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Mucosal lymphatic-derived $\gamma\delta$ T cells respond early to experimental *Salmonella* enterocolitis by increasing expression of IL-2R α

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Abstract

To better understand the roles of $\gamma\delta$ T cells in mucosal infection, we utilized *Salmonella enterica* serovar Typhimurium (*Salmonella* serovar Typhimurium) infection in cattle as it closely approximates *Salmonella* serovar Typhimurium-induced enterocolitis in humans. Protein and gene expression in $\alpha\beta$ and $\gamma\delta$ T cells derived from lymphatic ducts draining the gut mucosa in *Salmonella* serovar Typhimurium infected calves were analyzed. In calves with enterocolitis, general gene expression trends in $\gamma\delta$ T cells suggested subtle activation and innate response, whereas $\alpha\beta$ T cells were relatively quiescent following *Salmonella* serovar Typhimurium infection. An increase in IL-2R α expression on $\gamma\delta$ T cells from infected calves and results from *in vitro* assays suggested that $\gamma\delta$ T cells were primed by *Salmonella* serovar Typhimurium LPS to better respond to IL-2 and IL-15. Together with gene expression trends *in vivo*, these data support early priming activation of target tissue $\gamma\delta$ T cells during *Salmonella* serovar Typhimurium infection.

Keywords

$\gamma\delta$ T cells; mucosal; IL-2R α ; cell proliferation; *Salmonella* serovar Typhimurium; gene expression; lymphatic; priming

Introduction

Depending on the serotype and host, *Salmonella* can cause bacteremia, typhoid fever or enterocolitis. Bacteremia induced by infection with *Salmonella enterica* serovars Choleraesuis (swine adapted) and Dublin (bovine adapted) is relatively rare in humans [1]. Typhoid fever caused by *Salmonella enterica* serovar Typhi in humans is virtually eradicated in the United States, but remains a problem in regions of the developing world with poor sanitation practices [1]. In contrast, *Salmonella*-induced enterocolitis in humans, primarily caused by *Salmonella enterica* serovar Typhimurium (*Salmonella* serovar Typhimurium), is extremely common and is the most common cause of death by food-borne illness in the U.S. [1,2]. The role of T cells in infection with *Salmonella* species has largely been investigated in mice [3]. However, while infection of mice with *Salmonella* serovar

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Typhimurium is an excellent model for human typhoid fever, it does not induce enterocolitis [1,3]. In contrast, *Salmonella* serovar Typhimurium infection in calves induces enterocolitis that closely mirrors disease in humans and is also the most common *Salmonella* strain associated with ill cattle in the United States [1].

$\gamma\delta$ T cells localize to the gut mucosa in all animals, including humans, express the $\gamma\delta$ TCR of limited diversity, recognize unprocessed antigen, and respond rapidly to infection, in part by rapid recruitment to infected sites [4,5]. Whereas $\alpha\beta$ T cells appear to be more important than $\gamma\delta$ T cells in the clearance of *Salmonella* serovar Typhimurium in the typhoid fever model in mice [1,3], the roles of $\gamma\delta$ T cells in the gut mucosa of a highly relevant model of enterocolitis have not been investigated. Since $\gamma\delta$ T cells localize to the gut mucosa and are thought to participate in innate immune responses, they are likely involved in early protective responses to *Salmonella* serovar Typhimurium infection [6]. The phenotype of T cell populations in blood, peritoneal fluid, or lymphoid tissues, (the sources of lymphocytes in several murine and human investigations [3,7,8]), does not necessarily reflect that of those responding to initial infection in the target tissue. We hypothesized that $\gamma\delta$ T cells derived from the gut mucosal lymphatic ducts provide an early innate response during *Salmonella* serovar Typhimurium enterocolitis. To address this hypothesis, cells in lymphatic fluid draining from the intestine were collected at early intervals during *Salmonella* serovar Typhimurium-induced enterocolitis in naïve bovine calves. T cells were analyzed by FACS and microarray to determine their protein and gene expression phenotypes. Among other indicators of activation, IL-2 receptor α (IL-2R α) transcripts and protein increased in expression on $\gamma\delta$ T cells during *Salmonella* serovar Typhimurium infection *in vivo* whereas $\alpha\beta$ T cells appeared minimally stimulated. In an *in vitro* functional assay, highly purified $\gamma\delta$ T cells stimulated with *Salmonella* serovar Typhimurium LPS had enhanced proliferation in response to IL-2 and IL-15. Our results indicate an early priming activation of $\gamma\delta$ T cells, relative to a less dramatic early role of $\alpha\beta$ T cells in an infrequently studied but highly relevant model of *Salmonella* serovar Typhimurium induced enterocolitis.

Materials and Methods

Surgery and experimental infection

Four to six week old calves with no evidence of prior *Salmonella* infection were used for the surgery and infection. Catheters were inserted into the mesenteric efferent lymphatic vessel following standard surgical procedures. Calves recovered from surgery for approximately 20 hours, then the time 0 lymphatic fluid and blood were collected just prior to infection with 4.7×10^7 to 4×10^8 CFUs of a natural calf isolate of *Salmonella* serovar Typhimurium (Type O, Group B) or mock infection with an equivalent volume of sterile Luria broth (LB) media. Prior to infection, 100 μ l of *Salmonella* serovar Typhimurium stock was added to 5ml of LB media and shaken for 6 hours at 37°. Bacterial cell growth was determined by absorbance reading and comparison to a standard growth curve for the same strain. CFU counts were verified by plating serial dilutions of bacterial suspensions on LB agar plates. Blood and lymphatic fluid were collected at intervals post infection during which the calves were closely monitored. Enterocolitis was defined as fever ($>105^\circ$ F) and diarrhea between 24 and 48 hours post infection. At this time fecal samples were submitted to the Montana State Veterinary Diagnostic Service and those from experimentally infected calves tested positive for the input strain of *Salmonella* serovar Typhimurium and negative for other infectious agents. All animal protocols were reviewed and approved by the MSU Institutional Animal Care and Use Committee.

