid 2 related factor 1
CATGAGTGGGTACC	0.99	18	18.2	18	17.9	4	12	2.9	7	1.6	No matches
CATGAGCAGCAAAA	2	28	15.6	67.4	37.7	7	12	1.7	28	3.9	No annotation
CATGAGTATTCACA	0.99	24	24.2	14	14.3	1	0.99	0.7	9	6.6	No annotation
CATGCTGTGTGATG	0.99	18	18.2	14	14.3	3	0.99	0.3	21	7.4	Integrin, alpha X (antigen CD11C)
CATGGATATCCGCA	0.99	18	18.2	0.99	1.0	0.99	0.99	1.0	0.99	1.0	No matches
CATGGCTGGTTCCC	0.99	16	16.2	11	10.7	0.99	5	4.9	7	7.1	No matches
CATGTTTAAAACTT	0.99	16	16.2	7	7.2	4	7	1.7	9	2.2	SclB protein
CATGACCATCAGCC	0.99	16	16.2	7.1	7.2	6	29	5.1	9	1.6	No matches
CATGCACTCCTGTG	0.99	16	16.2	4	3.6	0.99	7	7.4	2	2.4	No annotation
CATGAGTAACCCCA	0.99	16	16.2	4	3.6	4	22	5.1	2	0.5	No matches
CATGTCATAGGGCA	2	28	15.6	14	7.9	0.99	47	47.1	21	21.4	No matches
CATGAGGAGGAGGA	2	26	14.5	145	81.3	0.99	1	1.0	33	33.2	Calreticulin (Calregulin)
CATGCGGTTTGAAC	2	16	8.9	78	43.6	4	1	0.2	45	10.4	No matches
CATGCCTCACCGAT	0.99	0.99	1.0	43	43.0	0.99	2	2.5	7	7.1	No annotation
CATGAGATCTATAC	0.99	2	2.0	39	39.4	3	2	0.9	14	4.9	DEC-205/CD205 protein
CATGGCACAGTTTA	0.99	14	14.1	32	32.3	3	12	4.3	7	2.5	No matches
CATGCGGGACGCAC	0.99	8	8.1	32	32.3	1	5	3.4	21	14.8	Autoantigen NGP-1

(Table 2.7 Continued)

CATGGCAAGAAGTT	0.99	6	6.1	32	32.3	0.99	2	2.5	16	16.6	No annotation
CATGTGCCGCAACT	0.99	4	4.0	32	32.3	0.99	0.99	1.0	7	7.1	Multiple matches
CATGAAAACAGTAG	2	8	4.5	57	31.7	4	2	0.6	26	6.0	Multiple matches
CATGAGGAGTGTAT	0.99	12	12.1	28	28.7	7	0.99	0.1	23	3.3	No annotation
CATGCGGGCAAAGA	0.99	0.99	1.0	28	28.7	1	0.99	0.7	2	1.6	No matches
CATGCTGCAATACG	0.99	0.99	1.0	28	28.7	1	0.99	0.7	5	3.3	Multiple matches
CATGAAAAAATCTT	0.99	10	10.1	25	25.1	0.99	2	2.5	12	11.9	Multiple matches
CATGGAAGATGTGG	2	8	4.5	43	23.8	1	2	1.7	19	13.1	Nuclear ubiquitous casein and cyclin-dependent kinases substrate (P1)
CATGTGGGGCTGGC	2	4	2.2	43	23.8	0.99	2	2.5	7	7.1	No annotation
CATGGTTCGTGCCA	4	0.99	0.3	82	22.7	6	2	0.4	12	2.1	60S ribosomal protein L35a
CATGTTCAAAGTTT	0.99	12	12.1	21	21.5	3	7	2.6	2	0.8	No matches
CATGTGTATCTGGT	0.99	8	8.1	21	21.5	1	0.99	0.7	26	18.1	Immunoglobulin heavy chain binding protein (BiP)
CATGGGGAGAGGCT	0.99	6	6.1	21	21.5	0.99	0.99	1.0	2	2.4	No annotation
CATGGCGGAGAGGG	0.99	6	6.1	21	21.5	0.99	0.99	1.0	12	11.9	No matches
CATGATGGGGGCGG	0.99	2	2.0	21	21.5	3	2	0.9	0.99	0.3	No annotation
CATGGCCGTTTTGG	0.99	2	2.0	21	21.5	0.99	0.99	1.0	5	4.7	No annotation
CATGTGTAGTTTCA	0.99	2	2.0	21	21.5	3	0.99	0.3	5	1.6	SCF complex protein (Skp1)
CATGGATGCTGCCA	0.99	2	2.0	21	21.5	1	2	1.7	9	6.6	Ribosomal protein L22
CATGGCAAGAAGCC	0.99	2	2.0	21	21.5	0.99	0.99	1.0	12	11.9	Vacuolar sorting protein4 A
CATGAGACAAGAGT	2	0.99	0.6	35	19.8	0.99	0.99	1.0	0.99	1.0	No matches
CATGAGCAGTAAAA	4	30	8.4	67	18.8	11	47	4.1	28	2.5	No matches
CATGTACTCTTGGC	0.99	10	10.1	18	17.9	4	0.99	0.2	7	1.6	Heterogeneous nuclear ribonucleoprotein L
CATGATAAATTGAA	0.99	8	8.1	18	17.9	0.99	0.99	1.0	2	2.4	BTG3 protein (Tob5 protein)
CATGAGGCAGATGA	0.99	8	8.1	18	17.9	0.99	5	4.9	2	2.4	No matches

(Table 2.7 Continued)

CATGCACAGCACAT	0.99	6	6.1	18	17.9	0.99	5	4.9	5	4.7	No annotation
CATGTAGTGTTC	0.99	6	6.1	18	17.9	0.99	10	9.9	40	40.3	High mobility group-like nuclear protein 2 homolog 1
CATGTGCAAGGACA	0.99	4	4.0	18	17.9	1	0.99	0.7	9	6.6	Bcl-2-related protein A1
CATGGGTGTATGAG	0.99	4	4.0	18	17.9	0.99	7	7.4	9	9.5	No annotation
CATGCAGTGTGGT	0.99	4	4.0	18	17.9	0.99	0.99	1.0	21	21.4	Multiple matches
CATGTGAAATATTT	0.99	4	4.0	18	17.9	1	0.99	0.7	31	21.3	CD69
CATGTAGTAGTTTG	0.99	2	2.0	18	17.9	0.99	0.99	1.0	0.99	1.0	Protein-tyrosine phosphatase 1B
CATGGATAGATCTA	0.99	2	2.0	18	17.9	0.99	0.99	1.0	0.99	1.0	No annotation
CATGATCAGAAAAG	0.99	2	2.0	18	17.9	0.99	0.99	1.0	0.99	1.0	Multiple matches
CATGTTTCAGATTG	0.99	2	2.0	18	17.9	0.99	0.99	1.0	5	4.7	Multiple matches
CATGGAAGTAGAGG	0.99	2	2.0	18	17.9	0.99	2	2.5	7	7.1	Multiple matches
CATGTTCTGTGAAA	0.99	2	2.0	18	17.9	1	0.99	0.7	12	8.2	Multiple matches
CATGGTGACCACGG	2	8	4.5	32	17.8	14	5	0.3	26	1.8	Multiple matches
CATGGAGTGAAAAA	2	0.99	0.6	32	17.8	0.99	0.99	1.0	2	2.4	50S ribosomal protein L25
CATGCTTTTCACTT	2	24	13.4	28	15.9	14	5	0.3	35	2.5	RBX1 protein
CATGAAGGCCCAGG	2	6	3.4	28	15.9	4	0.99	0.2	2	0.5	No matches
CATGAGACGGTGGT	4	12	3.3	57	15.8	0.99	2	2.5	31	30.8	YY1 transcription factor
CATGTTCAAGCAAG	2	6	3.4	25	13.9	6	5	0.9	7	1.2	Multiple matches
CATGGTTGGAGGGG	0.99	6	6.1	11	10.7	1	5	3.4	26	18.1	Prefoldin subunit 2

