A NOVEL ROLE FOR γδ INTRAEPITHELIAL LYMPHOCYTES

IN ANTIBACTERIAL DEFENSE OF THE INTESTINE

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Dedicated to Mum and Dad

for their

love, support, and inspiration

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The mammalian intestine has coevolved with a highly complex population of enteric bacteria. For the most part, mammals and their intestinal microbiota maintain a mutually beneficial relationship. However, the symbiotic nature of this relationship depends on strict sequestration of intestinal microbes in the gut lumen, and damage to intestinal surfaces by chemical agents or microbial pathogens poses a serious threat of inflammation and sepsis. Therefore, the cells populating the intestinal epithelium have evolved strategies to maintain the integrity of the intestinal epithelium and to limit bacterial invasion. γδ intraepithelial lymphocytes (γδ IEL) are unconventional T cells that intercalate under epithelial tight junctions of the intestine. While γδ IEL are

numerically the most abundant T cell population in the body, their biology in intestinal tissues has remained obscure. The work in this thesis seeks to understand the role of γδ IEL in maintaining homeostasis with symbiotic intestinal microbes and in protecting against bacterial pathogens. My findings disclose that intestinal bacteria provide critical regulatory input to γδ IEL in the small and large intestine, and direct the production of proinflammatory and antibacterial factors in γδ IEL. Additionally, my *in vivo* studies disclose a novel role for γδ IEL in antibacterial defense of the intestine, revealing that γδ IEL protect the mucosal barrier in two general ways. First, γδ IEL protect against opportunistically invading commensals immediately after mucosal damage. Next, they also function to limit dissemination of invasive bacterial pathogens. My work suggests that a unique feature of γδ IEL relative to other intestinal immune cells is their early role in providing protection against invading bacteria immediately after challenge. Taken together, these findings disclose that γδ IEL participate in multifaceted antibacterial responses to promote beneficial host-microbial relationships in the intestine.

TABLE OF CONTENTS

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LIST OF FIGURES

. .

LIST OF TABLES

LIST OF ABBREVIATIONS

α – anti

Ab – antibody

Abx- antibiotics

APC – antigen presenting cell

APC- Allophycocyanin

B. theta- Bacteroides thetaiotaomicron

BrdU- 5-bromo-2 deoxyuridine

BSA – bovine serum albumin

CCL – chemokine (C-C motif) ligand

cDNA – complementary DNA

cfu – colony forming unit

convD- conventionalized

convL- conventional

cRNA- copy RNA

CXCR – chemokine (C-X-C motif) receptor

CXCL – chemokine (C-X-C motif) ligand

Cy3- cyanine 3

DC – dendritic cell

DNA – deoxyribonucleic acid

DETC- dendritic epidermal T cell

DSS- dextran sulfate sodium

DTT- dithiothreitol

FACS- flow activated cell sorting

FBS – fetal bovine serum

FITC- fluorescein isothiocyanate

GF- germ-free

HSP- heat shock protein

IBD- inflammatory bowel disease

IEL—intraepithelial lymphocyte

Ig – immunoglobulin

IL – interleukin

IGF-1- insulin growth factor-1

KGF- keratinocyte growth factor

LPS—lipopolysaccharide

MAMP – microbe-associated molecular pattern

MHC – major histocompatibility complex

MIP – macrophage inflammatory protein

MLN- mesenteric lymph node

mRNA- messenger RNA

MyD88- Myeloid differentiation primary response gene (88)

NK – natural killer cell

NF-κB – nuclear factor κB

NOD – nucleotide-binding oligomerization domain

PAP- Pancreatitis-associated protein

PBS – phosphate buffered saline

PCR – polymerase chain reaction

PE – phycoerythrin

PRR- pattern recognition receptor

qPCR – quantitative real-time polymerase chain reaction

Reg- Regenerating

SFB- segmented filamentous bacteria

SPI-1- Salmonella pathogenicity island 1

SSC-side scatter

TCR – T cell receptor

TJ- tight junction

TLR – Toll-like receptor

Unt- untreated

Wt- wild-type

CHAPTER ONE
Review of Literature

BACTERIAL CONTRIBUTIONS TO INTESTINAL

EPITHELIAL BARRIER INTEGRITY

The literature review presented in this chapter has been published in the *American Journal of physiology gastrointestinal and liver physiology*, volume 289, pages g779-g784. This work is reproduced with the permission of the *American Journal of Physiology*.

Introduction

Mammals have coevolved with vast populations of commensal bacteria. The majority of these microbes are found in the intestine, where they are in constant contact with gut epithelial surfaces. The gut's bacterial consortia colonize starting at birth, eventually reaching population levels as high as 10^{12} organisms. Given the size of this population and its intimate contact with intestinal surfaces, it is likely that these bacteria profoundly influence many aspects of intestinal physiology. For the most part, we share a mutually beneficial relationship with our prokaryotic counterparts. Humans and other mammals depend on intestinal microbes to extract maximum nutritional benefit from their diets [1]. Gut bacterial societies are metabolically active, degrading dietary substances that otherwise would be indigestible by the host [2]. The microbes derive benefit from these associations as well, as they are given a protected, nutrient-rich habitat in which to

multiply. In an environment where nutrients are in short supply, natural selection would likely favor such host-microbial associations, which may explain why these interactions evolved and have been maintained.

Although indigenous bacterial populations provide important metabolic benefits to their hosts, such host-bacterial relationships remain "friendly" only as long as these microbes are effectively corralled in the gut lumen. However, the magnitude of the intestinal surface area renders the underlying tissues highly vulnerable to microbial incursions that can lead to inflammation or sepsis. The intestine is thus faced with challenges that are unique relative to other organs in the body. On the one hand, the gut must accommodate large luminal bacterial populations without mounting an overzealous inflammatory response that could cause collateral damage to host tissues. On the other hand, the gut must be poised to trigger such responses if luminal microbes invade the epithelial barrier. As a result of these challenges, intestinal surface epithelia have evolved several key functions that facilitate a peaceful coexistence with luminal microbial populations while maintaining immunological vigilance against invading microbes. These functions include modulation of proinflammatory signaling pathways, expression of key antimicrobial proteins that actively defend epithelial surfaces, and initiation of epithelial repair after mucosal injury. Work in this thesis focuses on demonstrating that resident bacteria play an important role in shaping the functions of a specialized population of mucosal T cells, the γδ intraepithelial lymphocytes (γδ IEL). My results underscore the central role of commensal microbes in the development and maintenance of epithelial barrier integrity. Furthermore, my findings have led to the discovery that intestinal γδ IEL play a critical role in protecting the host against

opportunistic penetration by intestinal commensals and limit the invasion of pathogenic bacteria.

Cellular makeup of the intestinal epithelial barrier

The intestine's internal tissues are separated from the microbe-filled lumen by a single epithelial layer that is only ~20 μm thick. Far from being a homogeneous cell population, however, gut epithelial surfaces are composed of several distinct cell types, each of which contributes in a unique way to mucosal defense and the maintenance of barrier integrity. The enterocyte is the most abundant cell type at both small and large intestinal epithelial surfaces (Fig. 1). Enterocyte membranes, as well as the tight junctions that are formed between these cells, form an important physical barrier to microbial penetration. However, enterocytes also assume a more active role in defending epithelial surfaces by secreting a variety of antimicrobial proteins [3]. Gut surfaces harbor other less-abundant epithelial cell lineages that also help to protect mucosal surfaces from bacterial invasion. Goblet cells, found in both the small and large intestines, secrete large quantities of mucin, which is composed of highly glycosylated proteins that form a protective layer of gel-like mucus over the surface epithelium. In the small intestine, Paneth cells are the key effectors of antimicrobial defense. These specialized epithelial cells are situated at the base of small intestinal crypts (Fig.

1) and harbor secretory granules containing several microbicidal proteins including α-defensins, lysozyme, and members of the RegIII family of microbicidal C-type lectins.

Representatives of the adaptive immune system are also important components of epithelial surfaces. Intraepithelial T lymphocytes (IELs) populate the human small intestine at a frequency of about 10 cells per 100 villus epithelial cells (Fig. 1), and are also present in the large intestine, although in smaller numbers. IELs form intimate contacts with epithelial cells, thus constituting an important part of the physical barrier separating luminal microbes from deeper host tissues. Prior to studies described in this thesis, very little was known about the role of members of the intraepithelial niche in maintaining homeostasis at the host-microbial interface of the intestine. Their predominance in tissues that provide the first line of defense between the 'outside' world and the host suggests that these unconventional T cells play a role in host protection against bacterial invasion. A protective role is also suggested by features including secretion of lytic proteins such as perforins and granzymes which kill infected epithelial cells and may also be involved in the direct killing of bacteria. Furthermore, the γδ IEL subset contribute to epithelial barrier integrity by promoting epithelial regeneration following mucosal injury [4]. However, the biological functions of these cells *in vivo* have remained poorly characterized, in part due to technical limitations preventing experimental manipulation of these cells *in vitro*.

The continuous and intimate contact between gut bacteria and intestinal mucosal surfaces suggests that indigenous microbes profoundly influence neighboring host cell

functions. Consistent with this prediction, a growing body of experimental evidence reveals that luminal bacteria drive key intestinal cell functions that help to maintain barrier integrity. These interactions are likely important for keeping inflammatory processes in check, thus preserving a mutually beneficial relationship with the gut's indigenous microbial populations. Thus, the various cells composing the intestinal barrier are united in an effort to prevent bacterial incursions. However, the manner in which they accomplish this is specific to each cell type, and is dictated by the various bacterial sensing mechanisms discussed below.

Bacteria-sensing mechanisms

Activation of key epithelial defensive mechanisms requires sensing of bacterial proximity by intestinal surface cells. Recognition of bacteria by cells of the intestine is mediated by host-encoded receptors that bind to conserved molecular patterns unique to prokaryotes. These molecular patterns include bacterial cell wall components such as lipopolysaccharide (LPS) and peptidoglycan or protein components of specialized bacterial structures such as flagella. Ligand binding to pattern recognition receptors activates signaling cascades that control transcription of defensive or proinflammatory genes [5[5].

Toll-like receptors (TLRs) are a key group of pattern recognition receptors (PRRs) in mammals. To date, 11 mouse and 10 human TLRs have been identified. Each family member recognizes a distinct set of molecules derived from viruses, bacteria, or fungi. At least four TLRs are specific for bacterial patterns. TLR2 and TLR4 recognize

the bacterial cell wall components lipoteichoic acid and LPS, respectively. TLR5 detects flagellin, a major protein component of gram-negative flagella. TLR9 binds to unmethylated CpG DNA, which is found in bacteria but not eukaryotic cells. Although TLR9 is localized intracellularly [6], TLR2, 4, and 5 are expressed predominantly on the cell surface, where they likely detect extracellular bacteria [7]. Upon ligand binding, each of these TLRs initiates a signaling cascade which directs expression of various proinflammatory genes such as IL-8 and tumor necrosis factor. These signaling cascades involve recruitment of intracellular molecules such as MyD88, a common signaling adaptor for several TLRs, which triggers the activation and nuclear translocation of the transcription factor NF-κB.

A second major group of PRRs is the nucleotide-binding oligomerization domain (NOD) family, a group of cytoplasmic proteins. NODs are thought to recognize intracellular microbial components, likely derived from invading bacteria. The best-characterized members of this family are NOD1 and 2, which bind to muramyl peptide, a constituent of peptidoglycan. Whereas NOD1 recognizes muramyl tripeptides from gram-negative bacteria only [8], NOD2 binds a specific muramyl dipeptide common to both types of bacteria [9]. Like TLRs, NOD ligand binding activates cytoplasmic signaling cascades leading to NF-κB activation and proinflammatory gene transcription [10]. NF-κB is thus a nexus of proinflammatory signaling, receiving and coordinating inputs from multiple pattern recognition receptors.

Intestinal modulation of proinflammatory responses

The gut avoids mounting an inflammatory response against its prokaryotic residents despite the proximity of these large bacterial populations to host intestinal tissues. This is underscored by the fact that neutrophil infiltrates, which are hallmarks of clinically significant inflammation, are virtually absent in healthy intestine. One possibility for this observation might be the strategic compartmentalization of host PRRs within the gut to prevent luminal bacterial populations from triggering uncontrolled inflammation [5, 11, 12]. Consistent with this idea, TLR5 is localized to the basolateral surfaces of epithelial cells and promotes an inflammatory response only when bacteria penetrate the tight junctions between epithelial cells [13].

On the other hand, two key studies suggest that certain nonpathogenic gut bacteria actively suppress inflammatory responses. First, studies in model epithelia have revealed that nonpathogenic *Salmonella* strains attenuate the transcription of inflammatory cytokines in surface epithelial cells. A second anti-inflammatory mechanism restricting production of proinflammatory cytokines has been elucidated in the case of *Bacteroides thetaiotaomicron (B. theta)*, a prevalent commensal anaerobic bacterium found in the intestines of mice and humans. Interestingly, the mechanisms revealed by both studies involve direct interference with the master proinflammatory transcription factor NF-κB [14, 15].

These findings strongly underscore the idea that commensal bacteria engage in active cross talk with epithelial cells, profoundly affecting epithelial integrity and mucosal health. The role of indigenous gut bacteria in shaping mucosal barrier function suggests that antibiotic use may also severely compromise the maintenance of epithelial

health. By disrupting the composition and stability of indigenous microbial populations, antibiotics likely interfere with the beneficial cross talk required to develop and maintain a robust epithelial barrier. This could facilitate both pathogenic and commensal insults to mucosal surfaces and lead to inflammation and sepsis.