Flow Cytometry and FACS

RBCs in whole blood were lysed in ACK (0.15M ammonium chloride, 1mM potassium carbonate, 0.1mM EDTA disodium salt) buffer and lymphatic fluid cells were washed twice with PBS with 2% horse serum. A small volume of cells from blood and lymphatic fluid was stained with the following mouse anti-bovine antibodies (specificity): GD3.8 (pan $\gamma\delta$ T cell), ILA29 (WC1, $\gamma\delta$ T cell subset), CC21 (B cells), CC42 (CD2, $\alpha\beta$ T cells and $\gamma\delta$ T cell subset), BN180 (monocytes), BN115 (neutrophils), CACT116A (IL-2R α ; VMRD Inc., Pullman, WA). Flow cytometry was performed following standard protocols as previously described [9]. Because the changes in cell percentages between 0 and 6 hours post infection were highly variable, and were potentially residual fluctuations that occurred post surgery, cell percentages at these two time points were averaged to arrive at a new time 0 post infection value represented on the graphs.

For one mock infection (calf 156) and two experimental *Salmonella* serovar Typhimurium infections (calves 112 and 162), the lymphatic cells were stained with GD3.8 directly conjugated to FITC, washed, and sorted on a Vantage SE cell sorter (BD Immunocytometry Systems) as previously described [9]. Percent purities of the sorted cells were as follows: calf 112; 0 hour $\alpha\beta$ T cell 81%, 0 hour $\gamma\delta$ T cell 97%, 6 hour $\alpha\beta$ T cell 85%, 6 hour $\gamma\delta$ T cell 93%, 24 hour $\alpha\beta$ T cell 78%, 24 hour $\gamma\delta$ T cell 96%, 48 hour $\alpha\beta$ T cell 89%, 48 hour $\gamma\delta$ T cell 97%, 72 hour $\alpha\beta$ T cell 95%, 72 hour $\gamma\delta$ T cell 91%, calf 156; 0 hour $\alpha\beta$ T cell 98%, 0 hour $\gamma\delta$ T cell 89%, 48 hour $\alpha\beta$ T cell 82%, 48 hour $\gamma\delta$ T cell 89%, calf 162; 0 hour $\alpha\beta$ T cell 87%, 0 hour $\gamma\delta$ T cell 88%, 48 hour $\alpha\beta$ T cell 81%, 48 hour $\gamma\delta$ T cell 86%. The $\alpha\beta$ T cell populations were contaminated by a mixture of cells, mainly B cells and a few $\gamma\delta$ T cells, whereas $\gamma\delta$ T cells were mainly contaminated with $\alpha\beta$ T cells. Sorted $\gamma\delta$ and $\alpha\beta$ T cells were directly lysed in TRIzol reagent (Invitrogen; calf 112) or in Buffer RLT (Qiagen; calves 156 and 162) and genomic DNA sheared using Qiashredder columns, then frozen at -80°C .

RNA extraction, amplification, and microarrays

RNA was extracted following the manufacturer's protocol for TRIzol (Invitrogen) extraction, or RNeasy (Qiagen) column purification, assessed on a Bioanalyzer 2100 (Agilent Technologies), and amplified either using Affymetrix Two-cycle (calf 112) target labeling protocol with 100 ng total RNA or the One-cycle protocol (calves 156 and 162) with approximately 1.6 μg of total RNA as described in the GeneChip[®] Expression Analysis Technical Manual (June 2004). Hybridizations to Genechip[®] Bovine Genome Arrays (Affymetrix) were performed with 15 μg biotin labeled cRNA. Washing and staining was performed in the GeneChip[®] Fluidics Station 450 using the Midi_euk2v3 protocol. Chip scans were performed on the Affymetrix GeneChip[®] Scanner 3000. GeneChip[®] Operating Software (GCOS v.1.1, Affymetrix) [10,11] was used for data collection and analysis of gene lists using GCOS assigned detection calls and significance values of signal log ratios of >1 or <-1 (2 fold changes). Annotation of these lists was improved by approximately 50% by blasting the bovine genome dataset against the mouse and human datasets. Other analyses were done with Genespring (Agilent Technologies) software, applying RMA preprocessing to the Affymetrix CEL files. All data was normalized to time 0 post infection values and only genes with a raw value of greater than 100 expression level and that changed by at least 2 or 2.5 fold during the time course were considered significant.

Priming Assay

Vantage sorted bovine peripheral blood $\gamma\delta$ T cells ($>97\%$ purity) were washed with HANKS buffer and loaded with 0.25 μM carboxyfluorescein diacetate succinimidyl ester (CFSE) in the dark for 5 minutes. Cells were assayed in duplicate or triplicate wells, depending on the sort yield and primed with PBS, or phenol-extracted LPS from *E. coli*

O111:B4 (Sigma L2630) or *Salmonella* serovar Typhimurium (Sigma, L6511, 10 μ g/ml) in XVIVO (Cambrex, Walkersville, MD) media for 48 hours at 37°C. Priming medium was removed and replaced with medium containing IL-2 (1 ng/ml) or IL-15 (10 ng/ml) then cultured another 72 hours at 37°C. Cells were then read on a FACS Calibur HTS using high-throughput settings. Gates were placed on lymphocyte populations and GD3.8 positive cells, for analysis of CFSE staining.