^aAdjusted tag frequencies^bTags with a frequency of 0 were given a frequency of 0.99 to determine an approximate fold-difference

Both stimuli induced up-regulation of transcription factors, including nuclear erythroid 2 related factor 1. Integrin- α expression was also up-regulated in both populations. The activation marker CD69 was dramatically up-regulated in cells stimulated with PMA/ionomycin. ConA/IL-2 also induced increased expression of CD69 on $\gamma\delta$ T cells, but to a lesser extent, supporting the observation that PMA/ionomycin have a more robust effect on gene expression profiles (Table 2.7).

Like $\gamma\delta$ T cells, $\alpha\beta$ T cells responded to the mitogens by regulating the expression of different sets of genes. PMA/ionomycin also induced a greater change in gene expression profiles than did ConA/IL-2. $\alpha\beta$ T cells down-regulated the expression of approximately the same number of genes with respect to PMA/ionomycin and ConA/IL-2, as did $\gamma\delta$ T cells, 38 and 12 respectively (Fig. 2.8 and Table 2.8). However, $\alpha\beta$ T cells did not up-regulate nearly as many genes in response to the stimuli (PMA/ionomycin and ConA/IL-2) as did $\gamma\delta$ T cells, 22 and 5 respectively (Table 2.9). In fact, the three different $\alpha\beta$ T cell populations shared a much higher percentage of their expressed genes than did $\gamma\delta$ T cells, illustrating less of a transcriptional response to the stimulants.

$\alpha\beta$ T cells, similar to $\gamma\delta$ T cells, up-regulated the expression of transcription and translation factors in response to mitogenic stimulation (Table 2.9). Several genes encoding cytokines were up-regulated over 10-fold in PMA/ionomycin stimulated $\alpha\beta$ T cells, including IFN- γ , MIP-1 α and MIP-3 α (data not shown). Both stimuli also dramatically down-regulated some genes, including CD2 and connexin 43 (Table 2.8).

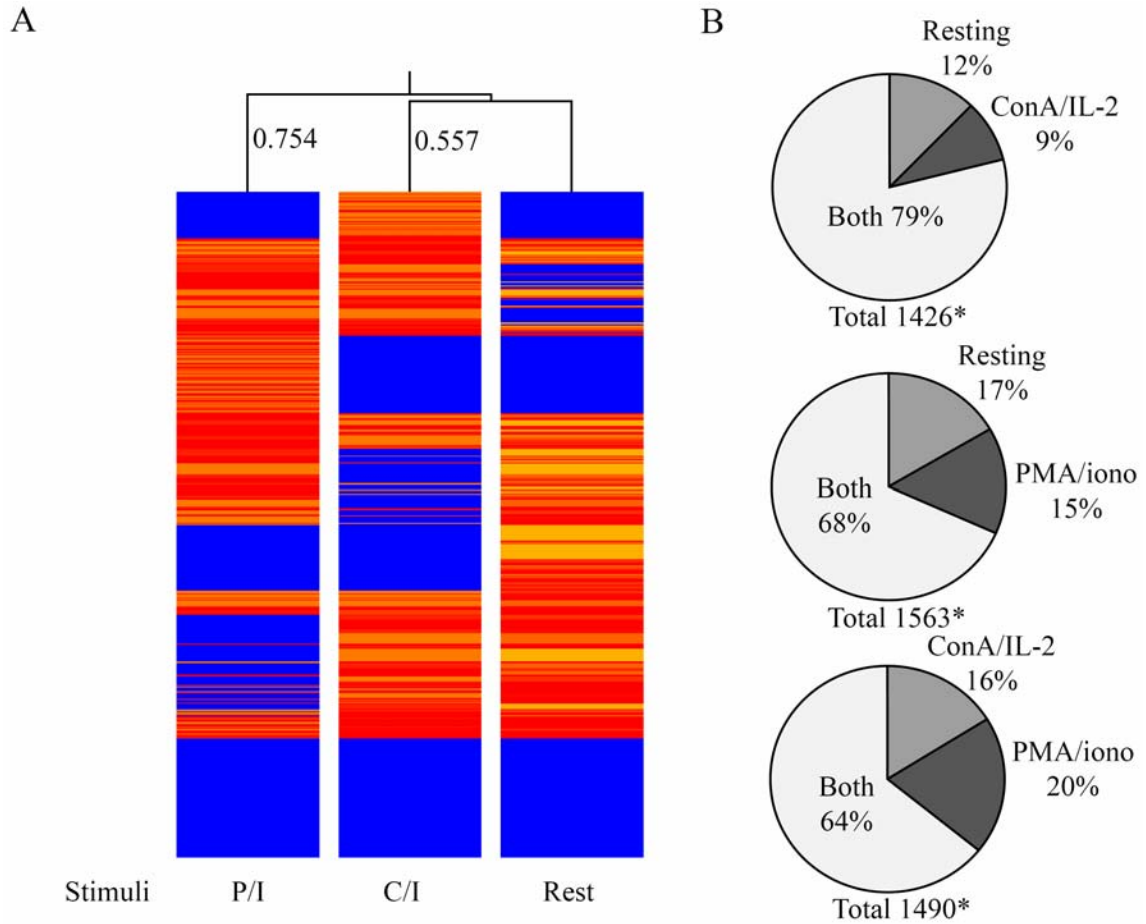


Fig. 2.8 FACS-sorted $\alpha\beta$ T cells respond by up- and down-regulating different sets of genes in response to ConA/IL-2 and PMA/ionomycin stimulation. A. Condition tree demonstrating the relatedness of the three blood $\alpha\beta$ T cell libraries based on the Pearson correlation of the gene expression profiles including only tags with an adjusted frequency of 10 or greater between all 12 libraries. B. Comparison of the libraries using unique tags (3 adjusted tags or more in a single library and not present in the compared library). *Total number of tags used in the comparisons.

Table 2.8 Tags expressed at a 10-fold or greater level in FACS-sorted resting blood $\alpha\beta$ T cells than in ConA/IL-2 and/or PMA/ionomycin stimulated blood $\alpha\beta$ T cells.