Bacterial modulation of gut antimicrobial defense

Although down-modulation of proinflammatory signaling undoubtedly contributes to mucosal tolerance to commensal bacteria, other compelling evidence suggests that under normal, healthy conditions, the systemic immune system is largely ignorant of noninvasive luminal bacteria. Mucosal secretions such as secretory IgA and antimicrobial proteins play a critical role in preventing luminal bacteria from crossing the epithelial barrier, where they can initiate adaptive immune responses and inflammation [16, 17]. Recent evidence suggests that indigenous gut bacteria collaborate with the host to maintain this state of immunological ignorance by inducing expression of a complex antimicrobial protein program in the intestine.

Previous studies have defined the extent to which indigenous microbes shape innate immune responses of the intestine [18]. Studies comparing germ-free mice with those harboring a diverse microbial flora have disclosed that epithelial antibacterial factor expression is triggered by commensal bacteria (Fig. 1) [19]. For example, these analyses led to the identification of RegIIIγ as a novel, microbe-regulated member of the epithelial antimicrobial arsenal. This C-type lectin is expressed throughout the intestinal epithelium, binds its bacterial targets through interactions with peptidoglycan

carbohydrate, and is directly bactericidal [20]. In addition to RegIIIγ, other screens have revealed that enteric bacteria direct intestinal expression of luminally directed proteins that include antibacterial factors [19, 21]. These studies have disclosed that intestinal microflora orchestrate expression of a complex antimicrobial program in cells of the intestinal epithelium (Fig. 1).

Recent findings have revealed that intestinal epithelial cells directly sense enteric bacteria through cell-intrinsic activation of TLRs. In support of the idea of direct bacterial sensing by epithelial cells, toll-like receptor agonists such as LPS are sufficient to induce intestinal expression of a key subset of antibacterial proteins [22]. Studies in Paneth cells have further elucidated that these cells directly sense enteric bacteria through a MyD88-dependent mechanism and initiate a complex antibacterial program that limits the numbers of enteric bacteria that associate with, and translocate through, the mucosal surface [22]. These findings have provided critical insight into the role for epithelial MyD88 in maintaining host-microbial homeostasis and limiting bacterial penetration of the mucosal interface. Collectively, these findings have revealed a role for the gut epithelium in sensing enteric bacteria at the mucosal surface and initiating antibacterial responses, disclosing a direct dialog between intestinal bacteria and the gut epithelium (Fig. 2) [22].

Bacterial stimulation of intestinal epithelial repair

The vast surface area of the gut epithelium is constantly exposed to ingested foreign substances as well as to luminal microbes. It is thus susceptible to damage by a variety of

factors, including environmental toxins and pathogenic bacteria. The presence of large indigenous microbial populations means that gut epithelial damage can quickly lead to bacterial penetration, inflammation, and sepsis. The intestinal mucosal surface must therefore be able to recognize and repair damage rapidly and efficiently. Interestingly, previous studies have revealed that luminal gut bacteria actively trigger mucosal repair through a mechanism involving TLR signaling [23, 24].

Numerous analyses of the effects of gut mucosal damage have relied on the dextran sulfate sodium (DSS)-induced model of epithelial injury. In this model, direct colonic epithelial injury is initiated in mice within a few days after *ad libitum* administration of DSS in drinking water. Epithelial damage is apparent through the appearance of focal colonic lesions, is accompanied by increasing mucosal permeability, and can be detected well in advance of an ensuing inflammatory response. Removal of DSS from drinking water initiates a complex tissue repair pathway that results in a vigorous enterocyte proliferative response and restoration of intact epithelium [4].

Recent work has revealed that efficient colonic epithelial repair requires the presence of resident gut bacteria. Using the DSS-induced injury model, Rakhoff-Nahoum et al. [24] found that mice lacking most of their gut microflora due to broad-spectrum antibiotic treatment are more susceptible to DSS-induced epithelial injury than fully colonized mice (Fig 3). However, recolonization of antibiotic-treated mice with commensal bacteria restores their ability to repair damaged mucosa.

Mice deficient in TLR signaling are unable to fully heal epithelial damage even in the presence of commensal bacteria. For example, mice lacking MyD88 show profound defects in their ability to repair DSS-induced mucosal damage. Bacterial

activation of TLRs in damaged mucosa moreover induces expression of several factors known to contribute to cellular protection, including IL-6, KC-1(IL-8), and heat shock proteins (21). Together, these results suggest that bacterial activation of TLR signaling pathways plays a critical role in directing colonic tissue repair processes (Fig 3).

The DSS injury model has also yielded important clues about which intestinal cell populations play a role in microbe-regulated mucosal repair. Pull et al. [23] found that bacteria are required for the proliferation of epithelial progenitor cells that fuel the replacement of damaged epithelium with new cells. As in the Rakhoff-Nahoum study, TLR signaling plays an essential role in this damage response, as MyD88-deficient mice exhibit profound deficiencies in epithelial progenitor proliferation. Moreover, studies in mice lacking various immune cell populations reveal that macrophages are required for the colonic epithelial proliferative response. After DSS-induced injury, colonic macrophages are recruited to sites of active epithelial proliferation where they become juxtaposed to epithelial progenitor cells and express factors involved in stimulating cellular proliferation (Fig 3) [23].

These results suggest a model in which epithelial repair is driven by bacterial activation of various host epithelial cells. The intact mucosal barrier likely prevents detection of bacteria by TLR-bearing cells such as macrophages. However, following epithelial damage, bacterial penetration may be detected by engagement of macrophage TLRs. Bacterial penetration of mucosal barriers could thus drive a damage response program that induces macrophage migration to injured areas and expression of mitogenic factors that stimulate epithelial cell proliferation. Therefore, microbial surveillance of

tissues with vast loads of bacteria likely plays an essential role in modulating appropriate host responses after tissue damage.

Although macrophages clearly play a key role in promoting epithelial healing after damage, the intestinal response to mucosal injury is likely to be a complex process involving many distinct cell populations. In the work outlined in Chapter 4, I show that γδ T cells also integrate microbial signals and express factors that promote epithelial regeneration and mucosal homeostasis. Furthermore, I provide new insights into the function of intestinal γδ IEL by showing that they limit opportunistic penetration of commensals across damaged mucosal surfaces.

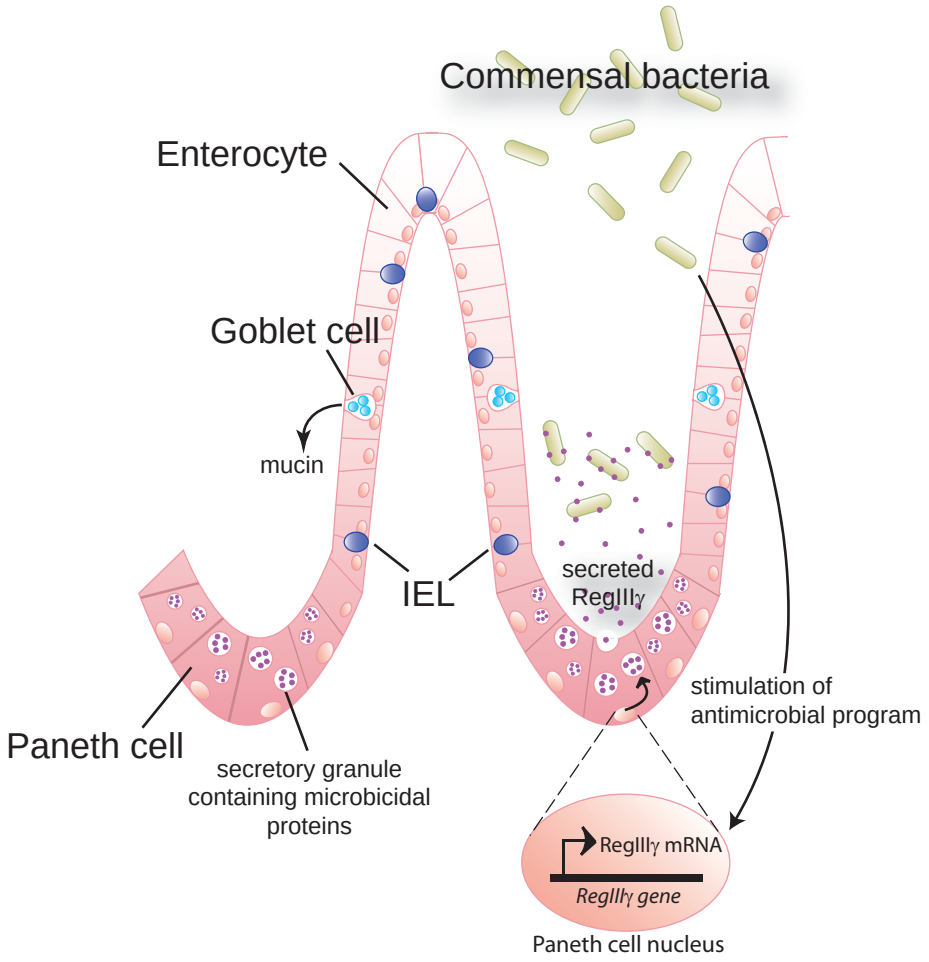

Figure 1: Intestinal mucosal surface cells and their interactions with the enteric microbiota. Mucosal surfaces are composed of several distinct cell types, including enterocytes, goblet cells, Paneth cells, and IEL, each of which contributes in a unique way to the maintenance of barrier integrity. Commensal bacteria directly trigger Paneth cell expression of RegIIIγ, a secreted directly bactericidal C-type lectin, through innate pattern recognition receptors. These findings suggest that commensal bacteria actively play a key role in shaping the composition of the intestinal antimicrobial arsenal.

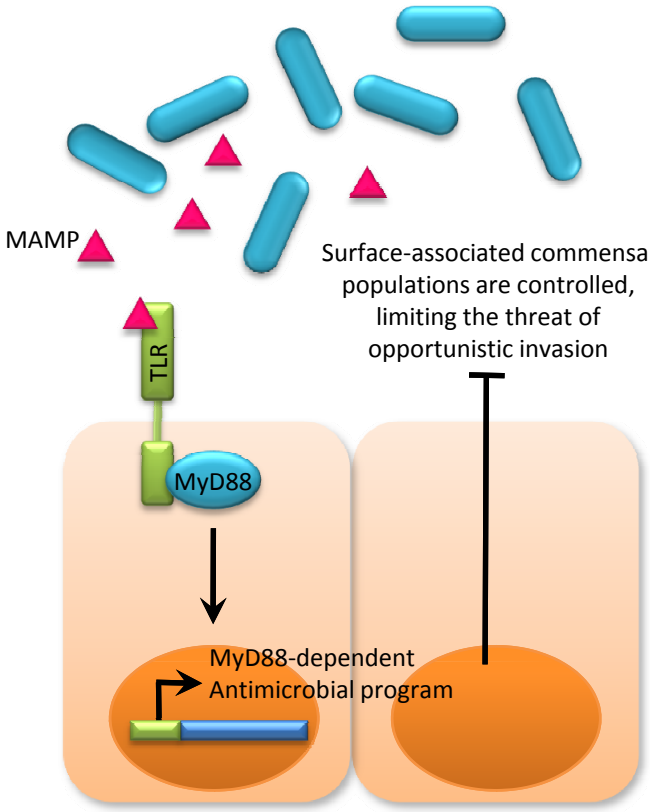

Figure 2: Enteric bacteria trigger antimicrobial responses in gut epithelial cells through activation of epithelial Toll-like receptors. (*Adapted from Vaishnava, et al. PNAS. 2008 Dec 30;105(52):20858-63. 2008*). Paneth cells directly sense enteric bacteria through cell-autonomous MyD88 activation and limit bacterial penetration of the mucosal surface. MyD88-dependent sensing triggers expression of a complex antimicrobial program that could function to limit the numbers of bacteria that localize at the mucosal surface, in or beneath the mucus layer.

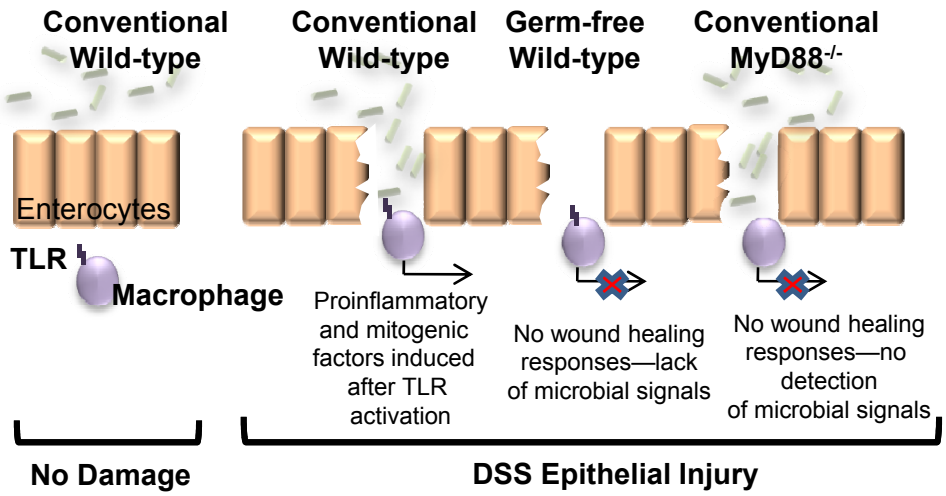

Figure 3: Model of bacterial stimulation of epithelial repair and protection. Bacterial detection by sub-epithelial macrophages is required for epithelial proinflammatory and mitogenic responses after intestinal damage. While the intact mucosal barrier likely prevents detection of bacteria by TLR-bearing hematopoietic cells such as macrophages, epithelial damage and subsequent bacterial penetration is detected by engagement of macrophage TLRs. Germ-free mice lack the microbial signals necessary to induce wound healing responses, while mice lacking the innate recognition adaptor MyD88 cannot detect the luminal bacterial populations to stimulate epithelial repair [23, 24].