Statistical analyses

Anova (Two factor with replication) analysis was used to calculate the significance of the increase in IL-2R α expression in $\gamma\delta$ T cells as compared to non- $\gamma\delta$ T cells. Statistical analyses of priming assay data were performed with a two-tailed paired *t* test. Mean values of percent of gated cells were calculated with 1 to 3 experimental replicates (sample wells) from each of at least 3 individual calves (biological replicates). Extensive experience with this assay in a high throughput screen setting indicates that the percent of gated cells follows an approximately normal distribution. Mean values of purified $\gamma\delta$ T cells treated with PBS were compared to those treated with *E. coli* LPS or *Salmonella* serovar Typhimurium LPS. The *n* values are as follows: for the set that proliferated in IL-2; PBS, 8; *E. coli* LPS, 4 and *Salmonella* serovar Typhimurium LPS, 9 for set that proliferated in IL-15; PBS, 8; *E. coli* LPS, 6 and *Salmonella* serovar Typhimurium LPS, 8.

Microarray Data

The microarray data and the supplemental Table 1 discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (GEO, <http://www.ncbi.nlm.nih.gov/geo/>) and are accessible through GEO Series accession number GSE3439.

Results

Blood and lymphatic fluid cell changes during *Salmonella* infection

The mesenteric lymphatic ducts of four different calves (35, 112, 149, and 162) were cannulated. The calves were then orally infected with *Salmonella* serovar Typhimurium and monitored for 72 hours. Four additional calves (86, 156, 200, and 217) were treated identically, mock infected and monitored for 48 hours. Calves 156, 200 and 217 were cannulated and monitored for a 48 hour mock infection, and then infected with *Salmonella*, providing both a mock and experimental infection in the same animal. All infected calves developed enterocolitis by 24 to 48 hours post infection whereas mock infected calves had no signs of infection.

Lymphocyte populations were monitored in the blood and lymphatic fluids of *Salmonella*- and mock- infected calves. Whereas there were no major changes in lymphocyte populations in the blood, changes in frequency of monocytes and neutrophils were detected, but followed no clear patterns (data not shown). In the lymphatic fluid, $\gamma\delta$ T cell frequency seemed to increase compared to $\alpha\beta$ T cells (Figure 1) but results were variable and not statistically significant. Percentages of the inflammatory WC1⁺, CD8⁻ $\gamma\delta$ T cell subset normally found in circulation [4,5] were also measured in the lymphatic fluid of *Salmonella* serovar Typhimurium- and mock-infected calves to determine the extent of recruitment during infection (data not shown). The increases in this subset followed similar trends as the total $\gamma\delta$ T cell population, and suggested some recruitment or induction of the WC1 antigen along with representation of the tissue resident subset in lymphatic fluid. Others have similarly observed an increase in gut mucosal resident CD8⁺ $\gamma\delta$ T cells (equivalent of many WC1⁻ $\gamma\delta$ T cells in cattle) in the intraepithelial lymphocytes at 3 to 10 days post infection in the murine typhoid fever model [12]. Lacking a definitive increase in $\gamma\delta$ T cells in the

lymphatic fluid as an indication of their early response, we sought to better characterize their response early in *Salmonella* serovar Typhimurium infection.

Microarray analysis of $\gamma\delta$ and $\alpha\beta$ T cells in lymphatic fluid

$\gamma\delta$ and $\alpha\beta$ T cells were sorted from the mucosal lymphatic fluid at 0, 6, 24, 48 and 72 hours post infection from calf 112. The cells were sorted and relative mRNA expression was analyzed by microarray. Figure 2 illustrates the genes that changed in expression level by at least 2.5 fold at one or more time points compared to expression level at time 0. Both T cell subsets had an initial response by 6 hours post infection that may reflect an ongoing response to surgery. At 48 hours after infection, after the animal responded clinically, the majority of gene changes occurred and differences in gene expression between the subsets emerged. Specifically, in $\gamma\delta$ T cells similar numbers of genes increased as decreased in expression, whereas in $\alpha\beta$ T cells genes more than twice as many genes decreased in expression than increased.

Given the apparent significance of 48 hours post *Salmonella* serovar Typhimurium infection, efforts were focused on the 0 and 48 hour post infection times in another infected calf (calf 162) and a mock infection (calf 156). The samples representing $\alpha\beta$ and $\gamma\delta$ T cells at 0 and 48 hour post infection were hybridized to microarrays. Figure 3 graphically illustrates the genes that changed by greater than 2 fold between 0 and 48 hours post *Salmonella* serovar Typhimurium (calves 112 and 162) or mock (calf 156) infection in the two major T cell subsets. There was a striking difference in the numbers of genes that changed between the mock and experimental infections. Specifically, in *Salmonella* infected calves, $\gamma\delta$ T cells responded by increasing expression (by at least 2 fold) of 220 and 224 genes and decreasing expression of 196 and 252 genes in calves 112 and 162 respectively. In contrast, in $\gamma\delta$ T cells in the mock infected calf, expression increased in only 57 genes, whereas 288 genes decreased in expression. An opposite effect was observed in $\alpha\beta$ T cells. In this case, mock infection or the surgery alone induced an increase in expression of 267 genes in $\alpha\beta$ T cells in calf 156 and a decrease in expression in 129 genes. Whereas *Salmonella* serovar Typhimurium infection induced increases in only 162 and 135 genes and far greater decreases; 368 and 375 genes in $\alpha\beta$ T cells in calves 112 and 162 respectively. Thus, in terms of the numbers of genes changing, $\alpha\beta$ and $\gamma\delta$ T cells seemed to respond very differently to *Salmonella* serovar Typhimurium infection.