Tag	Blood $\gamma\delta$ T cells					Blood $\alpha\beta$ T cells					Annotation
	Resting	ConA/ IL2	Fold diff.	PMA/ iono	Fold diff.	Resting	ConA/ IL2	Fold diff.	PMA/ iono	Fold diff.	
CATGGGTTTCGATCC	14 ^a	0.99 ^b	14.5	0.99	14.5	36	0.99	36.1	0.99	36.1	Multiple matches
CATGAAGGTGGAAG	23	0.99	23.6	0.99	23.6	21	0.99	21.7	0.99	21.7	Hepatocyte growth factor-regulated tyrosine kinase substrate
CATGTCAGATGTCA	0.99	0.99	1.0	0.99	1.0	20	0.99	20.2	0.99	20.2	T-cell surface antigen CD2
CATGGAAATGGAGA	7	0.99	7.3	0.99	7.3	20	0.99	20.2	0.99	20.2	No matches
CATGAACGTGGAGG	7	0.99	7.3	14	0.5	20	0.99	20.2	0.99	20.2	No matches
CATGTGGCTACTTA	14	0.99	14.5	0.99	14.5	44	2	18.1	0.99	44.8	No matches
CATGGGTGAGAGCG	4	0.99	3.6	0.99	3.6	17	0.99	17.3	0.99	17.3	Connexin 43
CATGCTGTGGATGG	5	4	1.3	0.99	5.4	16	0.99	15.9	0.99	15.9	Spectrin beta chain
CATGGCCCCTGAAG	13	6	2.1	18	0.7	36	0.99	36.1	28	1.3	Multiple matches
CATGAGCCAAGGGG	5	2	2.7	0.99	5.4	19	0.99	18.8	2	7.9	No annotation
CATGACCACTGCAT	2	6	0.3	7	0.3	17	0.99	17.3	9	1.8	No matches
CATGAGCGAGTTCC	0.99	0.99	1.0	0.99	1.0	16	0.99	15.9	2	6.7	No matches
CATGTGCCTTGTGC	36	8	4.5	0.99	36.2	113	54	2.1	0.99	114.1	Calpactin I heavy chain (p36)
CATGTGCAAATAGT	106	12	8.8	0.99	106.9	66	34	1.9	0.99	66.4	No matches
CATGCTTGCTTGGG	29	2	14.4	0.99	29.0	61	12	5.0	0.99	62.1	Tumor necrosis factor, alpha-induced protein 4
CATGGTGTTTAGCA	18	8	2.2	0.99	18.1	57	7	7.8	0.99	57.8	No matches
CATGAACTCTTTCT	31	2	15.3	0.99	30.8	41	7	5.6	0.99	41.9	No matches
CATGAAGTCCTCTC	2	0.99	1.8	0.99	1.8	39	10	3.9	0.99	39.0	No matches
CATGCGCTTGTACT	5	2	2.7	0.99	5.4	37	10	3.8	0.99	37.6	DNA-directed RNA polymerase II 14.5 kDa polypeptide
CATGTGTGTCAGTT	0.99	0.99	1.0	0.99	1.0	30	12	2.4	0.99	30.3	No annotation
CATGCTTCATTTCC	65	4	16.1	7	9.1	29	17	1.7	0.99	28.9	No matches
CATGAAAGTGAGG	18	10	1.8	7	2.5	27	7	3.7	0.99	27.4	No matches

(Table 2.8 Continued)

CATGTCAATCTGTG	22	2	10.8	0.99	21.7	27	34	0.8	0.99	27.4	Microtubule-associated protein 4 isoform 1
CATGTGCAACGACT	9	12	0.7	4	2.5	26	5	5.3	0.99	26.0	No matches
CATGGCTTGCTTGT	0.99	0.99	1.0	0.99	1.0	23	10	2.3	0.99	23.1	No annotation
CATGTGTGGCAGGA	2	2	0.9	0.99	1.8	23	10	2.3	0.99	23.1	No matches
CATGATCCTCTATC	13	2	6.3	0.99	12.7	23	10	2.3	0.99	23.1	TYL protein
CATGCTTCCCTAGG	9	2	4.5	0.99	9.1	20	5	4.1	0.99	20.2	No matches
CATGACTCAAAAGA	7	4	1.8	0.99	7.3	20	10	2.0	0.99	20.2	Mastermind-like 1
CATGGGATGATTTT	23	6	3.9	0.99	23.6	20	15	1.4	0.99	20.2	Multiple matches
CATGGTTTCGATCC	2	6	0.3	0.99	1.8	19	5	3.8	0.99	18.8	No annotation
CATGGAGGAGGAAG	188	12	15.7	7	26.5	412	56	7.3	23	17.5	CD52 antigen
CATGTGGGGAAAAA	0.99	0.99	1.0	0.99	1.0	17	7	2.3	0.99	17.3	T-cell receptor alpha chain
CATGTGCATATTTG	0.99	2	0.5	0.99	1.0	17	7	2.3	0.99	17.3	No matches
CATGGAGACCATAT	5	8	0.7	7	0.8	17	10	1.7	0.99	17.3	No matches
CATGAGTGTTGTGT	16	2	8.1	0.99	16.3	17	12	1.4	0.99	17.3	No matches
CATGCTGGGGGACC	13	4	3.1	0.99	12.7	17	12	1.4	0.99	17.3	PITPNM1 protein
CATGGCTCGGGCGG	18	8	2.2	0.99	18.1	17	12	1.4	0.99	17.3	Multiple matches
CATGCTTACCTGCC	22	20	1.1	0.99	21.7	17	27	0.6	0.99	17.3	No annotation
CATGGATCTTGGGT	5	2	2.7	0.99	5.4	16	5	3.2	0.99	15.9	No matches
CATGGAGCGGTGAG	22	18	1.2	4	6.1	16	34	0.5	0.99	15.9	Glycosyltransferase-like 1B
CATGGGAAGCGGCA	36	8	4.5	0.99	36.2	36	27	1.3	2	15.2	Galectin-9

^aAdjusted tag frequencies^bTags with a frequency of 0 were given a frequency of 0.99 to determine an approximate fold-difference

Table 2.9 Tags expressed at a 10-fold or greater level in FACS-sorted ConA/IL-2 and/or PMA/ionomycin stimulated blood $\alpha\beta$ T cells than in resting blood $\alpha\beta$ T cells.

Tag	Blood $\gamma\delta$ T cells					Blood $\alpha\beta$ T cells					Annotation
	Resting	ConA/ IL2	Fold diff.	PMA/ iono	Fold diff.	Resting	ConA/ IL2	Fold diff.	PMA/ iono	Fold diff.	
CATGTCATAGGGCA	2 ^a	28	15.6	14	7.9	0.99 ^b	47	47.1	21	21.4	No matches
CATGGAGATCCACA	0.99	10	10.1	14	14.3	0.99	29	29.7	0.99	1.0	No matches
CATGAACCTCAGAG	0.99	0.99	1.0	7	7.2	0.99	25	24.8	7	7.1	Cytochrome b
CATGTAAGAATGAC	0.99	4	4.0	0.99	1.0	0.99	17	17.3	0.99	1.0	Helicase like protein 2
CATGGGAAGCTGGA	7	0.99	0.1	0.99	0.1	1	25	17.1	2	1.6	Beta-2-glycoprotein I
CATGAGATAGAGAA	0.99	26	26.3	64	64.5	0.99	5	4.9	63	64.1	No matches
CATGACCCGCCGGG	5	4	0.7	67	12.5	0.99	2	2.5	45	45.1	Multiple matches
CATGTAGTGTTC	0.99	6	6.1	18	17.9	0.99	10	9.9	40	40.3	High mobility group-like nuclear protein 2 homolog 1
CATGAGGAGGAGGA	2	26	14.5	145	81.3	0.99	0.99	1.0	33	33.2	Calreticulin (Calregulin)
CATGAGACGGTGGT	4	12	3.3	57	15.8	0.99	2	2.5	31	30.8	YY1 transcription factor
CATGTGAAATAAAC	4	10	2.8	50	13.8	1	5	3.4	40	27.9	Nucleophosmin
CATGGTCATAATGG	22	30	1.4	0.99	0.0	0.99	12	12.4	21	21.4	No matches
CATGTATGAACTGG	0.99	4	4.0	7	7.2	0.99	0.99	1.0	21	21.4	Cytochrome c oxidase polypeptide VIIa
CATGCAGTGTGGT	0.99	4	4.0	18	17.9	0.99	0.99	1.0	21	21.4	Multiple matches
CATGGCAGAGTTCG	2	8	4.5	28	15.9	1	7	5.1	31	21.3	Multiple matches
CATGTGAAATATTT	0.99	4	4.0	18	17.9	1	0.99	0.7	31	21.3	CD69
CATGAAAGCTTACA	2	4	2.2	4	2.0	0.99	2	2.5	19	19.0	Endozepine precursor
CATGGTTGGAGGGG	0.99	6	6.1	11	10.7	1	5	3.4	26	18.1	Prefoldin subunit 2
CATGTGTATCTGGT	0.99	8	8.1	21	21.5	1	0.99	0.7	26	18.1	Immunoglobulin heavy chain binding protein (BiP)