CHAPTER TWO

THE UNCONVENTIONAL PROPERTIES OF γδ T CELLS

Introduction

Since their discovery, γδ T cells have challenged well-defined models of lymphocyte function. These unconventional T cells, which bear the γδ T Cell Receptor (TCR), exhibit characteristics of both the adaptive and the innate immune system, fueling controversies surrounding their exact roles in the immune response to infection[25] . While studies in mice lacking γδ T cells have revealed that γδ T cells, like other lymphocytes, are involved in immune regulation, it is clear that they participate in immune functions through roles that are distinct from those of αβ T cells. For example, although αβ T cells occupy a similar niche in the intestinal epithelial layer, they are unlikely to fulfill the same role as γδ T cells, since they are recruited into the epithelial layer under the direction of bacterial signals, unlike γδ T cells which are constitutively present in intestinal epithelia [26]. Additionally, while studies in γδ T cell-deficient mice have focused on their role in immunoregulation during autoimmunity and infectious disease, their exact role has remained obscured primarily due to the heterogeneity found in this population. This heterogeneity possibly stems from the immediate

environment in which individual γδ T cell populations are housed, their antigen specificity, and their tissue distribution [27].

γδ T cells exhibit characteristics that distinguish them from other T cells, primarily the αβ T cells. First, the majority of γδ T cells have a highly restricted tissue distribution as they are highly enriched in various epithelial organs[27]. Second, despite the fact that their epithelial location exposes them to an immense variety of antigens, γδ T cells are remarkably limited in their TCR repertoire when compared to canonical αβ T cells[28, 29]. Third, γδ T cells are constitutively activated and can mount an immune response rapidly without requirements for antigen presentation [30-32]. These effector functions include those typically associated with innate immune responses to infection, including production of chemokines and proinflammatory cytokines that recruit other immune mediators to areas of tissue damage and infection [33]. Of note, these responses often precede αβ T cell responses by as much as hours and days, suggesting that γδ T cells may be regulated through pathways that are distinct from those of αβ T cells[34]. Finally, γδ T cells are not restricted by the same MHC class I or II interactions to which other conventional T cells must adhere, and have been noted to function as professional antigen-presenting cells to αβ T cells[35, 36].

γδ T cells have been implicated in early clearance of food-borne pathogens such as *Listeria monocytogenes* [37-39], supporting the idea that they function in regulation and resolution of inflammatory responses associated with infectious diseases. In these studies, αβ T cell responses were observed much later after γδ T cell responses, suggesting that γδ T cells function specifically at early times after the initial infection [37, 38]. However, several studies have clearly defined a role for γδ T cells in other responses not directly involved in immune responses against pathogens. These include production of tissue-specific growth factors, control of αβ T cell responses, promotion of isotype switching in B cells, and anti-tumor responses [4, 40, 41]. Taken together, these features collectively suggest that γδ T cells are important contributors to the immune response and likely play a critical role in areas where conventional T cells are underrepresented.

These innate-like characteristics are hallmark features distinguishing γδ T cells from other T cells and have been the focus of many studies focused on elucidating γδ T cell function. However, despite the similarities that γδ T cells share with innate immune cells, they retain inherent characteristics of T cells such as the ability to be activated through their TCR and through the CD3 signaling complex[42, 43]. The dichotomy between their innate-like characteristics as well

as their adaptive features has made study of γδ T cells challenging and has fueled many controversies surrounding their function.

γδ T cell distribution

T cells bearing the γδ T cell receptor develop before any other T cell population in the body[44]. While most T cells in the blood and periphery express the αβ T cell receptor, a minority express the non-canonical γδ T cell receptor. This population of cells is distinct in its tissue distribution and a unique property of γδ T cells is their ability to preferentially home to epithelial tissues such as the skin, lung, intestine, and genitourinary tract [45]. A distinguishing feature of γδ T cells is that subsets with distinct antigen receptor repertoires are associated with certain organs and may determine the unique distribution associated with these cells. For example, murine epidermal T cells, commonly referred as dendritic epidermal T cells (DETCs) because of their dendritic morphology, are almost entirely Vγ5Vδ1 γδ T cells[46]. γδ T cells found in the lung express Vγ4Vδ5/6 [47], while those found in the reproductive tract are encoded by Vγ6Vδ1[45]. Alternatively, γδ intraepithelial lymphocytes (γδ IEL) that are housed within the intestinal epithelium, primarily use the Vγ7 chain[48]. Ligand recognition by the various γδ TCRs, while still not clearly understood, includes stress-induced molecules found

on damaged cells or in tumor-bearing tissues[49]. While there is a clear tissue preference for the various γδ T cell subsets, how this tissue distribution is coordinated still remains unclear. Nonetheless, the γδ TCR restriction in specific organs may allow recognition of ligands that are specifically expressed in these anatomical locations, allowing for a rapid response of antigen-specific γδ T cells.

Study of γδ T cell functions during infection has been slow due to conflicting studies reporting a both beneficial or, alternatively, deleterious roles for these cells against various pathogens[50]. Interestingly, the kinetics of γδ T cell responses could explain these conflicting reports and may determine the role of individual γδ T cell repertoires during infection. Nevertheless, these studies have elucidated a role for γδ T cells in early proinflammatory responses followed by regulatory roles after resolution of infection.

A role for γδ T cells in immunoregulation and tumor surveillance

γδ T cells have often been attributed a regulatory role within epithelial tissues given their relative abundance in these areas. Supporting this regulatory role, skin γδ T cells have been shown to maintain tissue integrity and regulate epidermal homeostasis through production of various growth factors such as insulin growth factor-1 (IGF-1) and Keratinocyte growth factor (KGF) [4, 51, 52]. Expression of

these growth factors is thought to occur after the Vγ5Vδ1 TCR recognizes antigens that are expressed by stressed and damaged keratinocytes[52]. Furthermore, the relative contribution of these γδ T cell-specific growth factors has been confirmed through studies showing amelioration of tissue damage after addition of these factors to areas of injury[51]. Finally, in support of a critical role for epidermal γδ T cells in immunoregulation, mice that lack all γδ T cells have severe defects in wound healing and tumor rejection[4, 40].

An intriguing facet of γδ T cell biology is that in addition to their non-canonical TCR, they also express a variety of receptors with established roles in immunosurveillance, including NKG2d, the activating receptor on NK cells and other CD8 T cells[53]. The ligands for NKG2d are induced through environmental stresses and are also often upregulated on various tumor cells. Increased incidence of malignant epidermal keratinocytes has been noted in mice lacking γδ T cells, suggesting a role for these cells in tumor immunosurveillance [40]. Furthermore, studies have found that γδ T cells require engagement of both the NKG2d and the TCR in order to directly lyse tumors expressing NKG2d stress ligands, suggesting a costimulatory role for the NKG2d receptor in γδ T cell activation [40].

A role for γδ T cells in microbial surveillance and antibacterial defense

While the exact function of the γδ T cell receptor (TCR) is not well understood, most well-characterized ligands for this receptor suggest that γδ T cells are acutely responsive to antigens that are induced in cells by stress. Studies in wounded skin have revealed that dendritic epidermal γδ T cells (DETCs) respond to antigen expressed by stressed keratinocytes, producing epithelial growth factors through a TCR-dependent mechanism [46, 52, 54, 55] (Fig 4). Other studies have disclosed that interactions between γδ T cells and monocytes drive critical responses during tissue inflammation. These responses were regulated through activation of the γδ TCR by microbe-derived metabolites [34]. While these studies do not directly address the interactions between γδ T cells and bacteria, they do provide evidence for a functionally active, microbe-responsive γδ TCR. Additionally, studies in human patients have disclosed a significant expansion of circulating peripheral γδ T cells after infection with opportunistic pathogens such as *Streptococcus pneumoniae*, suggesting a role for circulating γδ T cells in antimicrobial surveillance of the immune system [56-58]. However, these studies did not address the role of γδ T cells in regulation of immune responses to tissue-resident populations of commensal microbes. In the work outlined in Chapter 4, I show that γδ IEL display an acute and dynamic response to enteric bacteria and furthermore show that γδ IEL limit dissemination of *Salmonella typhimurium*

immediately following bacterial challenge. These studies constitute the first evidence that γδ IEL function in antibacterial defense in the intestine.

Understanding the relationship between mucosal T cells and indigenous bacteria in mucosal barrier function may additionally shed light on the fundamental causes of inflammatory bowel disease (IBD). IBD denotes a group of disorders characterized by chronic gut inflammation and includes both Crohn's disease and ulcerative colitis. IBD is frequently characterized by an abrogation of tolerance toward luminal bacterial antigens, resulting in an excessive inflammatory response [59]. A number of studies now implicate dysregulated epithelial barrier functions and dampened intraepithelial lymphocyte function in IBD pathogenesis [16, 60]. This makes sense, as strict confinement of commensal bacteria to the luminal side of the mucosal barrier is likely essential for keeping inflammation in check. Interestingly, the rising incidence of IBD in the United States has been linked to increased antibiotic use [61] and excessive hygiene [18], suggesting that factors that disrupt normal host-bacterial cross talk may compromise barrier integrity and lead to increased invasion of indigenous bacteria with ensuing inflammation.

Thesis objective

The biology of γδ IEL has remained enigmatic despite their numerical prominence within various tissues such as the skin and the gut. Their unique location in the intraepithelial niche of the intestine (Fig 5) has fueled much discussion regarding their role in protection against pathogens at the intestinal interface. However, apart from expansion in γδ T cell populations in the periphery, previous studies have failed to show significant γδ IEL responses to pathogenic bacteria [30]. Thus, it has not been clear whether intestinal γδ IEL play a role in antibacterial defense.

In this thesis, I provide new insight into the functions of γδ IEL in the intestine. Indigenous microbes actively stimulate antimicrobial defenses and promote gut epithelial repair, suggesting a profound intertwining of microbial and host biology in the intestine. Given the fact that commensal microbiota direct the antibacterial responses of epithelial cells, I reasoned that indigenous microbes might also shape the biology of γδ IEL. In my thesis, I have indeed shown that γδ IEL require regulatory input from commensal bacteria for expression of key proinflammatory and antibacterial factors following intestinal injury. My work reveals that γδ IEL play a complex and dynamic role at the host-bacterial interface of the gut and reveals the essential role that intestinal microbiota play in regulating γδ IEL responses.

In addition to elucidating how γδ IELs respond to enteric bacteria, my work has uncovered a novel functional role for these cells in host-bacterial homeostasis. My finding that commensal bacteria regulate γδ IEL proinflammatory and antibacterial responses suggested that γδ IEL might function to protect intestinal mucosa against

bacterial invasion. In my thesis, I show for the first time that intestinal γδ IEL protect against opportunistic penetration of commensal bacteria following injury, and furthermore limit dissemination of *Salmonella typhimurium* immediately following bacterial challenge. These studies constitute the first evidence that γδ IEL function in antibacterial defense in the intestine. Together, my work reveals the existence of a dialog between the microbiota and a mucosal T cell population that is essential for intestinal homeostasis.

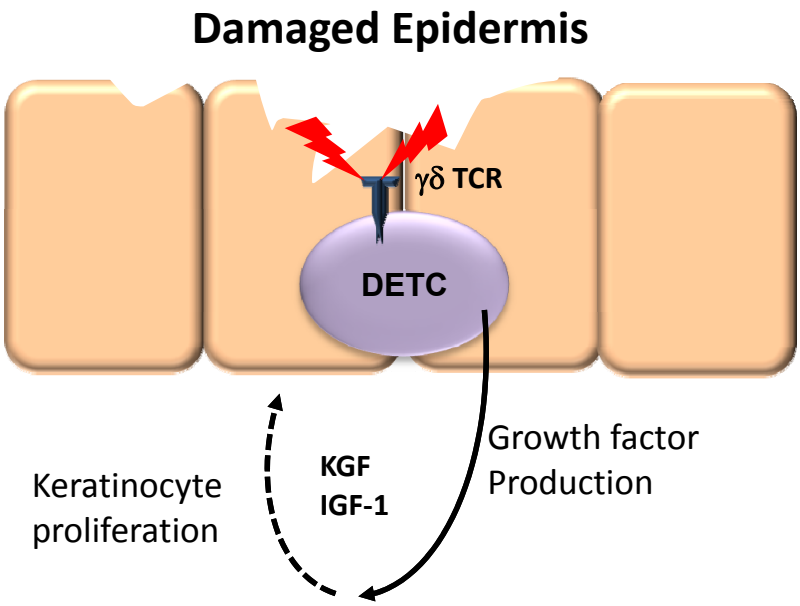

Figure 4: Dendritic epidermal γδ T cell function. Dendritic epidermal γδ T cells (DETCs) detect skin injury through TCR-dependent stress signals produced by stressed keratinocytes. This γδ TCR-dependent signal stimulates DETC to produce epithelial growth factors such as Keratinocyte growth factor (KGF) and Insulin growth factor-1 (IGF-1) to promote keratinoctye proliferation and epidermal restitution after damage to the skin [4, 32, 42].