As to be expected in genetically dissimilar individuals with varied backgrounds, the genes that changed in response to *Salmonella* infection were not exactly the same. However, some general trends emerged from these experiments. A number of genes consistent with subtle activation and innate response to infection increased in expression in $\gamma\delta$ T cells whereas gene changes specific to $\alpha\beta$ T cells were largely decreases in expression or were indicative of quiescence (Table 1, supplemental data, <http://www.ncbi.nlm.nih.gov/geo/>). Specific gene changes in response to *Salmonella* serovar Typhimurium infection in lymphocytes derived from the mucosal lymphatic ducts suggested distinct responses to infection for $\alpha\beta$ and $\gamma\delta$ T cells.

Increase in IL-2R α expression on $\gamma\delta$ T cells

Included in the genes that were increased in expression in $\gamma\delta$ T cells during *Salmonella* serovar Typhimurium infection was a marker for activation on $\gamma\delta$ T cells, transcripts encoding the IL-2R α , the private receptor for IL-2. To further investigate this change and determine if changes in gene expression were reflected in the phenotype, an IL-2R α -specific antibody was used to detect changes in protein expression on cells from experimental (subsequent to calf 112) and mock infections. Fluorescent intensity of labeled IL-2R α increased significantly ($p=0.008$) on $\gamma\delta$ T cells in lymphatic fluid during experimental

Salmonella serovar Typhimurium enterocolitis compared to non- $\gamma\delta$ T cells (Figure 4). IL-2R α remained at low level expression on $\gamma\delta$ T cells in the negative control calves and on non- $\gamma\delta$ T cell lymphocytes in all calves. In addition to increases in fluorescence, the percent of cells expressing IL-2R α also increased following infection in most calves (data not shown). These data indicate that, consistent with the gene expression data, expression of IL-2R α increased on $\gamma\delta$ T cells in the mucosal lymphatic duct during early *Salmonella* infection.

***In vitro* priming**

The increase in IL-2R α expression *in vivo* suggested that $\gamma\delta$ T cells could be rendered more sensitive to further activation by IL-2. To determine if this response could be due to a direct response of $\gamma\delta$ T cells to *Salmonella* serovar Typhimurium molecular patterns, a functional proliferation assay was performed in which purified (>97% pure) bovine $\gamma\delta$ T cells were pre-treated with PBS, *E. coli* LPS or *Salmonella* serovar Typhimurium LPS and then stimulated with IL-2 and allowed to proliferate. Even though the response to IL-15 is unrelated to IL-2R α , it is known to promote $\gamma\delta$ T cell proliferation [13] and was also included. *Salmonella* LPS alone had no impact on $\gamma\delta$ T cell proliferation (data not shown); however as shown in Figure 5, it was particularly effective in priming a major proportion of the $\gamma\delta$ T cells to proliferate in response to both IL-2 and IL-15. Despite great variation between individual calf donors, the proliferation response was statistically significant. These data underscore the functional relevance of the increase in IL-2R α expression following *Salmonella* infection, suggest a functional increase in the IL-15 receptor, and support priming of $\gamma\delta$ T cells in the target tissue early in infection by *Salmonella* serovar Typhimurium molecular patterns.

Discussion

In this investigation the early transcriptional activities of mucosal lymphatic T lymphocyte subsets in *Salmonella* serovar Typhimurium-induced enterocolitis have begun to be elucidated, revealing substantial differences in the responses of naive $\alpha\beta$ T cells and $\gamma\delta$ T cells to this infection. The bovine model of *Salmonella* serovar Typhimurium-induced enterocolitis directly reflects the pathogenesis and progression of human *Salmonella* serovar Typhimurium-induced disease. Others have detected a $\gamma\delta$ T cell response to *Salmonella* infections in mice [6,12], but the relevance of this response to enterocolitis is unclear. Analysis of peripheral blood $\gamma\delta$ T cell subsets in chickens 14 days after immunization with *Salmonella* serovar Enteritidis also identified increases in IL-2R α transcripts, but this was not confirmed on a protein expression or functional level [14]. Our data suggests that similar, very early protein changes in mucosal lymphatic-derived bovine $\gamma\delta$ T cells in response to *Salmonella* infection occur and the responses likely have functional significance. Specifically, we found that $\gamma\delta$ T cells were subtly activated, or primed, early in *Salmonella* serovar Typhimurium-infected calves, as evidenced by the increase in IL-2R α on cells derived from intestinal lymphatic ducts. Minimal changes in $\gamma\delta$ T cells were observed in the blood of infected calves, consistent with responses seen in human *Salmonella* serovar Typhimurium induced enterocolitis [8]. The functional significance of increased expression of the IL-2R α receptor using purified $\gamma\delta$ T cells *in vitro* was demonstrated. Our data suggests that in this enterocolitis model, which is highly relevant to human disease, $\gamma\delta$ T cells are primed early in infection to later expand in response to secondary signals.