(Table 2.9 Continued)

CATGGGACCACTTA	7	16	2.2	92	12.8	4	2	0.6	73	17.0	60S ribosomal protein L3
CATGTCGTCTTCTT	0.99	0.99	1.0	0.99	1.0	0.99	5	4.9	16	16.6	No matches
CATGGCAAGAAGTT	0.99	6	6.1	32	32.3	0.99	2	2.5	16	16.6	No annotation
CATGGGGATCCGCA	4	14	3.9	0.99	0.3	0.99	0.99	1.0	16	16.6	No matches
CATGTGCATAAAAT	4	24	6.7	28	7.9	1	10	6.9	23	16.4	No annotation
CATGGCTGATTTC	5	6	1.1	18	3.3	1	2.45	1.7	23	16.4	Proteasome subunit alpha type 7
CATGGCTAGGATCC	2	38	21.2	57	31.7	4	10	2.3	70	16.4	Nuclear factor erythroid 2 related factor 1

^aAdjusted tag frequencies^bTags with a frequency of 0 were given a frequency of 0.99 to determine an approximate fold-difference

In comparing $\gamma\delta$ and $\alpha\beta$ T cell gene expression patterns, predictions based on the spleen analysis held true. For example, the majority of expressed genes were the same between blood T cell populations (81% in the resting populations; Fig. 2.9). However, upon stimulation with either ConA/IL-2 or PMA/ionomycin, the lymphocyte populations became dramatically more dissimilar and shared only 71 and 59% of their expressed genes, respectively. Figure 2.10 demonstrates the more robust response to mitogenic stimulation by $\gamma\delta$ T cells than $\alpha\beta$ T cells in that in all three conditions $\gamma\delta$ T cells shared only 29% of their expressed genes, whereas $\alpha\beta$ T cells shared 41%. This study also uncovered nearly 20 genes that were potentially specific to $\gamma\delta$ T cells and another 20 that were potentially specific to $\alpha\beta$ T cells (Fig. 2.11 and Table 2.10). These tags were represented with an adjusted frequency of 15 or greater in the $\gamma\delta$ T cell libraries (regardless of activation state) and were not present in any of the $\alpha\beta$ T cell libraries, or vice versa. The transcript that was expressed at the highest level in $\alpha\beta$ T cells, but was not present in the $\gamma\delta$ T cell population was the alpha chain of the TCR. Another confirmatory gene was the specificity of CD2 to the $\alpha\beta$ T cell library, as the large majority of bovine $\gamma\delta$ T cells do not express the CD2 molecule (212). Other $\gamma\delta$ T cell-specific genes worthy of note included VEGF, prohibitin (involved in repression of cell proliferation; 43,202), and a low-density lipoprotein receptor.

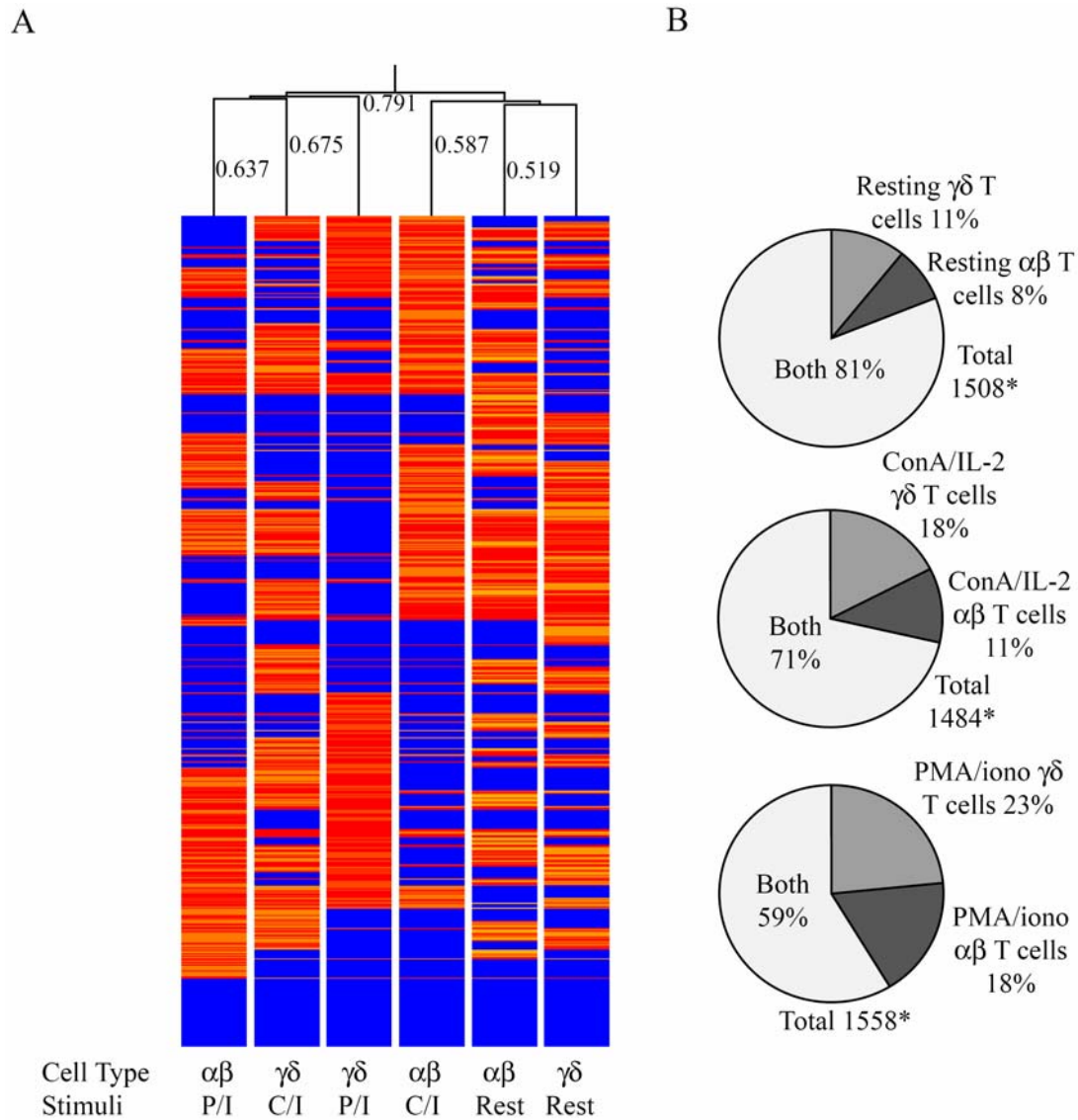


Fig. 2.9 FACS-sorted blood $\gamma\delta$ and $\alpha\beta$ T cells share the majority of their expressed genes, however $\gamma\delta$ T cells express a greater percentage of unique genes. A. Condition tree demonstrating the relatedness of all 6 FACS-sorted libraries based on the Pearson correlation of the gene expression profiles including only tags with an adjusted frequency of 10 or greater between all 12 libraries. B. Comparison of the libraries using unique tags (3 adjusted tags or more in a single library and not present in the compared library). $\alpha\beta$: pure $\alpha\beta$ T cells, $\gamma\delta$: pure $\gamma\delta$ T cells, C/I: ConA/IL-2, P/I: PMA/ionomycin. *Total number of tags used in the comparisons.