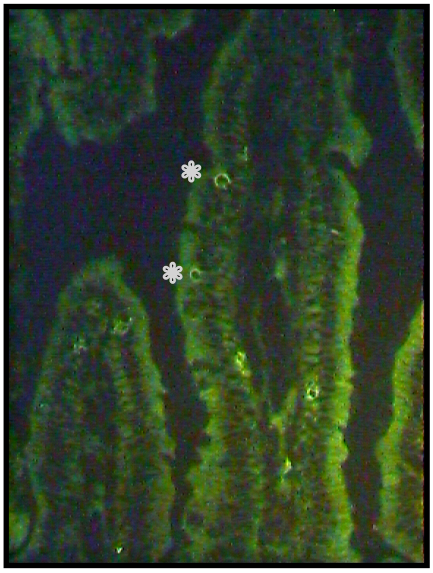

Figure 5: γδ IEL localize to the intestinal epithelium. Immunostaining was performed on fresh frozen small intestinal tissues to localize γδ IEL within the intestinal epithelium. For every 10 enterocytes, there is approximately 1 γδ IEL. Biotinylated α-TCRδ antibody (GL3) was used followed by a streptavidin-conjugated FITC. γδ IEL are indicated by stars.

CHAPTER THREE
Methodology

Animals. Conventionally-raised wild-type and MyD88$^{-/-}$ C57/B6 mice, (from Jackson Laboratories) and TCR$\delta^{-/-}$ mice were maintained in the barrier facility at the University of Texas Southwestern Medical Center at Dallas. Germ-free C57/B6 mice were maintained in plastic gnotobiotic isolators as previously described [21]. All mice were maintained under a 12-h light cycle and were fed the same autoclaved chow diet. 6 to 10 week old mice were used for all experiments. For conventionalization studies, germ-free mice were colonized with microflora from conventional mice 72 hrs prior to treatment with DSS. Dilution plating of luminal bacteria confirmed that these mice were reconstituted to conventional levels. All experiments were performed using protocols approved by the Institutional Animal Care and Use Committees of The University of Texas Southwestern Medical Center.

DSS treatment. Groups of 5-8 C57BL/6 mice received 2% DSS (molecular weight 40,000; ICN Biomedicals) in drinking water *ad libitum* for the indicated periods of time. For examination of mucosal healing, mice were treated with 2% DSS for five days, then were returned to regular drinking water for an additional 3 days. The amount of DSS

water consumed per animal was noted and there were no marked differences between experimental groups. Control mice received water alone.

γδ IEL isolation. Intestinal tissues were removed and were flushed with PBS to remove luminal contents. The tissues were inverted and were washed in ice cold PBS three times. After washing, 25 mL IEL extraction buffer (1mM EDTA, 1%BSA, 1mM DTT, 1X PBS) was added to each intestinal segment. Tissues were incubated for a total of 30 minutes at 37^0 Celsius, with gentle shaking on an orbital shaker. IEL were shaken off the intestinal lining by vigorous shaking/vortexing after 30 minute incubation at 37 degrees. Cells were filtered through two consecutive steps, first with a 100 μM cell strainer and next with a 40 μM strainer. Resulting cells were put over a glass wool column and were counted. Total IEL were stained with PE-labeled anti-TCRδ (GL3; BD Biosciences), and γδ IEL were purified on a BD MoFlo cell sorter in the UT Southwestern Flow Cytometry Core Facility. The purity of isolated γδ IEL was assayed post-sorting and was always ≥ 98%.

RNA double amplification. Total colon IEL were pooled from 5 mice and γδ IEL were isolated as described above. Total RNA was isolated from purified γδ IEL using the Arcturus PicoPure RNA isolation kit and was subjected to mRNA amplification with the Arcturus RiboAmp kit. cDNAs were generated from the mRNAs using random primers.

Microarray analysis. Total RNAs were isolated from purified γδ IEL using the PicoPure RNA isolation kit (Arcturus). For each experimental condition, RNA was isolated from γδ IEL recovered from two independent groups of 5-8 mice. Yields of total RNA were typically 10 ng per group. 5 ng of total RNA was amplified using the Arcturus RiboAmp HS kit. Biotinylated cRNAs were generated by substituting the Enzo T7 BioArray Transcript Kit during the last step and hybridized to Affymetrix Mouse Genome 430 2.0 GeneChips in the UT Southwestern Microarray Core.

To identify genes whose expression was altered by DSS treatment, I performed two-way comparisons between untreated and DSS-treated groups, with untreated samples designated as baseline. Raw data were imported into Affymetrix GeneChip software for analysis, and previously established criteria were used to identify differentially expressed genes (4). Briefly, a ≥2-fold difference was considered significant if three criteria were met: 1) The GeneChip software returned a Difference Call of Increased or Decreased; 2) the mRNA was called Present by GeneChip software in either untreated or DSS-treated samples; 3) the difference was observed in duplicate microarray experiments. GeneChip quality and amplification linearity were assessed using poly-adenylated spike-in control transcripts and oligo-B2 hybridization control (Affymetrix). Heatmaps to visualize signal intensities were generated using GeneTraffic software (Iobion).

To identify DSS-induced genes whose expression is governed by intestinal microflora, I performed a microarray analysis on γδ IEL isolated from untreated and DSS-treated germ-free mice. The list of 272 genes altered by DSS treatment in conventional mice was used to recover the corresponding signal intensities from the germ-free dataset. Signal intensity data were converted to Z-scores [$z = (x - \mu)/\sigma$ where

x = signal intensity, μ = mean signal intensity for all samples, σ = standard deviation across all samples] and subjected to unsupervised hierarchical clustering using GeneTraffic software. The cluster analysis was used to identify the subset of probe sets where the signal intensity in at least 3 of 4 germ-free samples fell at or below the mean signal intensity averaged across all 8 arrays.

Quantitative Real-time PCR (Q-PCR). Total RNA was isolated from purified $\gamma\delta$ IEL using the Arcturus PicoPure RNA isolation kit and was subjected to mRNA amplification with the Arcturus RiboAmp kit. cDNAs were generated from the mRNAs using random primers and were used as a template for Q-PCR with gene-specific primers and SYBR Green Master Mix (Invitrogen). Expression levels were calculated relative to GAPDH or 18S. The sequences of all Q-PCR primers are given in Table 5.

BrdU incorporation studies. Mice were treated for 5 days with 2% DSS followed by 3 days on regular drinking water to initiate epithelial repair. Cells undergoing DNA replication *in vivo* were labeled with 5-bromo-2 deoxyuridine (BrdU; 120 mg/kg of body weight) from a fresh stock solution dissolved in PBS (BD Biosciences). BrdU was administered by intraperitoneal injection to groups of 3-5 mice (DSS treated or untreated) 90 minutes prior to sacrifice. Colons were fixed in Bouin's fixative and paraffin embedded for histology. 5 μm sections were probed with rat anti-BrdU (AbCam) and Cy3-conjugated goat anti-rat secondary antibody (Biomeda). BrdU incorporation was

detected by fluorescent microscopy, and BrdU-labeled cells were quantitated by unbiased counting of all well-oriented crypts, regardless of whether they resided in damaged or undamaged areas. BrdU was injected 90 minutes before sacrifice and was detected by anti-BrdU immunohistochemistry (red). Cell nuclei are stained with Hoescht dye (blue). BrdU-labeled cells were quantitated by unbiased counting of all crypts, regardless of whether they resided in damaged areas. (N= 4-5 mice per group).

Flow Cytometry. For surface staining, isolated IEL were suspended in FACS Buffer (PBS + 0.5% BSA) and stained for 20 minutes with PE-conjugated anti-TCRδ (BD Biosciences) and washed twice. For intracellular staining, cells were then fixed in 5% formaldehyde in PBS, and permeabilized with PBS containing 0.5% BSA and 0.1% saponin. Staining was done with rabbit anti-lysozyme (Chemicon) or rabbit anti-RegIIIγ (4) followed by FITC-labeled goat anti-rabbit secondary antibody (Jackson Immunoresearch). Cells were analyzed on a FACSCalibur flow cytometer (BD Biosciences) in the UT Southwestern Flow Cytometry Core Facility.

Evaluation of DSS-induced colon damage: Colon tissues were fixed in Bouin's fixative and were submitted for paraffin embedding to the UT Southwestern Histopathology core. Representative H&E images of colon tissue in adult C57BL/6 wild type and TCR$\delta^{-/-}$ mice treated with 2% DSS in drinking water for indicated time periods. Sample areas of normal and ulcerated mucosa after 3, 4, and 5 days of DSS treatment are shown.

Immunostaining: Fresh frozen, OCT-embedded small intestines were cut into 5 um sections and were fixed with methanol for 20 minutes at room temperature. Tissues were washed in 1X PBS prior to being probed with a biotin-labeled mouse α-TCRδ GL3 antibody (BD biosciences) followed with a streptavidin-conjugated FITC secondary antibody. $\gamma\delta$ IEL were visualized by fluorescent microscopy.

Bacterial colonization. For monocolonization experiments, age-matched germ-free C57 mice were orally gavaged with stationary phase bacterial culture of the following strains: *Bacteroides thetaiotaomicron* strain VPI-5482, *Salmonella typhimurium* strain SL1433 and its isogenic mutant SPI-1. Mice were sacrificed 48 hours post inoculation and $\gamma\delta$ IEL were isolated as previously described. Small intestinal colonization levels were measured by dilution plating of luminal contents. Bacterial levels in spleen and mesenteric lymph nodes were determined by dilution plating of homogenized tissue.

Assays for bacterial translocation. Conventional C57/B6 wild-type and TCR$\delta^{-/-}$ mice were co-housed 5 days prior to initiation of DSS-treatment to control for microflora differences between mouse strains. Groups of 3-6 C57/B6 wild-type and TCR$\delta^{-/-}$ mice received 2% DSS (molecular weight 40,000; ICN Biomedicals) in drinking water *ad libitum* for up to five days. In regeneration studies, mice were returned to regular drinking water for an additional 3 days after 5 days DSS treatment. Control mice received water

alone. Mice were sacrificed and bacterial translocation to mesenteric lymph nodes was determined by dilution plating of homogenized tissue. Intestinal colonization levels were measured by dilution plating of luminal contents.

Salmonella typhimurium translocation. Conventional C57/B6 wild-type and TCR$\delta^{-/-}$ mice were co-housed 5 days prior to initiation of *Salmonella* infection to normalize for microflora differences between mouse strains. Groups of 3-6 C57BL/6 wild-type and TCR$\delta^{-/-}$ mice were orally infected with wild-type *Salmonella typhimurium* strain SL1433 for 3 hours. Mice were sacrificed and bacterial levels in the spleens of infected mice were determined by dilution plating of homogenized tissue.

Antibiotic treatment. Age matched conventional C57 wild-type mice were given ampicillin (1g/L; Sigma), vancomycin (050 mg/L; Sigma), neomycin sulfate (1g/L; Sigma) and metronidazole (1g/L; Sigma) in drinking water for 4 weeks as described previously. Depletion of the commensal microbiota was verified by aerobic and anaerobic culture of feces. At the end of the treatment, mice were sacrificed and $\gamma\delta$ IEL were isolated as described. Depletion of small intestinal microbiota was verified by aerobic and anaerobic culture of luminal contents following sacrifice.

Bone Marrow Reconstitution. Wild-type C57 Ly5.1 recipient mice were gamma irradiated at 9 Gy and reconstituted with $5x10^6$ bone marrow cells from MyD88$^{-/-}$ Ly5.2 donor mice (6-8 weeks of age). 8 weeks post transfer, mice were tested for chimerism.

γδ IEL of Ly5.1 (wild-type) and Ly5.2 (MyD88$^{-/-}$) origin were isolated by flow cytometry as previously described. Expression of RegIIIγ in each population of γδ IEL was determined by quantitative RT-PCR and intracellular flow cytometry.

CHAPTER FOUR

RECIPROCAL INTERACTIONS BETWEEN γδ INTRAEPITHELIAL LYMPHOCYTES AND COMMENSAL BACTERIA DURING MUCOSAL INJURY

The work presented in this chapter has been published in The *Journal of Immunology*, volume 182, pages 3047-3054.

Introduction

The intestinal immune system has coevolved with a vast non-pathogenic luminal microflora. These indigenous bacteria do not pose a significant threat to host health as long as they remain confined within the intestinal lumen. However, the epithelium can be injured by environmental factors such as toxins, rendering the host susceptible to opportunistic invasion by commensals. Thus, it is essential that the intestine be able to defend against opportunistic penetration of commensal bacteria across injured mucosal surfaces.

Intraepithelial lymphocytes that bear γδ T cell receptors (γδ IEL) promote repair of injured gut epithelia [4]. γδ IEL are intercalated between intestinal epithelial cells, residing on the basolateral side of epithelial tight junctions. Although rare in the circulation, γδ T cells are prominent at intestinal surfaces where they are endowed with a number of properties that distinguish them from conventional T cells. These include the ability to secrete epithelial growth factors and to produce innate cytokines and

chemokines that recruit inflammatory cells [4, 51]. Analysis of mice lacking γδ T cells has revealed that γδ IEL play an essential role in promoting epithelial restitution following mucosal injury [4, 62]. This function has been linked to upregulated expression of keratinocyte growth factor (KGF) which stimulates proliferation of colonic epithelial progenitors [4]. Consistent with their unique role in tissue repair, KGF expression is a distinctive feature of γδ IEL and does not occur in other mucosal T cell populations, including αβ IEL [4].

Despite the unique contributions of γδ IEL to mucosal healing, the molecular details of the γδ IEL response to intestinal injury remain poorly defined. Furthermore, little is known about the factors that regulate this response. This is due in large part to inherent experimental challenges posed by these cells, including the fact that they are refractory to experimental manipulation outside of their intestinal niche. In this report, I uncover new insights into the role of γδ IEL in maintaining intestinal homeostasis following mucosal injury. I first used a genome-wide analysis to elucidate a DSS-induced transcriptional program in colonic γδ IEL that includes orchestrated expression of factors involved in epithelial protection, antibacterial defense, and inflammatory cell recruitment. I next showed that commensal microbes direct key elements of the γδ IEL injury response, revealing a dialog between commensal bacteria and γδ IEL. Finally, I discovered that γδ T cells are essential for controlling bacterial penetration across injured mucosal surfaces. These results suggest that intestinal γδ IEL play a multifaceted role in maintaining mucosal homeostasis following injury, and reveal the existence of a dynamic and reciprocal cross-talk between the intestinal microbiota and γδ T cells.