The difference in gene expression between $\gamma\delta$ and $\alpha\beta$ T cells suggested priming of $\gamma\delta$ T cells and a minimal response of $\alpha\beta$ T cells. Infection did not increase gene expression in $\alpha\beta$ T cells over that of the negative control, rather, *Salmonella* serovar Typhimurium infection appeared to have a dampening effect on this T cell subset. Considering that B cells express PAMP receptors, this contaminating cell population in the sorted $\alpha\beta$ T cells might have

skewed gene expression toward activation, but this was not detected. $\alpha\beta$ T cells were the main contaminating cell in the $\gamma\delta$ T cell populations, but they clearly had little to contribute to changes in gene expression. The collection of lymphatic fluid during *Salmonella* infection is a technically difficult feat with inherent variability, especially considering individual calf's genetics and experience. Thus, although lack of sufficient biological repetition preclude discussion of the significance of changes in individual genes *in vivo* in this study, these analyses indicated distinct responses to early *Salmonella* serovar Typhimurium infection by mucosal lymphatic-derived $\gamma\delta$ and $\alpha\beta$ T cells that were critical in directing, and are supported by, subsequent investigations.

Our working model is that the rapid *in vivo* response of $\gamma\delta$ T cells early in infection with *Salmonella* is a response to pathogen associated molecular patterns (PAMPs), such as LPS or flagella, generated by bacterial infection in the gut. Earlier microarray and real time RT-PCR analysis of purified human and bovine $\gamma\delta$ T cells stimulated *in vitro* with *E. coli* LPS documented increases in mRNAs encoding IL-2R α and other genes indicative of priming [15], similar to those upregulated *in vivo* after *Salmonella* infection. Here we show that IL-2R α protein upregulation on $\gamma\delta$ T cells could be reproduced *in vitro* with *Salmonella* serovar Typhimurium LPS alone. Using highly purified and largely naïve (derived from neonatal animals) $\gamma\delta$ T cells we demonstrated, in part, the functional significance of this priming event, namely, enhanced expansion in response to IL-2 or IL-15. This response is similar, in part, to an antigen driven response, except it occurs with a large fraction of the naïve $\gamma\delta$ T cell population. Also, though it is widely accepted that $\gamma\delta$ T cells recognize microbial nonpeptidic phosphorylated molecules [16-18] and alkylamines [19,20] in a TCR-dependent manner, LPS has not, to our knowledge, been described as a TCR ligand. While we have not precisely determined the mechanism of LPS detection, transcripts encoding many TLRs and other pattern recognition proteins are readily detected in $\gamma\delta$ T cells [15,21-25]. Other groups have shown that TLR agonists have co-stimulatory effects in combination with TCR engagement on $\gamma\delta$ T cells, similar in many respects to the priming effect described here [21,25]. A similar costimulatory effect of the TLR2 agonist with TCR engagement that is enhanced by IL-2 on regulatory T cells in mice, and lack of effect of LPS on non- $\gamma\delta$ T lymphocytes [26] is also consistent with our results. An additive response of *Salmonella* serovar Typhimurium LPS and IL-2 in the absence of TCR engagement on regulatory T cells has also been observed [27]. Finally, a similar priming event has been well defined for macrophages, where prior exposure to LPS dramatically increases subsequent responses to secondary signals [28].

The priming event described here is subtle and in some instances may primarily be reflected at only the transcriptional level in PAMP-exposed $\gamma\delta$ T cells [15]. Priming alone does not lead to proliferation unless IL-2 or IL-15 is present, which may vary widely between animals, potentially explaining the inconsistent changes in $\gamma\delta$ T cells in the lymphatic fluid within 48-72 hours of *Salmonella* infection. Priming also leads to a rather restrictive pattern of cytokine production by $\gamma\delta$ T cells, reflected mainly in secretion of select chemokines, such as MIP1- α , GM-CSF and RANTES [15]. We have no evidence that prototypic cytokines of inflammatory $\gamma\delta$ T cells, such as IFN- γ or TNF- α , are induced by this mechanism in bovine cells (data not shown), although this may be a downstream effect following secondary signals [29]. Nonetheless, downstream proliferation is likely, as we have demonstrated *in vitro*. Increased lymphatic $\gamma\delta$ T cells in calves correlated with resistance to *Salmonella* infection (data not shown), and would contribute to dendritic cell maturation [30], potentially present antigen [31] and may interact with monocytes to resolve inflammation [32].

We define a unique, rapid response by bovine $\gamma\delta$ T cells to *Salmonella* serovar Typhimurium infection that is defined as a priming event leading to increased