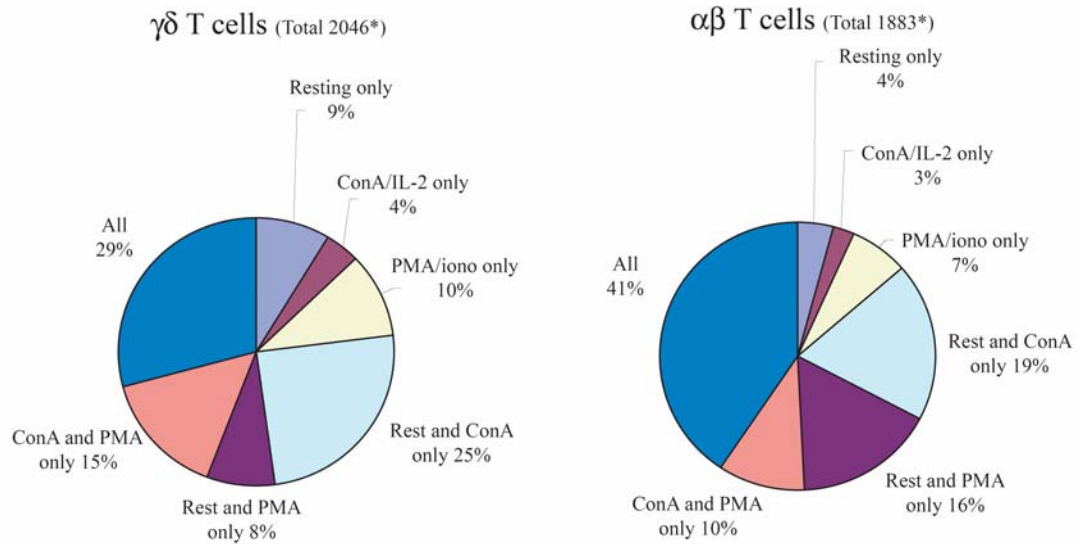


Fig. 2.10 FACS-sorted blood $\gamma\delta$ T cells respond more robustly to mitogenic stimulation than do $\alpha\beta$ T cells. Tags that are represented by an adjusted frequency of 3 or more and not in any of the other conditions are represented in each section. Only tags that were expressed at a level of 10 or more between all 12 libraries were included in the analysis. *Total number of tags used in the comparisons.

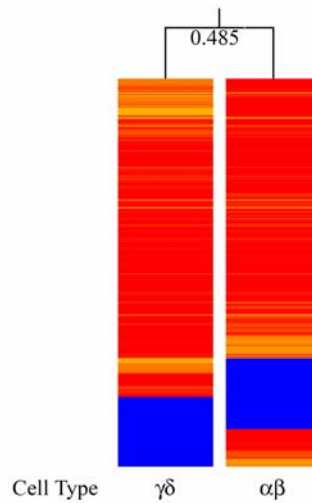


Fig. 2.11 The majority of FACS-sorted blood $\gamma\delta$ and $\alpha\beta$ T cell transcripts are the same, but both populations express genes that are potentially unique to that cell type. The relatedness of the gene expression profiles of the two cell types was determined using the Pearson correlation including only tags with an adjusted frequency of 10 or greater between all 12 libraries.

Table 2.10 Blood $\gamma\delta$ and $\alpha\beta$ T cell-specific transcripts

Tag	Blood $\gamma\delta$ T cells				Blood $\alpha\beta$ T cells				Annotation
	Resting	ConA/ IL2	PMA/ iono	Total	Resting	ConA/ IL2	PMA/ iono	Total	
CATGAGACAAGAGT	2 ^a	0	35	37	0	0	0	0	No matches
CATGTTCTCACGA	25	4	0	29	0	0	0	0	No matches
CATGTTTGACAGAA	5	2	18	25	0	0	0	0	NAD(P)-dependent steroid dehydrogenase
CATGAGGTTTTCTA	2	6	14	22	0	0	0	0	Embryonic ectoderm development protein
CATGAATTACTGAG	18	4	0	22	0	0	0	0	Isocitrate dehydrogenase [NAD] subunit alpha
CATGCCACCGTCTC	18	4	0	22	0	0	0	0	No matches
CATGACACGGGCTT	14	4	4	22	0	0	0	0	No matches
CATGTTGAGTTGGT	2	4	14	20	0	0	0	0	Mak3p homolog
CATGGATAGATCTA	0	2	18	20	0	0	0	0	No annotation
CATGTAGTAGTTTG	0	2	18	20	0	0	0	0	Protein-tyrosine phosphatase 1B
CATGATCAGAAAAG	0	2	18	20	0	0	0	0	Multiple matches
CATGGATATCCGCA	0	18	0	18	0	0	0	0	No matches
CATGAATCCTGTGA	4	0	14	18	0	0	0	0	Dehydrogenase/reductase SDR family member on chromosome X
CATGTTTGAAGAAT	4	0	14	18	0	0	0	0	No annotation
CATGCTCTAATTTT	11	6	0	17	0	0	0	0	No matches
CATGTGTGGTTCTG	0	2	14	16	0	0	0	0	Immunoglobulin heavy chain V region
CATGTAATTCAAAA	5	10	0	15	0	0	0	0	No annotation
CATGATTGGCTTAA	0	8	7	15	0	0	0	0	Prohibitin
CATGCTTGCTGGTT	0	0	0	0	53	22	7	82	T-cell receptor alpha chain
CATGAATGTTTGGG	0	0	0	0	13	42	0	55	Protease inhibitor 15 preproprotein
CATGTGTGTCAGTT	0	0	0	0	30	12	0	42	No annotation
CATGTGTGTCAGTT	0	0	0	0	30	12	0	42	No annotation

(Table 2.10 Continued)

CATGGGGTCGCAGA	0	0	0	0	6	12	19	37	Multiple matches
CATGGCTTGCTTGT	0	0	0	0	23	10	0	33	No annotation
CATGCTCTGTGTGC	0	0	0	0	6	2	21	29	No annotation
CATGTGGGGAAAAA	0	0	0	0	17	7	0	25	T-cell receptor alpha chain
CATGAGAGATGAGA	0	0	0	0	16	2	5	23	Immune associated nucleotide protein
CATGGAGCAAGGGC	0	0	0	0	1	0	21	23	Low affinity immunoglobulin gamma Fc region receptor III
CATGTCGTCTTCTT	0	0	0	0	0	5	16	21	No matches
CATGAAAATGGGCC	0	0	0	0	9	5	7	21	Signal recognition particle 54 kDa protein
CATGGCTGTGGGGA	0	0	0	0	6	10	5	20	Lymphotoxin-beta (Tumor necrosis factor C)
CATGTCAGATGTCA	0	0	0	0	20	0	0	20	T-cell surface antigen CD2
CATGGGAAAAAAAT	0	0	0	0	7	12	0	19	Neurofilament L subunit
CATGCAACATCAGC	0	0	0	0	1	10	7	18	B52-prov protein
CATGAGCGAGTTCC	0	0	0	0	16	0	2	18	No matches
CATGAGCCCATTAC	0	0	0	0	0	15	2	17	No matches
CATGTGCAGACTGC	0	0	0	0	6	5	5	15	LOC161577 protein