Microarray analysis of colon γδ IEL

Intestinal γδ IEL present a number of unique experimental challenges that do not exist for other T cell populations. A key experimental roadblock is the fact that these cells readily undergo spontaneous apoptosis when cultured outside of the gut [63], and therefore cannot be studied extensively *in vitro*. Insights into the characteristics of γδ IEL relative to other T cell populations have been obtained through *in vivo* functional genomics studies of γδ IEL isolated from the small intestine [20, 64]. However, the relatively low numbers of colonic γδ IEL have precluded genome-wide analysis of this population. This has posed a serious problem for analyzing how γδ IEL respond to injury, as key mucosal damage models, such as the dextran sulfate sodium (DSS) model, specifically damage colonic epithelia [23]. As I detail below, I have used mRNA amplification techniques and genome-wide microarray analysis, previously used for analysis of rare epithelial cell populations [21], to gain new insight into the biological functions of colon γδ IEL.

Prior studies have shown that γδ IEL play a unique role in tissue repair through localized epithelial growth factor expression following colonic epithelial injury [4]. I hypothesized that this is representative of a more complex response to tissue damage. DNA microarrays were used to gain a genome-wide view of the γδ IEL transcriptional response to epithelial damage. I chose the DSS model of colonic epithelial injury for these studies as it has been used previously to elucidate the contributions of γδ IEL to mucosal repair in mice [4]. DSS treatment results in colon-specific epithelial damage that is characterized by focal lesions [23]. As previous studies of γδ IEL function were performed on cells isolated during recovery after DSS-induced damage [4], analyses were carried out on colonic γδ IEL purified from mice treated orally for 5 days with 2% DSS,

followed by 3 days' recovery (Fig 6). Epithelial cell proliferation was quantitated using 5-bromo-2'-deoxyuridine (BrdU), which is incorporated into the DNA of actively dividing cells. Consistent with prior studies [23], DSS-treated mice showed expanded zones of proliferation in crypts bordering ulcerated areas (Fig. 12), indicating active epithelial regeneration.

To study the transcriptional program expressed by γδ IEL during mucosal repair, I used flow cytometry to isolate pure γδ IELs from untreated and DSS-treated C57BL/6 mice. Consistent with prior results [65, 66], I obtained about 10,000 cells per mouse colon, and the numbers of cells isolated from untreated and DSS-treated colons were virtually identical (Table 1). The small numbers of γδ IEL present in the mouse colon initially presented a major obstacle to my study, as total RNA yields (~10 ng from pooled cells isolated from 5 mice) were insufficient for direct functional genomic analyses. I addressed this limitation by performing linear amplification of mRNAs in order to generate sufficient cRNA for hybridization to Affymetrix Mouse Genome 430 2.0 arrays (Fig. 7). To confirm the purity of my cells, I assessed the resulting transcriptional profiles for transcripts representative of other colon intraepithelial and subepithelial cell populations. While the TCRγ transcript was abundantly present in all samples, the TCRα transcript was undetectable (Table 2), indicating an absence of αβ IEL. Moreover, there was no detectable expression of the macrophage-specific transcript nitric oxide synthase 2, or of RELMβ, an epithelial cell-specific mRNA (Table 2), confirming the absence of macrophages and epithelial cells in my isolated cell populations.

DSS-induced epithelial damage elicits a complex transcriptional response in γδ IEL. Comparison of the DSS-treated and -untreated transcriptional profiles revealed that DSS treatment elicits a complex γδ IEL transcriptional response. Expression of 272 transcripts was altered 2-fold or more in γδ IEL isolated from DSS treated mice (Fig. 8A). 217 of these transcripts were enriched, while 55 showed reduced abundance. Prior studies have established that DSS treatment leads to increased expression of KGF, which is critical for promoting repair of epithelial lesions [4]. Although I was unable to detect KGF transcripts in γδ IEL from either DSS-treated or untreated mice, I observed enhanced expression of transcripts encoding other factors with established cytoprotective properties, including heat shock proteins [67] and the chemokine KC [68] (Fig. 8B). The microarray also revealed enhanced expression of βig-h3 (Fig. 8B), a secreted transforming growth factor β (TGFβ)-induced factor that supports keratinocyte proliferation and wound healing [69]. These findings were substantiated by real-time quantitative PCR (Q-PCR) analysis of amplified mRNAs from independently isolated cell populations (Fig. 8A), suggesting that γδ IEL regulate epithelial regeneration through multiple factors.

A large proportion of DSS-induced transcriptional changes occurred in genes involved in immunoregulation and inflammatory cell recruitment (Fig. 8B). This substantiates the fact that γδ T cells recruit inflammatory mediators into injured tissues [70, 71], and is consistent with prior observations of skin γδ T cells [33]. Q-PCR analyses of independently generated samples verified that DSS treatment elicited enhanced expression of the cytokines KC, IL1-β, MIP2α, Cxcl9, and Cxcl16 (Fig. 8B), which are chemotactic for neutrophils, macrophages, and $CD4^+$ T cells [72-74].

A second prominent category of DSS-regulated transcripts encompassed factors involved in innate immune responses to bacteria. These included the microbial pattern recognition receptors Toll-like receptor 1 (TLR1), which dimerizes with TLR2 to recognize bacterial lipopeptides [7], and CD14, which participates in lipopolysaccharide recognition in complex with TLR4. DSS treatment also enhanced expression of transcripts encoding directly bactericidal proteins, including complement components 1qa and 1qb and lysozyme (Fig. 8B). Increased lysozyme expression was verified by Q-PCR analysis of amplified mRNA (Fig. 10B). I also observed increased expression of pancreatitis-associated protein (PAP), a member of the RegIII family of C-type lectins that includes RegIIIγ (Fig 10A). Although the biological function of PAP remains undefined, we have previously shown that RegIIIγ is directly bactericidal for gram-positive organisms [20]. As PAP and RegIIIγ are co-expressed in intestinal epithelial cells [20], I tested whether RegIIIγ expression is also enhanced by DSS treatment. Q-PCR analysis of amplified mRNA revealed a 4.5-fold increase in RegIIIγ expression following DSS treatment (Fig. 10B).

Since production of directly bactericidal proteins is a previously unappreciated function of γδ IEL, I further analyzed the expression of both RegIIIγ and lysozyme by flow cytometry. The number of γδ IEL expressing RegIIIγ increased 6.6-fold after DSS treatment, and the number of cells expressing lysozyme underwent a 5-fold increase, in agreement with the Q-PCR analysis (Fig. 11). The fact that antimicrobial protein expression is induced in a limited subset of the cell population is consistent with the prior

observation that γδ IEL are activated to express KGF in a localized manner, specifically at focal sites of injury [51].

These findings reveal that DSS treatment elicits a complex transcriptional program in intestinal γδ IEL that includes coordinate expression of cytoprotective, immunomodulatory, and antibacterial factors. This suggests that γδ IEL orchestrate multiple responses that restore epithelial integrity, recruit inflammatory cells, and maintain host-microbial homeostasis following intestinal damage.

Commensal bacteria direct γδ IEL responses to mucosal injury

Several studies have revealed that commensal microbiota play an essential role in maintaining homeostasis during acute DSS-induced colonic injury. As a result, mice lacking intestinal microbes have increased susceptibility to colonic epithelial damage [23, 24]. While bacteria are known to govern many key functions of epithelial cells [20, 21], there has been a lack of experimental support for the idea that intestinal microbes significantly alter the properties of γδ IEL. Challenge experiments suggest that pathogenic bacteria do not trigger significant changes in γδ IEL gene expression [30]. However, there have been no studies addressing the role of indigenous intestinal bacteria in modulating the global properties of γδ IEL. Therefore, I sought to test the hypothesis that indigenous intestinal bacteria govern elements of the γδ IEL injury response.

I tested this idea by assessing expression of the complex DSS-induced transcriptional program in germ-free mice. BrdU incorporation studies disclosed that germ-free C57BL/6 mice exhibit markedly reduced epithelial proliferation during the

repair phase following injury (Fig. 12), in agreement with prior studies [23]. I isolated γδ IEL from DSS-treated germ-free C57BL/6 mice and untreated germ-free controls and determined that the numbers of γδ IEL isolated from both groups were similar to the numbers obtained from conventional mice (Table 1). Duplicate Affymetrix DNA microarray analyses were performed on pooled cells from DSS-treated and untreated germ-free mice using the same methods used for the analysis of conventional mice. I then used the list of 272 DSS regulated transcripts identified by the conventional analysis to recover the signal intensities for these transcripts from the germ-free microarray dataset. Signal intensity data were subjected to unsupervised hierarchical clustering and heatmap analysis.

The cluster analysis revealed a subset of genes that was regulated by damage only in conventional mice (68 genes; Fig. 13; Fig. 14). Commensal microbes were required for DSS-induced expression of a subset of transcripts involved in cytoprotection (e.g., heat shock protein transcripts). These results agree with studies showing that intestinal bacteria enhance heat shock protein and cytokine expression in intact colonic tissue during DSS-induced injury [24]. Although one probe set corresponding to βig-h3 clustered with the microbe-regulated subset of genes (Fig. 14), three other βig-h3 probe sets were excluded from this cluster. Q-PCR analysis of independently isolated γδ IEL populations revealed that βig-h3 transcripts were more abundant in γδ IEL from DSS-treated germ-free mice than from DSS-treated conventional mice (Fig. 15), correlating with the increased severity of epithelial wounding in germ-free mice [23]. Thus, DSS-induced expression of βig-h3 does not require microbial input, suggesting that a

component of the γδ IEL cytoprotective response is elicited independently of bacterial signals.

Remarkably, the microbiota were required for DSS-induced expression of the majority of the pro-inflammatory cytokine/chemokine transcripts upregulated by mucosal injury, including KC, IL-1β, MIP2, MIP2α, and CXCL9 (Fig. 14). Q-PCR analysis of independently isolated cells verified that the abundance of transcripts encoding the cytokines KC, CXCL9, IL-1β, and MIP2α was enhanced between 6- and 270-fold by DSS treatment of conventional as compared to germ-free mice (Fig. 16A). The fact that commensal microbes are required for DSS induced expression of these genes makes it unlikely that this transcriptional program is activated by the cell isolation procedure or as a non-specific response to DSS exposure.

The microarray analysis of germ-free mice further identified PAP mRNA as requiring bacterial signals for DSS-induced expression (Fig. 14). As bacterial signals induce coordinate expression of PAP and RegIIIγ in epithelial cells [20], I assessed whether DSS induced γδ IEL expression of the bactericidal lectin RegIIIγ also requires microbial input. Q-PCR analysis of amplified γδ IEL mRNA revealed that RegIIIγ transcripts were enriched 38-fold in cells isolated from DSS-treated conventional mice as compared to DSS-treated germ-free mice (Fig. 16B), establishing that a component of the DSS-induced antimicrobial response is governed by intestinal bacteria. In contrast, DSS-induced expression of lysozyme, another component of the γδ IEL antibacterial response, occurs independently of the intestinal microbiota.

I next assessed whether the defective injury response exhibited by γδ IEL in germ-free mice is reversible. Adult germ-free mice were colonized with a complete

ileal/cecal microflora harvested from conventionally-raised mice 72 hours prior to DSS exposure. Dilution plating of luminal bacteria established that colonization levels of the "conventionalized" mice were similar to those of conventionally-raised mice (~10^8 cfu/ml). Q-PCR analysis of amplified γδ IEL mRNA revealed that the conventionalized mice exhibited DSS-induced expression of KC, CXCL-9, IL-1β, MIP2α, and RegIIIγ (Fig. 17A,B). Thus, the defective injury response exhibited by γδ IEL in germ-free mice is reversible, and is unlikely to be due to an inherent developmental defect in γδ IEL from germ-free mice. Together, these results indicate that commensal bacteria provide critical regulatory input into the γδ IEL response to mucosal injury, eliciting orchestrated expression of directly antibacterial factors and chemotactic cytokines that function in inflammatory cell recruitment. These findings thus reveal for the first time that indigenous gut microbiota govern a complex transcriptional program in a mucosal T cell population.

Bacteria direct γδ IEL damage responses through MyD88-dependent and –independent pathways

I next sought to gain insight into the mechanisms by which bacteria direct γδ IEL responses to DSS-mediated epithelial injury. Several elements of the regenerative response to colonic damage, including production of cytoprotective factors [23, 24] and recruitment of activated macrophages to areas of damage [23], require the TLR signaling adaptor MyD88. To determine whether MyD88-dependent pathways govern microbe-

dependent responses to mucosal injury in γδ IEL, I analyzed cells isolated from the colons of untreated and DSS-treated conventional MyD88-deficient mice. Numbers of γδ IEL recovered from MyD88-deficient mice were similar to numbers recovered from wild-type mice (Table 1), in agreement with published data [75]. Analysis of amplified cellular mRNAs revealed that DSS treatment failed to trigger CXCL9 and KC expression in conventionally-raised MyD88-deficient mice, in contrast to conventional wild-type mice (Fig. 17A). Thus, CXCL9 and KC expression are induced in γδ IEL through a MyD88-dependent signaling pathway. In contrast, DSS treatment of MyD88-deficient mice elicited enhanced γδ IEL expression of IL-1β, MIP2α and RegIIIγ transcripts (17A, B). These results reveal that intestinal microbes regulate the γδ IEL response to mucosal damage through both MyD88-dependent and -independent pathways.