responsiveness to secondary signals. The two step process leads to expansion of $\gamma\delta$ T cells which is likely essential for induction of their full anti-microbial potential that results in resolution of and immunity to *Salmonella* enterocolitis. These studies are critical to our current efforts to develop therapeutic and/or prophylactic interventions that prime $\gamma\delta$ T cells to enhance protection from mucosal disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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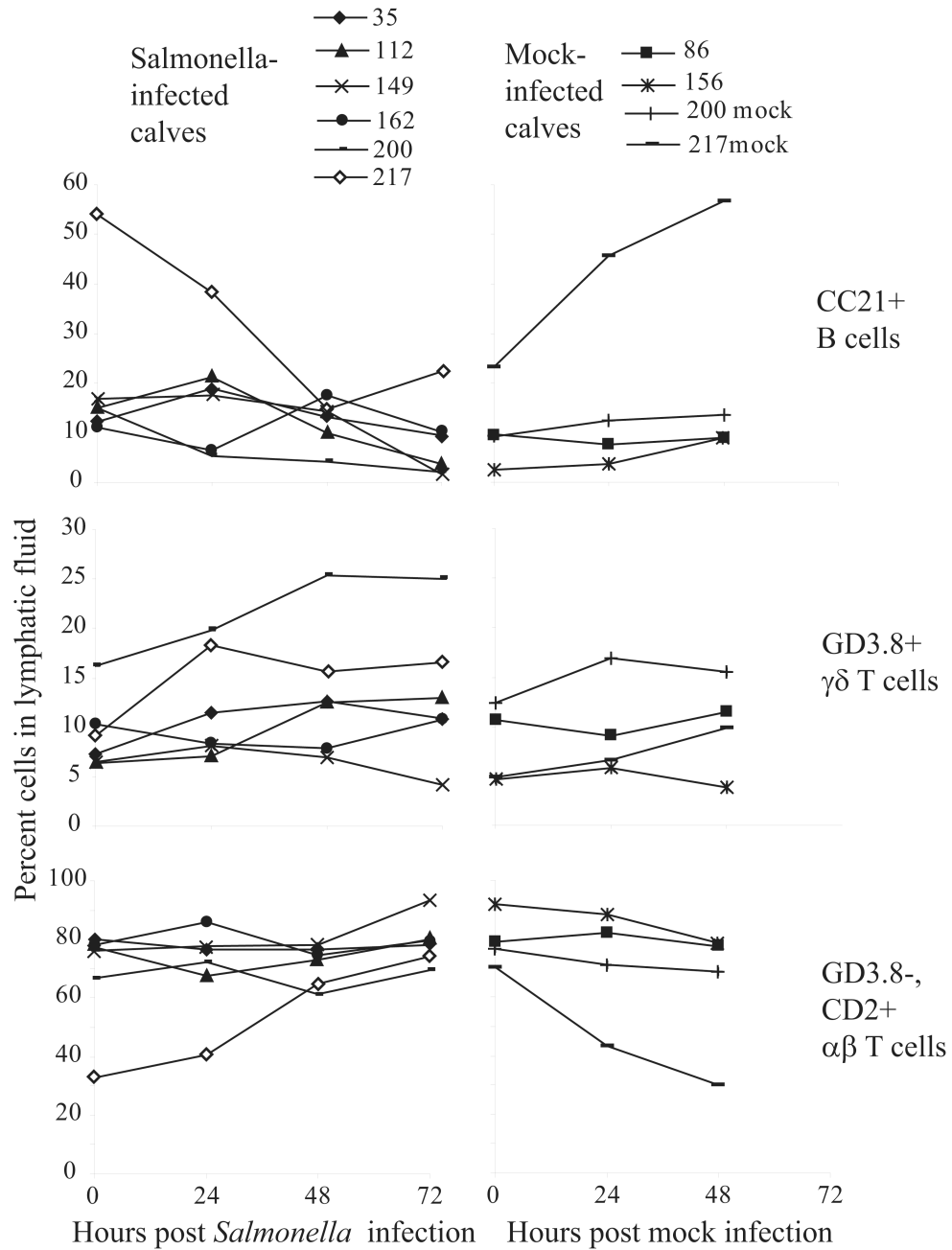


Figure 1. Analysis of cell populations in lymphatic fluid. Multi-color FACS analysis of leukocyte populations in lymphatic fluid during *Salmonella* serovar Typhimurium-induced enterocolitis (calves 39, 112, 149, 162, 200, 217, black lines) or mock infection (calves 86, 156, 200mock, 217mock, grey lines).

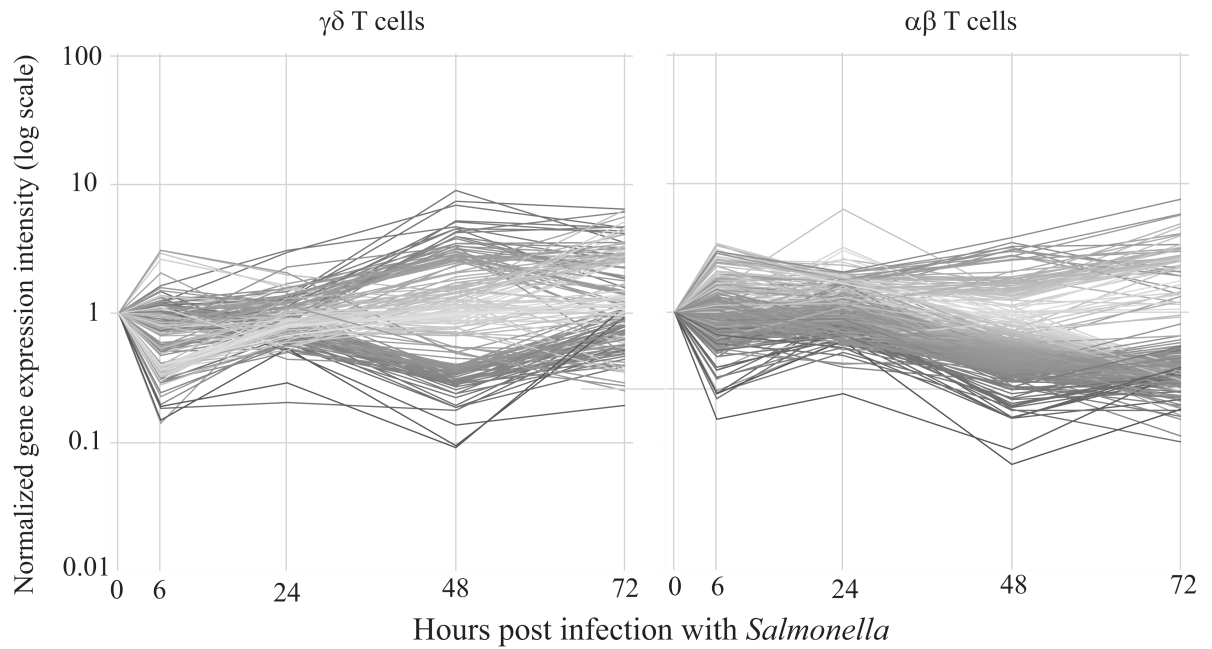


Figure 2.