^aAdjusted tag frequencies

Discussion

To gain a better understanding of unique $\gamma\delta$ T cell responses to mitogenic stimuli in different microenvironments and in comparison to $\alpha\beta$ T cells, and to provide new genome resources to those who study these cells, 12 new SAGE libraries were constructed. These libraries complement two previously published $\gamma\delta$ T cell SAGE libraries from our laboratory (128) and other global gene expression studies of $\gamma\delta$ T cells (55,179,79,154,108,81). An initial experiment was performed with MACS-sorted T cells to compare $\gamma\delta$ T cells from blood and spleen in both resting and ConA/IL-2 activation states and to a spleen $\alpha\beta$ T cell-enriched population. To test the conclusions drawn from the analysis of the MACS separated PBL and spleen $\gamma\delta$ and $\alpha\beta$ T cells and the impact of activation, another series of SAGE experiments were done using FACS to isolate more pure preparations of $\gamma\delta$ and $\alpha\beta$ T cells (>95%) from 3 animals. These 12 libraries will continue to expand as we sequence additional tags and the bovine genome sequence and annotation becomes more complete.

To facilitate the use of the available SAGE libraries in the bovine SAGE database, we present the results as uncorrected sequencing data and adjusted data in a web-based interface. The user can search for a specific gene of interest, identify highest abundance tags, identify uniquely expressed tags, and identify tags based on fold-expression differences (Table 2.11). Importantly, although the presented data were obtained from

only bovine cells, they are comparable across species because the data sets were annotated against human and mouse gene resources.

Occasionally, a SAGE tag with an interesting expression pattern cannot be annotated as a single gene. To help overcome this obstacle, a reference long SAGE library was constructed to provide additional sequence information for tags that could not be uniquely identified or for tags that did not match any known bovine sequence. In many cases, the additional seven base pairs of sequence obtained with a long SAGE tag can uniquely identify which gene a short tag represents (Table 2.12). In the case of short SAGE tags that do not match any known sequence, long SAGE can provide enough sequence information to perform reverse SAGE (158) or 3'-RACE to gain additional sequence for gene identification. This reference long SAGE library is only beginning to be sequenced, but is made available as a WEB resource as well.

$\gamma\delta$ T cells exhibit diverse responses to different infections, including immunoregulatory functions, direct anti-microbial responses or they can be seemingly irrelevant to infection clearance (76). To begin examining responses of $\gamma\delta$ T cells to mitogenic stimuli, we compared global gene expression profiles of MACS-sorted $\gamma\delta$ T cells in resting and ConA/IL-2 activation states, which revealed dramatic differences. These findings were confirmed by the SAGE libraries built from very pure FACS-separated blood $\gamma\delta$ T cells (in the absence of crosslinking by the anti-TCR mAb, as occurs in the MACS bead protocol) isolated from 3 animals. Additionally, as expected, these libraries demonstrated unique responses of $\gamma\delta$ T cells to ConA/IL-2 and PMA/ionomycin.

Table 2.11 Tools available on the bovine SAGE database web resource.*

Website Analysis Tools	Input	Output
Search for tags by gene name	Gene name	List of corresponding tags, frequencies and annotations
Find annotation and frequencies of specific tags	Tag Sequence	Tag frequencies and annotations
Find expressed tags in specific sequences	Gene sequence of interest	Tag sequences, locations and frequencies
Find most abundant tags between all libraries or in a specific library	Library of choice	List of tags, frequencies and annotations
Find tags with specific fold differences between two libraries	Choice of libraries and desired fold difference	List of tags, frequencies and annotations
Find tags unique to specific libraries	Choice of library	List of tags, frequencies and annotations
Find matching long SAGE tags for additional annotation confidence	Short SAGE tag	Long SAGE tags and annotations
Analyze sequencing plates for anomalies	Choice of library	Tag frequencies for each sequencing plate
Obtain text files of any/all libraries to use for comparison to any other SAGE library	Pick desired library	Export text file

*<http://vmbmod10.msu.montana.edu/vmb/jutila-lab/sagebov.htm>

Table 2.12 Long SAGE tags help to uniquely annotate short SAGE tags

Short SAGE Tag	Short SAGE Annotation	Additional bp from Long SAGE	Long SAGE Annotation
CATGAAGAAAAGAG	Multiple EST Hits	ACCCGCC	T200 leukocyte common antigen precursor
CATGAAGAGAACCT	ribosomal protein L37, L36 or L35	GAAGAC	Ribosomal protein L35
CATGACTGAGCGAC	Multiple EST Hits	TGAACT	Renal high affinity glutamate transporter BEAAC1
CATGAGAGCCCTGG	Multiple EST Hits	GATTTA	Cox17p
CATGCCCTTCTCCA	Multiple EST Hits	GGGGAT	Translation initiation factor IF-2
CATGCGGGAGCTGG	Multiple EST Hits	ACGACA	Non-muscle myosin heavy chain
CATGGACGGAGGAG	Multiple EST Hits	CCTGGTG	TIGR EST
CATGGAGTCAGGAT	Multiple EST Hits	GGTAGC	Coatomer; CGI-120 protein
CATGGATTAAACCA	Multiple EST Hits	GACTGA	Chemokine-like factor super family 3
CATGGGACCACTTA	ribosomal protein L3, L4, L5, or L6	AGAAGGA	Ribosomal protein L3
CATGGGGCTGGACG	Multiple EST Hits	GCTGCGC	Tumor necrosis factor receptor superfamily member 12
CATGGGGTCGCAAA	Multiple EST Hits	GAGTCG	Mitochondrial brown fat uncoupling protein 1 (Thermogenin)
CATGTACATTTCTA	Multiple EST Hits	GAATGA	T200 leukocyte common antigen precursor
CATGTCTGTGAAAA	Multiple EST Hits	GAGTATG	Stathmin
CATGTTGGTGAAGG	Multiple EST Hits	AGGAAGC	Thymosin beta-4
CATGTTTCTCTTTT	Multiple EST Hits	ATAATA	Chaperonin 10

Though both mitogens are powerful stimulants, they act on the cells through different mechanisms. ConA stimulates T cells by cross-linking a variety of cell surface proteins displaying α -D-mannosyl and α -D-glucosyl residues, including CD3, which signals through the TCR and induces changes similar to those seen when lymphocytes contact specific antigens (164). PMA/ionomycin enter the cell and directly induce calcium-dependent and protein kinase C pathways, thus activating multiple signal-transduction mechanisms (129). As expected, $\gamma\delta$ T cells responded with different gene expression profiles to the two stimuli, with the response to PMA/ionomycin being greater. However, the extent of the differences was astounding. This finding emphasizes the need to study $\gamma\delta$ T cells in response to various stimuli, both in vitro and in vivo.