γδ T cells limit opportunistic penetration of commensal bacteria at early time-points following mucosal injury

My finding that commensal bacteria orchestrate antimicrobial and pro-inflammatory responses in γδ IEL following mucosal injury suggested that γδ IEL may play a role in antibacterial defense of damaged surfaces. These cells inhabit the intraepithelial spaces on the basolateral side of epithelial tight junctions which inhibit paracellular crossing of luminal contents including bacteria [76]. However, the integrity of epithelial tight junctions is compromised by DSS-induced injury [77], leaving the host vulnerable to opportunistic invasion by members of the commensal microbiota. Thus, γδ IEL are

ideally situated to sense epithelial damage and/or bacterial penetration immediately following injury, and to orchestrate direct antibacterial defenses with recruitment of additional immune cell populations. Based on these observations, I hypothesized γδ IEL may play a role in limiting opportunistic penetration of commensals across damaged mucosal surfaces. To test this idea I compared numbers of mucosa-penetrant commensal bacteria in wild-type and TCR$\delta^{-/-}$ mice, which lack γδ T cells [78].

Commensal bacteria that penetrate the intestinal barrier spread to the mesenteric lymph nodes (MLN), which confine them to the mucosal immune compartment and prevent their further penetration to the systemic immune system [79, 80]. I therefore monitored bacterial penetration of the intestinal mucosa by quantitating MLN bacterial loads. I initially examined mice treated with the 5 day DSS/3 day recovery regimen that was used for my transcriptional analyses. Consistent with prior results [4], I observed increased mucosal damage and delayed epithelial repair in TCR$\delta^{-/-}$ mice (Fig. 19). However, the numbers of MLN bacteria recovered from TCR$\delta^{-/-}$ mice were not significantly different from wild-type mice using this treatment regimen (Fig. 18). I reasoned that this might be due to the fact that colonic injury elicits a complex cellular response [23], and that by 5 days of DSS treatment and 3 days of recovery, other recruited immune cell populations (e.g., neutrophils) might be present in sufficient numbers to limit opportunistic penetration of commensals even in the absence of γδ IEL. Thus, I reasoned that γδ T cells may function to control bacterial penetration during acute versus prolonged exposure to a damaging agent.

To test this idea, I quantitated bacterial numbers at earlier time-points following DSS-induced injury. In agreement with prior studies [4, 23], a time course of DSS treatment resulted in detectable mucosal damage within 3 days of treatment, and this damage was augmented in TCR$\delta^{-/-}$ mice compared to wild-type mice (Fig. 19). Numbers of MLN bacteria recovered from TCR$\delta^{-/-}$ mice were significantly higher than in wild-type mice after 3 days of DSS-treatment (Fig. 20A), paralleling the development of mucosal damage. I found no significant differences in the numbers of MLN bacteria prior to 3 days of DSS treatment, suggesting that overt damage to the intestinal mucosa is necessary for increased bacterial penetration (Fig. 20A). Numbers of MLN bacteria were further elevated after 4 days of DSS treatment, with significantly more bacteria recovered from MLN of TCR$\delta^{-/-}$ mice than wild-type mice. However, by 5 days of DSS treatment, numbers of MLN bacteria were reduced in both TCR$\delta^{-/-}$ and wild-type mice, and were not statistically different between the two groups (Fig.20A). I detected no differences in the overall numbers of colonizing anaerobic bacteria between wild-type and TCR$\delta^{-/-}$ mice at any time during the DSS treatment time course (Fig 20B), indicating that differences in numbers of penetrating bacteria were not due to intestinal bacterial overgrowth. These results indicate that $\gamma\delta$ T cells help to limit opportunistic penetration of commensals across injured mucosal surfaces, and that they carry out this function specifically at early time-points following exposure to an epithelium damaging agent.

Discussion

While γδ IEL constitute a major intestinal T cell population, their exact biological functions have remained unclear. In this report I have demonstrated that γδ IEL mount a complex response to mucosal injury, and that commensal bacteria direct key elements of this response, including expression of immunomodulatory and antibacterial factors. Furthermore, I have shown that γδ T cells are essential for controlling opportunistic penetration of commensal bacteria immediately following damage. In combination with prior studies showing that γδ IEL promote epithelial repair [4], our findings suggest that γδ IEL play a multifaceted role in restoring homeostasis after epithelial damage.

Because low absolute numbers of γδ IEL are present in the mouse colon, and because these cells are difficult to manipulate *ex vivo*, there has been little information regarding the molecular details of how intestinal γδ IEL respond to mucosal injury. Through the application of mRNA amplification techniques, I was able to gain a comprehensive view of the colonic γδ IEL injury response that revealed orchestrated expression of cytoprotective factors, immunomodulatory factors, and directly bactericidal proteins. The regulated production of microbicidal proteins such as RegIIIγ [20] indicates a previously unappreciated function for γδ IEL. Furthermore, this finding suggests that γδ IEL make diverse contributions to antibacterial defense of damaged mucosal surfaces by producing proteins that directly target invading bacteria and simultaneously initiating a secondary line of defense through recruitment of additional immune cells (Fig. 21).

By analyzing the γδ IEL injury response in germ-free mice, I have discovered that commensal bacteria provide critical regulatory input to γδ IEL. Strikingly, intestinal

microbes are required for enhanced expression of both the antibacterial factor RegIIIγ and several chemotactic cytokines that function in inflammatory cell recruitment. I further found that γδ IEL injury responses can be restored in adult germ-free mice reconstituted with a conventional microbiota. Thus, γδ IEL from germ-free mice are not irreversibly defective in their ability to respond to mucosal injury, arguing against an inherent developmental defect in γδ IEL from germ-free mice. Rather, they lack the appropriate acute bacterial signals required to drive this program.

Bacterial signaling through the TLR adaptor protein MyD88 is critical for maintaining mucosal homeostasis during DSS-induced epithelial damage [23, 24]. Bacterial signals are detected through MyD88-dependent TLR signaling on subepithelial macrophages, which position themselves next to colonic epithelial progenitors and drive proliferation [23]. Similarly, I have shown that a component of the γδ IEL response to DSS-induced injury is governed by bacterial signaling through a MyD88-dependent pathway. This indicates that MyD88-dependent repair of mucosal damage is a complex process involving multiple cell types, including macrophages and γδ IEL. More importantly, it suggests a role for innate pattern recognition in activating γδ IEL responses to tissue damage. Again, a key question is whether γδ IEL can detect bacteria directly through innate mechanisms or whether bacterial signals are first detected by other cells (e.g., macrophages) and relayed to γδ IEL.

A critical remaining question is whether γδ IEL responses to epithelial injury are elicited by direct interactions between bacteria (or bacterial products) and γδ IEL, or whether other cells, such as epithelial cells, detect bacteria and then alter γδ IEL

responses through indirect mechanisms of cell-cell communication. Prior studies provide evidence consistent with both models. *In vitro* experiments suggest that circulating γδ T cells can respond directly to bacterial products [81], supporting the concept of direct bacterial detection. The idea that γδ IEL could directly sense the presence of invading bacteria also makes sense given that these cells inhabit the intraepithelial spaces on the basolateral side of epithelial tight junctions that prevent paracellular crossing of luminal contents [82]. While γδ IEL in healthy, intact epithelia should have limited contact with commensal populations that are confined to the gut lumen, they are strategically situated to detect penetration of bacteria through damaged epithelia.

There is also experimental support for the idea of indirect bacterial detection by γδ IEL. For example, studies in skin have revealed that epidermal γδ T cells respond to antigen expressed by stressed keratinocytes [51], indicating the possibility of an indirect detection mechanism. Analyses of intestinal IEL have shown that MyD88-dependent IL-15 production is crucial for γδ IEL development [83]. Future studies will be required to distinguish between the direct and indirect bacterial detection models with respect to intestinal γδ IEL mucosal injury responses and will also require development of mouse models that allow specific genetic manipulation of γδ IEL.

The results of my functional genomics studies prompted us to investigate whether γδ T cells might defend against opportunistic penetration of commensals across damaged mucosal surfaces. I found that γδ T cells were essential for limiting the spread of commensal bacteria to MLN immediately following the appearance of mucosal damage, suggesting that γδ T cells may be protective specifically following acute

epithelial insult. The cellular response to prolonged exposure to damaging agents such as DSS is complex [23], and thus other cell populations that are recruited to damaged areas under the control of γδ T cell-independent signals [84] could restrict the spread of commensals to MLN during chronic injury. A protective role for γδ IEL during acute damage furthermore makes sense given that these cells are well-situated to mount an immediate response designed to limit opportunistic penetration of bacteria through epithelial tears. This is likely to be especially important in otherwise healthy individuals where there may be frequent transient (as opposed to chronic) exposures to damage-inducing environmental factors such as toxins. However, the fact that I detected bacterial regulation of γδ IEL transcriptional responses after 5 days DSS/3 day recovery suggests that commensals impact γδ IEL function even after bacterial spread to MLN has been contained. This could result from continued exposure of γδ IEL to bacterial products and/or damaged epithelial cells, and is consistent with the fact that intestinal mucosa still show signs of damage at this stage (Fig.17).

The responses of γδ IEL uncovered by my study provide a plausible molecular explanation for the protective function of these cells. Through coordinated expression of antibacterial and immunomodulatory factors, γδ IEL could limit bacterial penetration through acutely injured mucosa and prevent bacterial spread to MLN. However, it remains to be determined experimentally whether the regulated production of antibacterial and chemotactic factors accounts for this protective function. Given the difficulties in experimentally manipulating γδ IEL *in vitro*, such studies will require development of animal models that allow cell-specific genetic manipulations of γδ IEL.

In this study I have shown that γδ IEL engage in a dynamic and reciprocal cross-talk with commensal bacteria. Commensal bacteria provide critical regulatory input to γδ IEL by directing the expression of key immunomodulatory and antibacterial responses following mucosal injury. While commensals were previously known to elicit complex gene transcription programs in epithelial cells [20], I have shown for the first time that intestinal bacteria also extensively regulate gene transcription in a mucosal T cell population. At the same time, I have found that γδ T cells defend against opportunistic penetration of commensal bacteria immediately following mucosal injury. Thus, γδ IEL make multifaceted contributions to restoring homeostasis after epithelial damage by both promoting epithelial repair and limiting opportunistic invasion of commensals through damaged mucosal surfaces. Together, these findings provide new insights into the role of γδ T cells in maintaining tissue homeostasis, and indicate that bacteria-lymphocyte cross-talk plays a critical role in mucosal immunity.

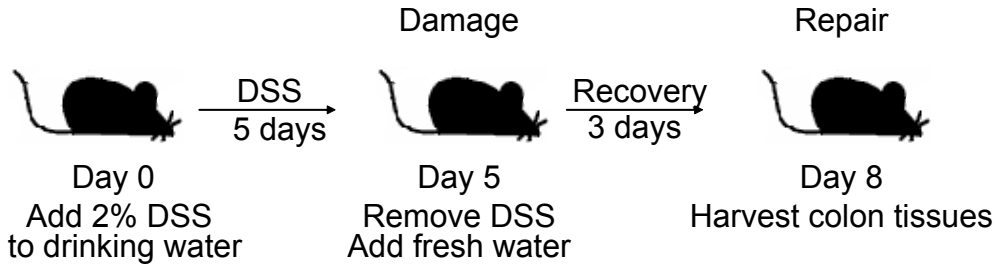

Figure 6: DSS treatment. Groups of 5-8 C57BL/6 mice received 2% DSS (molecular weight 40,000; ICN Biomedicals) in drinking water *ad libitum* for the indicated periods of time. For examination of mucosal healing, mice were treated with 2% DSS for five days, then were returned to regular drinking water for an additional 3 days.

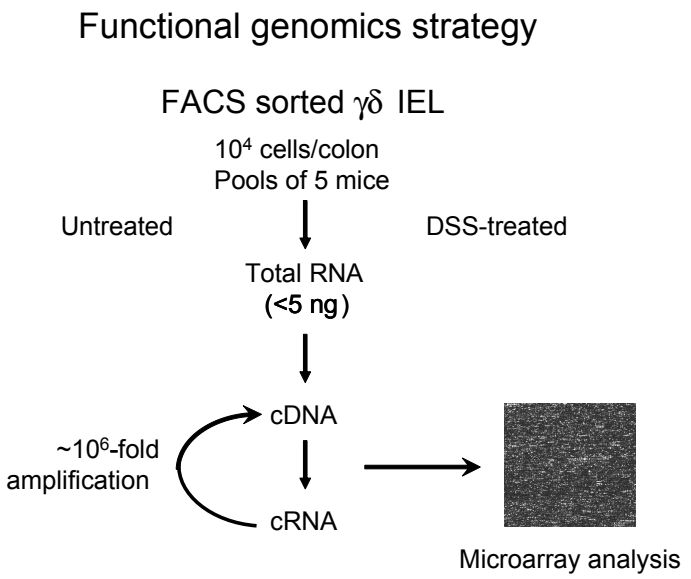

Figure 7: Functional genomics strategy. Total RNAs were isolated from purified γδ IEL using the PicoPure RNA isolation kit (Arcturus). For each experimental condition, RNA was isolated from γδ IEL recovered from two independent groups of 5-8 mice. Yields of total RNA were typically 10 ng per group. 5 ng of total RNA was amplified using the Arcturus RiboAmp HS kit. Biotinylated cRNAs were generated by substituting the Enzo T7 BioArray Transcript Kit during the last step and hybridized to Affymetrix Mouse Genome 430 2.0 GeneChips in the UT Southwestern Microarray Core.