Gene expression in $\gamma\delta$ and $\alpha\beta$ T cells during *Salmonella* serovar Typhimurium infection. Gene expression changes within $\gamma\delta$ and $\alpha\beta$ T cells in calf 112 were greatest 48 hours after infection. Genes with raw expression >100 and at least a 2.5 fold difference in at least one time point compared to time 0 are shown. The graphs are colored by expression levels at 48 hours post infection, greater changes in expression are indicated by a darker gray shade.

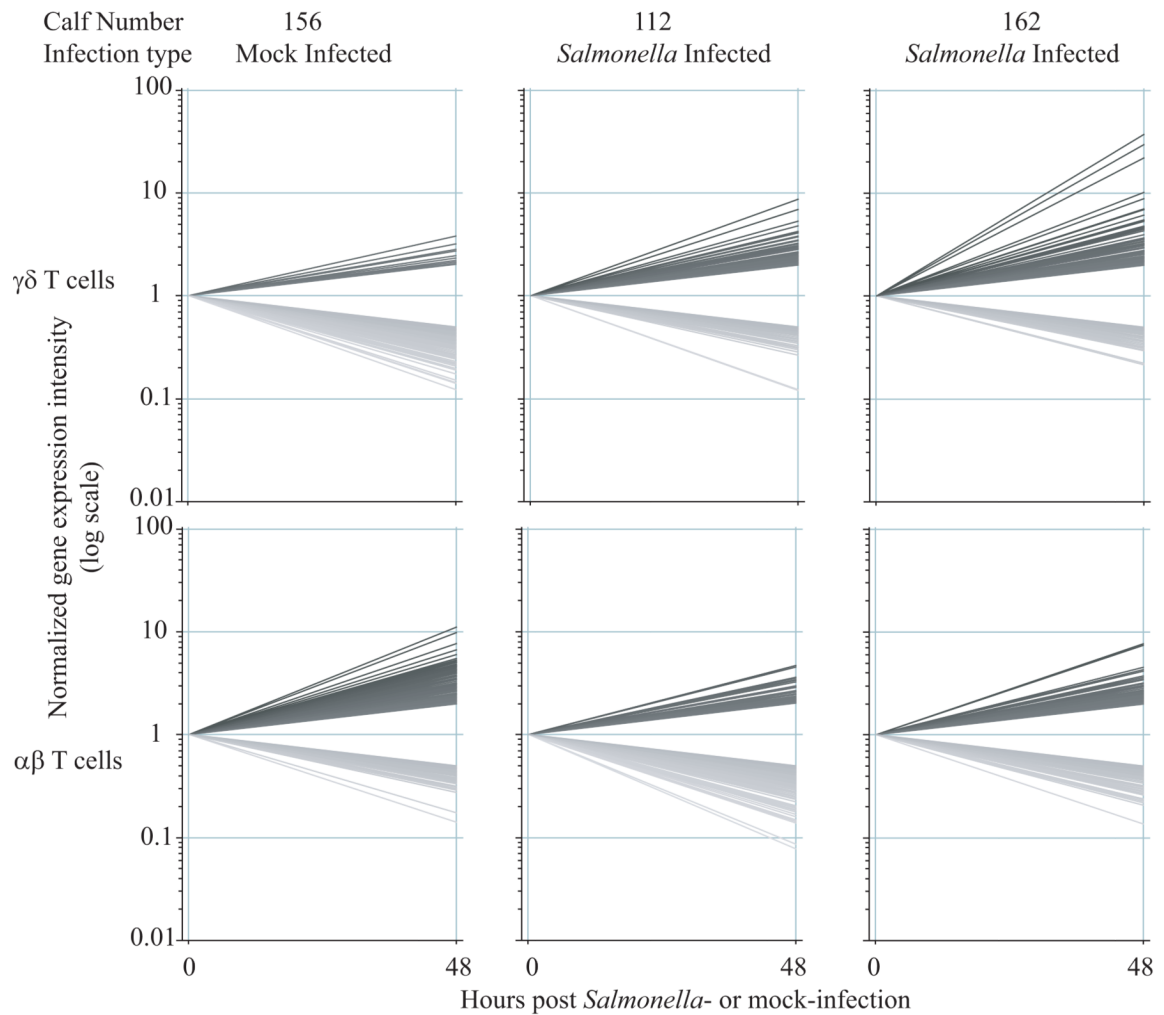


Figure 3.

Gene expression changes at 48 hours post-infection. Graphical representation of the genes that changed by at least 2 fold in $\gamma\delta$ and $\alpha\beta$ T cells between 0 and 48 hours post *Salmonella* serovar Typhimurium-infection (calves 112 and 162) or mock-infection (calf 156). Genes with increased expression are darkly shaded, and those with decreased expression are lightly shaded.