Unique subsets of $\gamma\delta$ T cells localize to specific tissues (76). To begin the investigation of how the microenvironment to which a $\gamma\delta$ T cell localizes affects gene expression profiles, we compared $\gamma\delta$ T cells from blood and spleen in both resting and ConA/IL-2 activated states. We chose to examine spleen $\gamma\delta$ T cells because our previous work demonstrated that these cells were different than circulating $\gamma\delta$ T cells (210,212). Specifically, bovine CD8⁺ $\gamma\delta$ T cells accumulate in large numbers in the spleen, unlike in the blood where $\gamma\delta$ T cells are predominately CD8⁻. Also, the CD8⁺ subset remains in the red pulp, whereas CD8⁻ $\gamma\delta$ T cells appear to quickly re-enter circulation (210). Upon comparing the blood and spleen $\gamma\delta$ T cells, it became immediately obvious that the spleen cells were in a more transcriptionally active state. Following ConA/IL-2 stimulation, blood $\gamma\delta$ T cell gene expression was more similar to that of spleen cells than to that of

resting blood cells. This observation is consistent with the fact that immune cells function within organs and tissues and use the blood simply to gain access to these sites. Once within the spleen, $\gamma\delta$ T cells are exposed to an array of environmental factors, including potential antigens, which drive significant changes in gene expression. Upon leaving the spleen, significant transcriptional repression likely takes place, leading to the profile seen in the resting blood $\gamma\delta$ T cells. Since many of the changes in gene expression are likely tissue-specific, it will be important to extend these types of analyses to cells harvested from other tissues in order to gain an even more comprehensive picture of the $\gamma\delta$ T cell transcriptome. However, these initial libraries provide a resource for identification of potential spleen- and blood-specific transcripts.

In support of the finding that gene transcription is suppressed in resting blood $\gamma\delta$ T cells, we have identified message for a transcriptional repressor, B lymphocyte-induced maturation protein-1 (Blimp-1; 197), which is expressed in resting blood $\gamma\delta$ T cells and is down-regulated upon activation (data not shown). Blimp-1 was identified for the first time in $\gamma\delta$ T cells by SAGE analysis (128). Further functional studies are needed to determine if Blimp-1 is a major transcriptional repressor in $\gamma\delta$ T cells, as it is in B cells, and if it contributes to the differences seen in the SAGE libraries. SAGE tags representing CD37 (involved in repression of T cell proliferation; ref. 198) were also identified in resting blood $\gamma\delta$ T cells, but not in stimulated blood $\gamma\delta$ T cells or either population of spleen $\gamma\delta$ T cells (data not shown). Therefore, Blimp-1 and CD37 gene

expression profiles correlate and their down-regulation is consistent with the transcriptionally activated profiles of spleen $\gamma\delta$ T cells.

Additional real time RT-PCR follow-up experiments have been performed on cells isolated from five additional calf spleens and indicate that expression profiles of specific genes vary between spleen preparations, unlike blood cell preparations, which are more consistent (data not shown). We have not determined whether the variation seen is due to calf-variability, the extensive cell-isolation procedure needed to generate the spleen cell preparations, or the variability in the purities of the cell preparations. Although the regulation of specific genes seen in these SAGE libraries may not hold true for all calf spleens, we believe that the global gene expression patterns are representative of lymphocytes in the spleen microenvironment.

Even though $\gamma\delta$ and $\alpha\beta$ T cells express distinct TCRs, localize in tissues differently, and likely perform distinct, yet complementary functions, the limited gene expression analyses done to date suggest that, overall, they express mostly the same genes. SAGE was previously used to compare $\gamma\delta$ and $\alpha\beta$ IELs, and very few differences between the two cell types and few transcripts representing cytokines were identified (179). An extensive extraction protocol was used prior to RNA isolation from the IELs for the SAGE library construction. We have previously found that lymphocytes dramatically down-regulate many genes, including those for cytokines, during even less extensive sorting protocols (128), which may be one reason few differences between cell types were identified. To overcome this inherent obstacle, we found that culturing sorted cells

overnight prior to stimulation or RNA extraction, allowed the lymphocytes to regain expected gene expression profiles (128). Here, we expand on these studies of IELs and provide a direct comparison of $\gamma\delta$ and $\alpha\beta$ T cells isolated from the same tissue (spleen) in both resting and activated states. We chose to examine spleen T cells because there are known differences between the two cell types within the spleen. For example, $\gamma\delta$ T cells localize to sites of T cell traffic within the spleen (marginal zone, red pulp and the marginal sinus), but are rarely found in the conventional T cell regions of the white pulp (82).

Due to the lack of quality reagents for isolating bovine $\alpha\beta$ T cells, the spleen $\alpha\beta$ T cell populations in this study were merely enriched for $\alpha\beta$ T cells (80% $\alpha\beta$ T cells; GD3.8 negative population) and contained B cells, residual $\gamma\delta$ T cells, and likely monocytes and/or NK cells. These contaminating cells confounded the interpretation of genes unique to $\alpha\beta$ T cells due to their inherent transcript repertoire. However, we were more interested in identifying expressed genes unique to $\gamma\delta$ T cells; therefore, comparing them to a less-pure population increases our confidence that the unique genes identified are truly unique to $\gamma\delta$ T cells. As expected, we found that the large majority of expressed genes were shared between spleen $\gamma\delta$ and $\alpha\beta$ T cells. In fact, the resting spleen populations were nearly identical, with only 1% of the transcripts being unique to $\gamma\delta$ T cells. Interestingly, total spleen $\gamma\delta$ and $\alpha\beta$ T cells became less similar when stimulated with ConA/IL-2 than they were in the resting state. The majority of the differences arose due to a selective 5-fold increase in $\gamma\delta$ T cell-specific transcripts. Though it might have

been predicted that stimulation via the TCR would lead to $\gamma\delta$ and $\alpha\beta$ T cell-specific gene expression profiles, the magnitude of the effect was striking.

To test the findings of the spleen T cell comparison, an additional experiment comparing SAGE libraries built from very pure FACS-separated blood $\gamma\delta$ and $\alpha\beta$ T cells was performed. This experiment confirmed the findings from the spleen libraries that $\gamma\delta$ and $\alpha\beta$ T cells share the majority of their expressed genes in the resting state and that the two populations become more dissimilar upon stimulation. Interestingly, the two T cell types did not respond by regulating the same sets of genes. Crosslinking of the TCRs by ConA likely induces lineage-specific signaling, thus resulting in the different transcriptional responses seen in this study. These SAGE data suggest that though PMA/ionomycin likely activates the same signal transduction pathways in both $\gamma\delta$ and $\alpha\beta$ T cells, lineage-specific responses occur. This could be due to differences in the genes initially expressed in each cell type or that the chromatin packaging of specific target genes vary between the cell types, thus preventing specific transcription of some genes.

Not only were different gene expression patterns identified between the two types of T cells, but several genes, aside from TCR genes, that are potentially specific to each population were identified in the very pure populations. These findings emphasize the need to compare $\gamma\delta$ and $\alpha\beta$ T cells in their resting states, and more importantly, following in vivo and in vitro activation. A more thorough analysis of various T cell agonists will likely reveal more examples of lineage specific gene expression patterns. Importantly, our current studies confirmed that even though most expressed genes are the same in the

two T cell populations, $\gamma\delta$ T cells have unique gene expression profiles compared to $\alpha\beta$ T cells, thus emphasizing unique roles for each cell type.