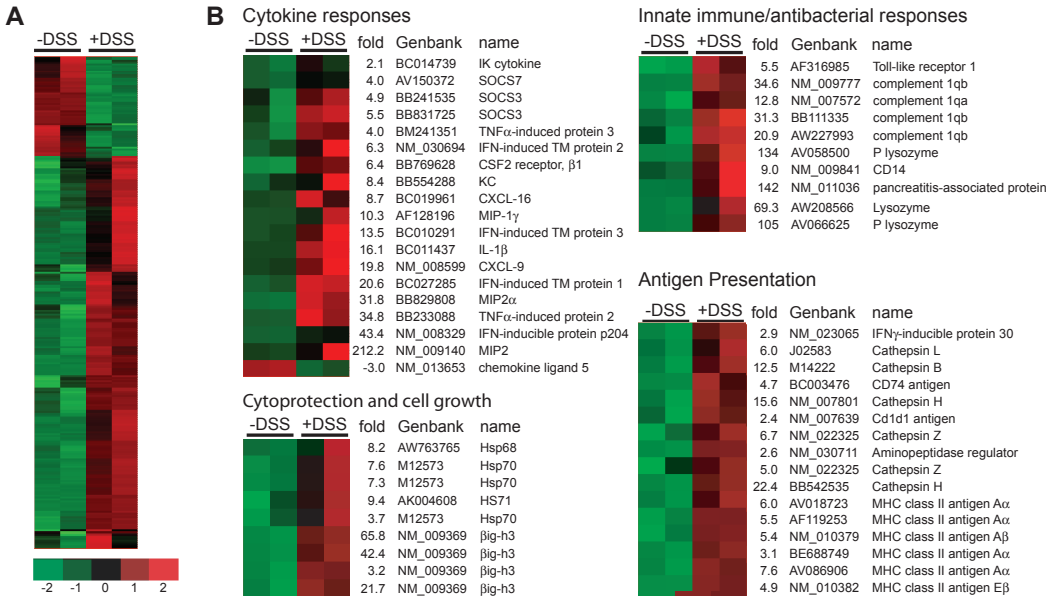

Figure 8: Colonic γδ IEL exhibit a complex transcriptional response to DSS-induced mucosal injury. (A) Affymetrix Mouse Genome 430 2.0 arrays were used to compare transcript abundance between γδ IEL from untreated and DSS-treated colons. Differentially expressed transcripts were identified as outlined in Materials and Methods, revealing 272 transcriptional changes between the DSS-treated and untreated groups. The differentially regulated genes are displayed as a heatmap in which expression level is defined by Z-score (defined in Materials and Methods). **(B)** Key functional groups were delineated using Gene Ontology (GO) terminology, and are displayed as heatmaps.

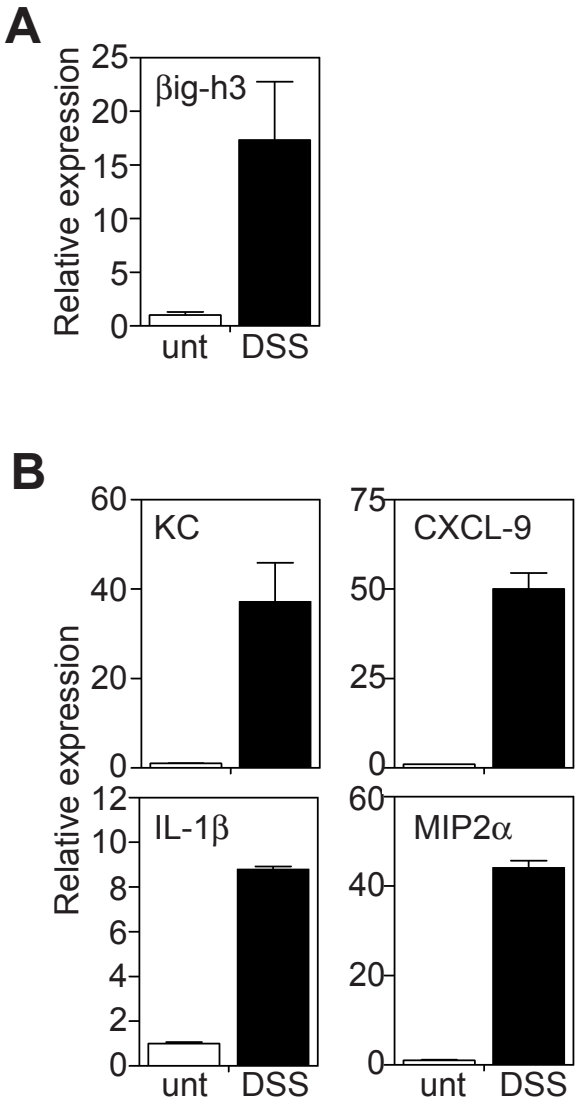

Figure 9: DSS treatement elicits coordinate expression of cytoprotective and immunomodulatory factors in γδ IEL. (A) RNA was isolated from sorted γδ IEL, amplified, and Q-PCR analysis was performed to quantitate expression of βig-h3, which stimulates epithelial proliferation during wound healing [51]. Assays were performed on γδ IEL isolated from pooled colons (n>5 mice), run in triplicate, and are shown as mean values normalized to GAPDH. Relative expression levels were calculated in relation to untreated samples. Results are representative of two independent experiments. unt, untreated; DSS, DSS-treated. Error bars, ±SEM. Q-PCR quantitation of **(B)** pro-inflammatory cytokine expression.

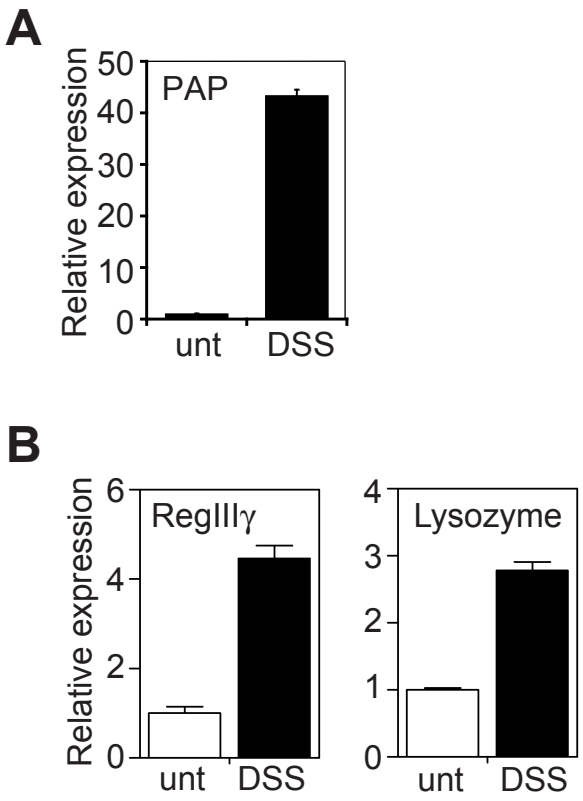

Figure 10: DSS treatment elicits coordinate expression of antibacterial factors in γδ IEL. RNA was isolated from sorted γδ IEL, amplified, and Q-PCR analysis was performed to quantitate expression of **(A)** PAP, a member of the RegIII family of C-type lectins with putative microbicidal function, **(B)** lysozyme, which is a microbicidal protein and RegIIIγ, a directly microbicidal C-type lectin. Assays were performed on γδ IEL isolated from pooled colons (n>5 mice), run in triplicate, and are shown as mean values normalized to GAPDH. Relative expression levels were calculated in relation to untreated samples. Results are representative of two independent experiments. unt, untreated; DSS, DSS-treated. Error bars, ±SEM.

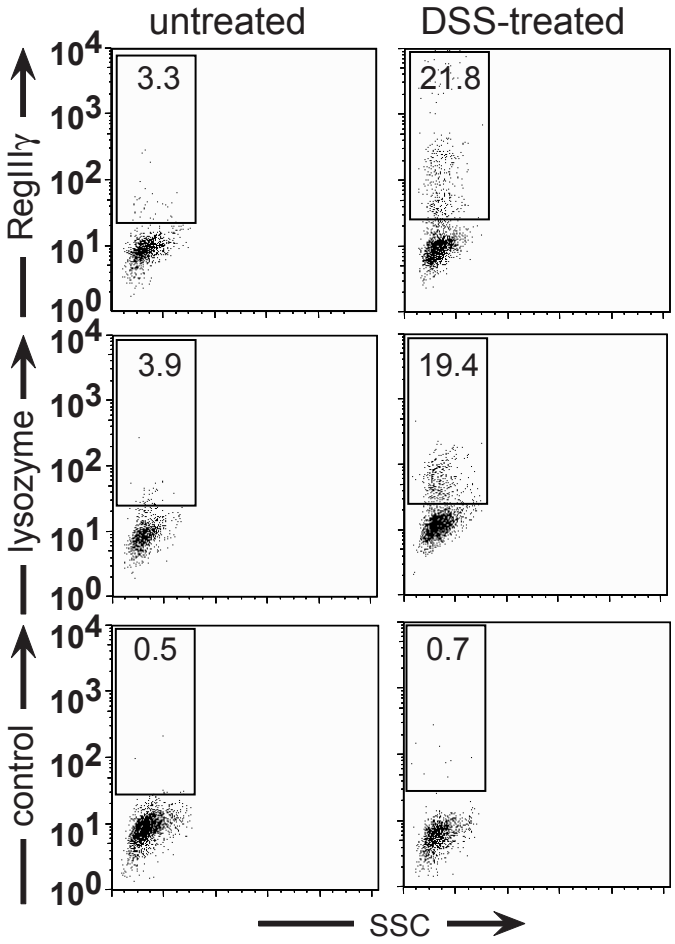

Figure 11: Bactericidal protein production in γδ IEL. Flow cytometry was performed on total IEL populations from untreated and DSS-treated colons. Intracellular staining was carried out with antibodies directed against lysozyme and RegIIIγ, as well as a control rabbit polyclonal antibody. Gated γδ IEL populations are shown, and percentages of the gated populations are given. SSC, side scatter.

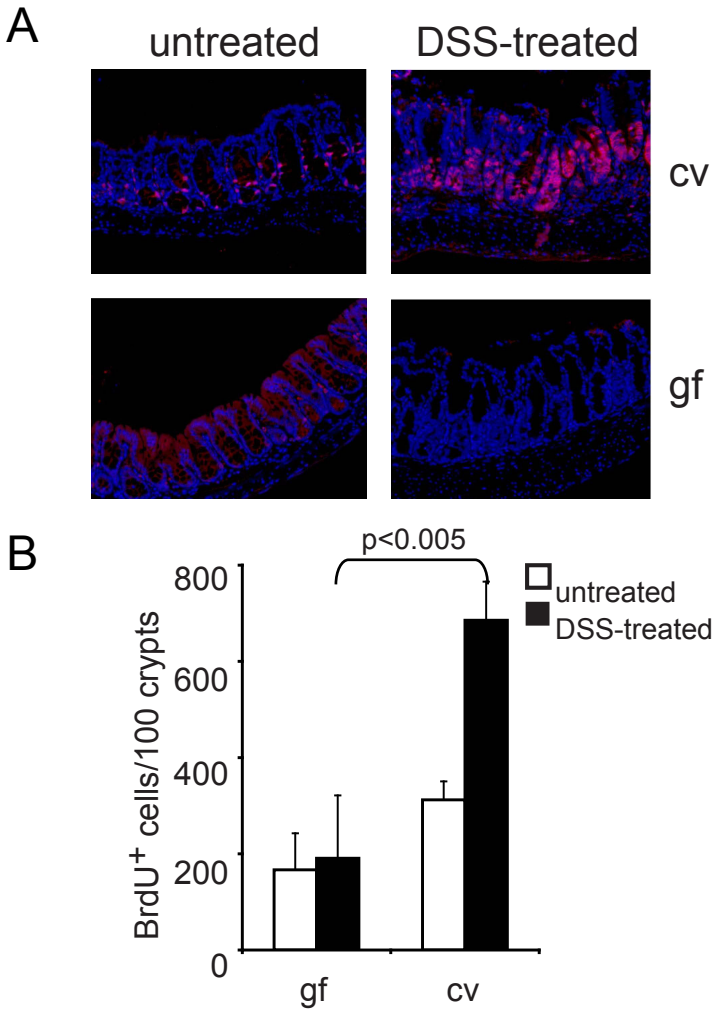

Figure 12. BrdU incorporation studies. Mice were treated for 5 days with 2% DSS followed by 3 days on regular drinking water to initiate epithelial repair. Cells undergoing DNA replication in vivo were labeled with 5-bromo-2 deoxyuridine (BrdU; 120 mg/kg of body weight) from a fresh stock solution dissolved in PBS (BD Biosciences). BrdU was administered by intraperitoneal injection to groups of 3-5 mice (DSS treated or untreated) 90 minutes prior to sacrifice. **(A)** Colons were fixed in Bouin's fixative and paraffin embedded for histology. 5 μm sections were probed with rat anti-BrdU (AbCam) and Cy3-conjugated goat anti-rat secondary antibody (Biomeda). BrdU incorporation was detected by fluorescent microscopy, and BrdU-labeled cells were quantitated by unbiased counting of all well-oriented crypts, regardless of whether they resided in damaged or undamaged areas. BrdU was injected 90 minutes before sacrifice and was detected by anti-BrdU immunohistochemistry (red). Cell nuclei are stained with Hoescht dye (blue). **(B)** BrdU-labeled cells were quantitated by unbiased counting of all crypts, regardless of whether they resided in damaged areas. (N= 4-5 mice