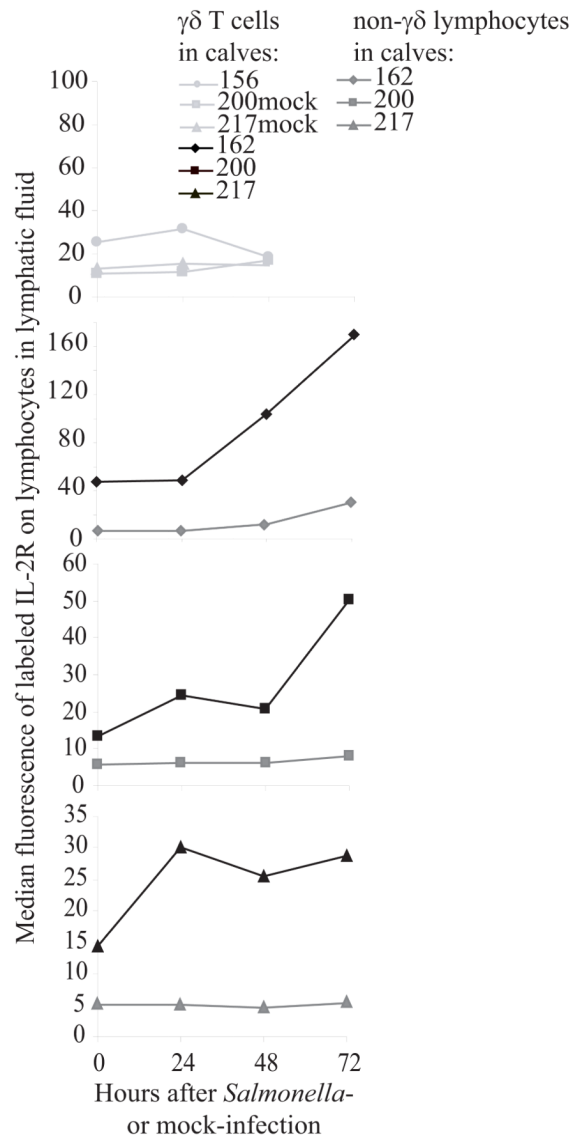


Figure 4. Increase in IL-2R α expression. Consistent with the gene expression data, in the lymphatic fluid of every *Salmonella* serovar Typhimurium infected calf (black lines), fluorescence of the anti-IL-2R α staining increased on $\gamma\delta$ T cells at 48 hours post infection. Expression did not increase in non- $\gamma\delta$ lymphocytes (dark grey lines) or in the mock-infected calves (light grey lines).

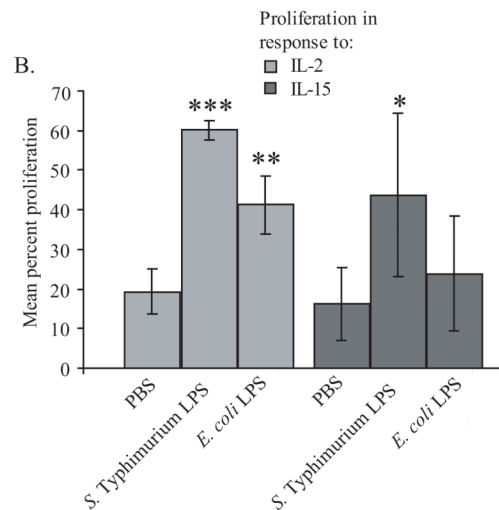
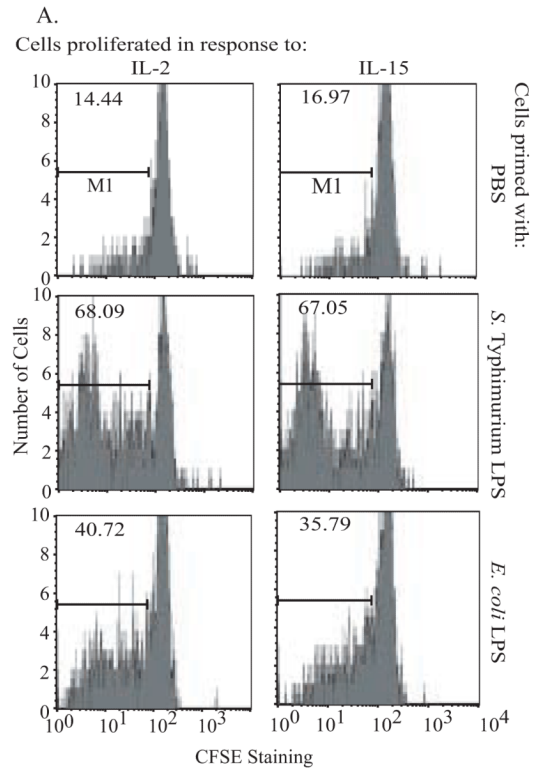


Figure 5.

$\gamma\delta$ T cells are primed by *Salmonella* serovar Typhimurium LPS. (A) Representative CSFE staining after priming of purified $\gamma\delta$ T cells with PBS, *Salmonella* serovar Typhimurium, or *E. coli* LPS proliferation in the presence of IL-2 or IL-15 is shown. (B) Mean value of percent of gated cells for multiple experimental replicates with cells from at least 3 individual calves, error bar denotes standard deviation. *** $p=0.0001$, ** $p=0.0095$, * $p=0.0015$.