An important consideration in any gene expression analysis of isolated cell populations is the impact of the isolation procedure on subset-specific gene expression. In comparing $\alpha\beta$ to $\gamma\delta$ T cells, differences could be due, in part, simply to the different approaches used to isolate the cells (for example, negative versus positive sorts, impact of different antibodies used in positive sorts, etc). Our initial experiment used cells sorted using the magnetic bead isolation procedure, which others have previously used in the analysis of bovine $\gamma\delta$ T cell gene expression (46), and we have found it does not drive a positive signal in the cell as measured by proliferation or IL-2 receptor up-regulation (data not shown). However, the potential cross-linking event caused by the magnetic bead could still be expected to alter basal level gene expression, generating artificial $\gamma\delta$ T cell-specific transcripts. Since this would only occur on $\gamma\delta$ T cells, conclusions from these experiments would be suspect. In testing conclusions from our magnetic bead sorts, we performed a second experiment using very pure flow cytometry-sorted cell populations in which the anti-TCR mAb was directly conjugated to FITC to minimize crosslinking. Interestingly, the trends and overall conclusions were similar regardless of what separation technique was used; however, there were differences in specific gene profiles associated with each technique (Fig. 2.12 and Table 2.13). Thus, in comparing gene expression data from different cell populations it is important that procedures used to isolate the cells are the same and, perhaps, multiple methods should be compared.

Because the two T cell populations are treated in a more similar fashion in the FACS versus the MACS bead protocol, we predict that subset-specific genes identified in these studies would be more reliable and predictive.

$\gamma\delta$ T cells clearly represent a diverse population of lymphocytes based on their tissue localization and activation states. As predicted, the analysis of these SAGE libraries has identified unique gene expression patterns of $\gamma\delta$ T cells in the various conditions studied, which has resulted in insights into these cells and has provided a foundation for more comprehensive studies of $\gamma\delta$ T cell gene expression. As additional gene expression studies are done, greater insight into $\gamma\delta$ T cells will be gained, allowing for a better understanding of the role of this enigmatic lymphoid cell.

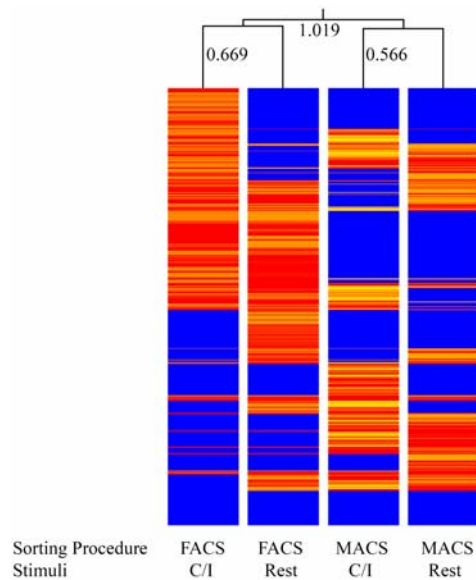


Fig. 2.12 The sorting procedure used to purify lymphocytes dramatically effects gene expression profiles. Condition tree demonstrating the relatedness of 4 comparable peripheral blood $\gamma\delta$ T cell populations (FACS-sorted or MACS-sorted resting and ConA/IL-2 stimulated) based on the Pearson correlation of the gene expression profiles including only tags with an adjusted frequency of 10 or greater between all 12 libraries.

Table 2.13 Effect of sorting procedures on gene expression profiles

Tag	MACS-sorted $\gamma\delta$ T cells			FACS-sorted $\gamma\delta$ T cells			Fold difference	Annotation
	Resting	ConA/ IL2	Total	Resting	ConA/ IL2	Total		
CATGAGTGCAGACT	2290	782	3072	8	2	10	306.0	IgM heavy chain C region
CATGTGGGGCAGGG	197	79	276	0	0	0	275.9	Multiple matches
CATGCAAAGGACAA	6	203	209	0	0	0	208.9	No annotation
CATGCAGGAGACGA	11	181	192	0	0	0	191.5	No annotation
CATGATGTAGTAGT	67	101	169	0	0	0	168.5	Multiple matches
CATGCACACACAGC	83	63	146	0	0	0	146.1	No annotation
CATGATTAAAGTAA	98	41	140	0	0	0	139.5	No annotation
CATGCAGAAGTCCA	94	42	136	0	0	0	135.9	IgG2a heavy chain C region
CATGTCAGAGGTGG	119	16	135	0	0	0	134.5	Multiple matches
CATGGAActCCGCC	3	118	121	0	0	0	121.4	No annotation
CATGGTCACCGAGC	72	35	107	0	0	0	106.7	IgM heavy chain C region
CATGGTCACCAGCT	73	23	97	0	0	0	96.6	Multiple matches
CATGATTCTTTGGT	315	332	647	3	4	7	92.9	60S ribosomal protein L23
CATGCTGAACATCT	123	41	165	0	2	2	89.9	Acidic ribosomal phosphoprotein P0
CATGGAAAAACATT	41	48	88	0	0	0	88.1	Heat shock cognate 71 kDa protein
CATGGTAGGGGAAT	16	127	142	2	0	2	86.8	No annotation
CATGAGAGATGAGA	75	6	81	0	0	0	81.3	Immune associated nucleotide protein
CATGCGAGTGGCGA	17	114	131	2	0	2	80.0	No annotation
CATGGCCTAGAGAC	5	126	130	0	2	2	71.2	No annotation
CATGCTGGGAAATT	429	183	612	0	9	9	66.7	Multiple matches
CATGGAGTGAAAAA	89	17	106	2	0	2	64.6	50S ribosomal protein L25
CATGGAAGATGCTA	47	17	64	0	0	0	63.7	Ribonucleoprotein
CATGGAGACCCGAC	2	102	104	2	0	2	63.4	No matches

(Table 2.13 Continued)

CATGGCCTAGAGGC	2	54	55	0	0	0	55.4	No matches
CATGAACAGGTCCC	973	752	1724	12	20	32	54.4	Ribosomal protein S25
CATGTGAACGATAC	33	21	54	0	0	0.0	53.9	Ornithine decarboxylase antizyme inhibitor
CATGAGAAGCCCCC	2	92	93	0	2	2	51.0	No annotation
CATGTGAAAAGAA	0	0	0	20	31	51	-50.9	Multiple matches
CATGATAACTCCAT	0	0	0	44	7	52	-51.7	No matches
CATGCCGTGTAAAA	0	0	0	28	24	52	-51.8	No matches
CATGCATTTTCCGT	0	0	0	31	22	53	-53.2	No matches
CATGTCGATGTTTG	0	0	0	10	46	56	-55.7	No matches
CATGATTCAGGCCC	0	0	0	25	33	58	-57.7	No matches
CATGCTTCATTCC	0	0	0	54	4	58	-57.9	No matches
CATGCAGTTCAATA	3	0	3	138	70	208	-66.6	No annotation
CATGTGCAAATAGT	2	0	2	95	11	106	-68.1	No matches
CATGAGAGTTCTTT	0	1	1	46	29	75	-71.1	No annotation
CATGTATTACCTTT	0	0	0	30	44	74	-73.6	No matches
CATGTCTGGTCGTG	0	0	0	38	37	74.5	-74.5	Histone H2a.2
CATGCTTTCCATTT	0	1	1	3	90	93	-87.9	No annotation
CATGTTTATAGATTTT	0	0	0	44	79	123	-123.2	No matches
CATGAAGAGCTGGG	0	0	0	163	86	249	-248.8	MHC class I antigen precursor

^aAdjusted tag frequencies

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