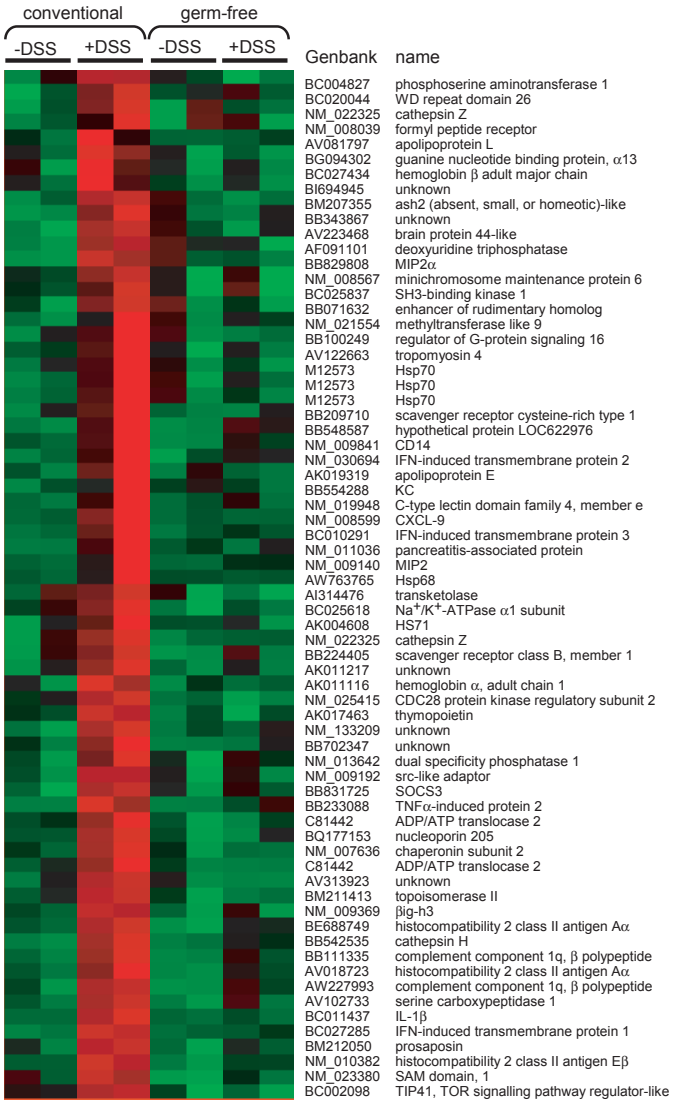

Figure 13. Complete list of DSS-induced γδ IEL transcripts that are regulated by commensal bacteria. Signal intensities of the 272 transcripts that were differentially regulated in γδ IEL from DSS treated conventional mice were compared to the corresponding signal intensities yielded by germ-free γδ IELs. These 272 genes were subjected to unsupervised hierarchical clustering, allowing identification of a subset of 68 genes that require commensal bacteria for enrichment following DSS-induced mucosal injury. Heatmap color is keyed to gene expression level as defined by Z-score. Red indicates increased expression relative to the mean expression level, while green indicates decreased expression. A subset of these bacteria-regulated transcripts is shown in Fig. 14.

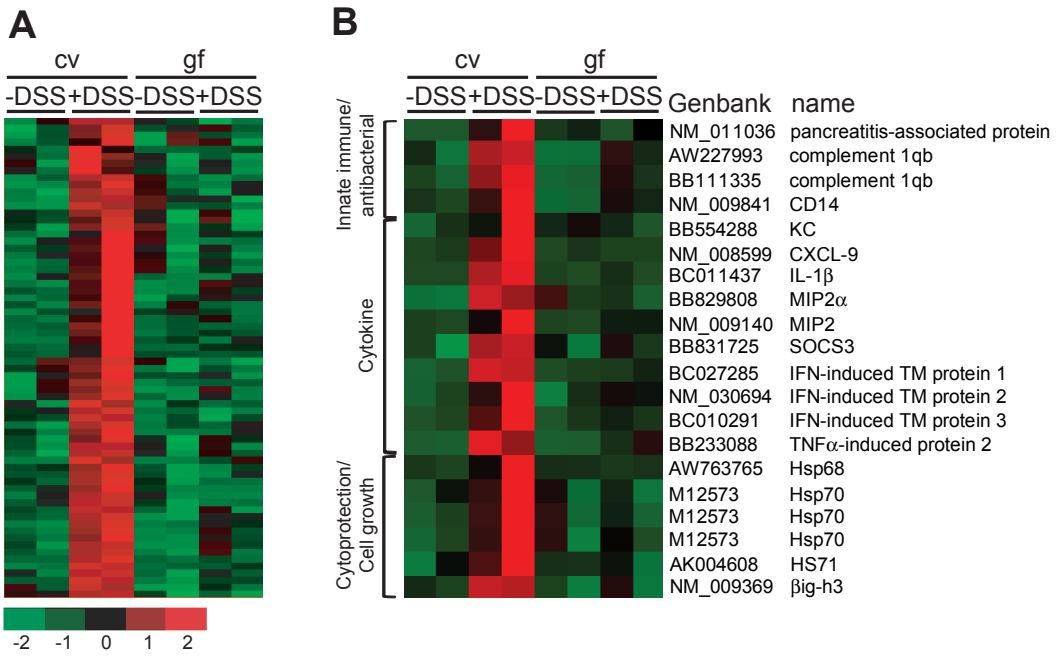

Figure 14: Commensal bacteria govern a component of the γδ IEL response to mucosal injury. γδ IEL from germ-free untreated and DSS-treated mice were analyzed by microarray. Signal intensities of the 272 transcripts that were differentially regulated in DSS-treated conventional mice were compared to the corresponding signal intensities from germ-free γδ IEL. **(A)** Unsupervised hierarchical clustering of the signal intensity data revealed a subset of transcripts that require commensal bacteria for enrichment after DSS-induced mucosal injury. **(B)** Bacteria-regulated transcripts that encode cytoprotective, immunomodulatory, and antibacterial factors. Heatmap color is keyed to gene expression level as defined by Z-score.

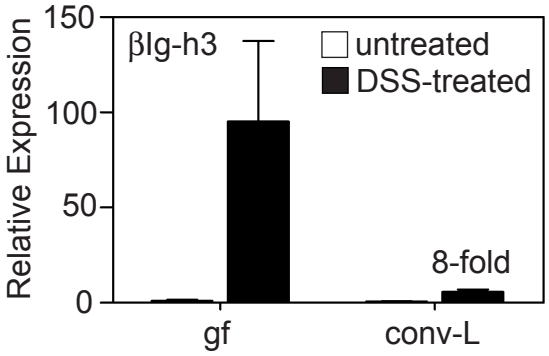

Figure 15: Commensal bacteria do not direct βig-h3 responses in γδ IEL following mucosal injury. βig-h3 levels were quantitated by Q-PCR of amplified γδ IEL mRNAs, revealing that bacterial signals are not required for expression of this cell proliferation-inducing factor. Assays were performed on γδ IEL isolated from pooled colons (n>5 mice), run in triplicate, and are shown as mean values normalized to GAPDH. Relative expression levels were calculated in relation to untreated gf samples. Results are representative of two independent experiments. gf, germ-free; conv-L, conventional from birth. Error bars, ±SEM.

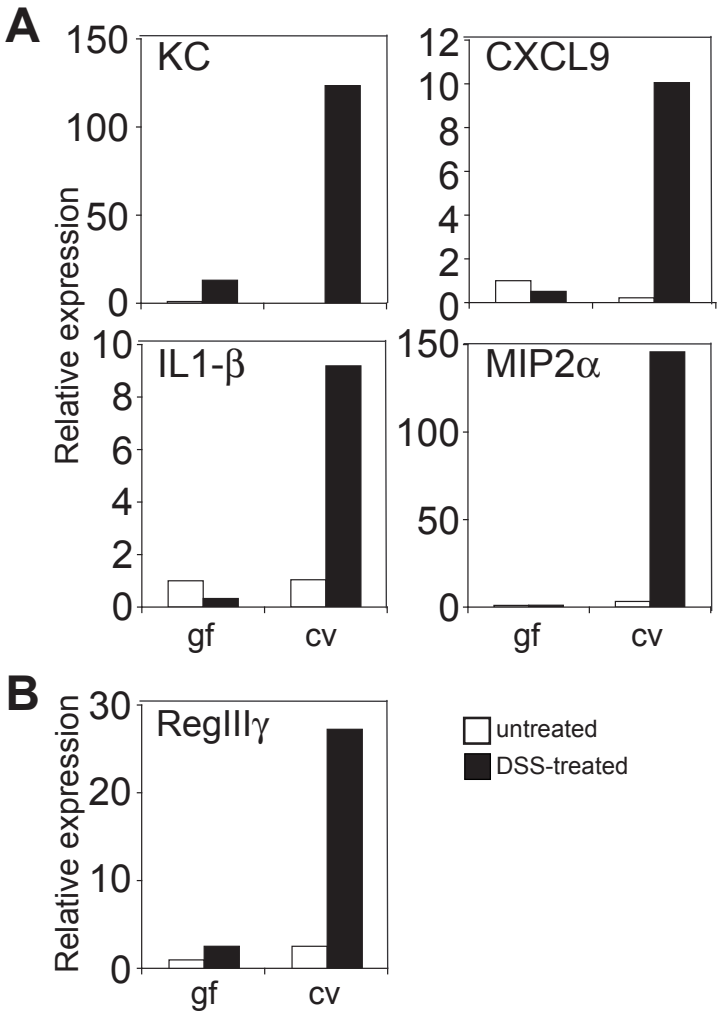

Figure 16: Commensal bacteria direct antibacterial and pro-inflammatory responses in γδ IEL following mucosal injury. (A) Pro-inflammatory cytokines and (B) the antibacterial lectin RegIIIγ were quantitated by Q-PCR of amplified γδ IEL mRNAs. Assays were performed on γδ IEL isolated from pooled colons (n>5 mice), run in triplicate, and are shown as mean values normalized to GAPDH. Relative expression levels were calculated in relation to untreated gf samples. Results are representative of two independent experiments. gf, germ-free; conv-L, conventional from birth. Error bars, ±SEM.

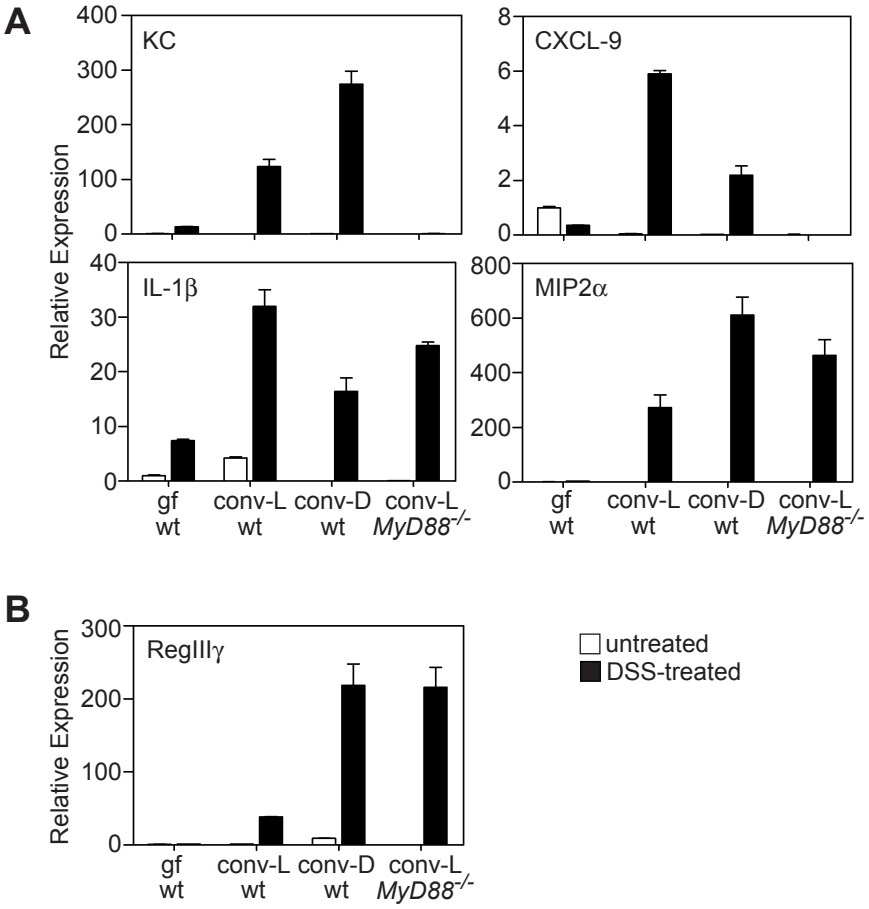

Figure 17: Regulation of antibacterial and pro-inflammatory responses in γδ IEL following mucosal injury. (A) Pro-inflammatory cytokines and **(B)** the antibacterial lectin RegIIIγ were quantitated by Q-PCR of amplified γδ IEL mRNAs. Q-PCR analysis additionally revealed MyD88-dependent and -independent regulation of pro-inflammatory cytokines and RegIIIγ. These responses were reconstituted in germ-free mice conventionalized with a normal microflora (A and B). Assays were performed on γδ IEL isolated from pooled colons (n>5 mice), run in triplicate, and are shown as mean values normalized to GAPDH. Relative expression levels were calculated in relation to untreated gf samples. Results are representative of two independent experiments. Error bars, ±SEM. conv-L, conventional from birth; conv-D, conventionalized for 72 hours; gf, germ-free.

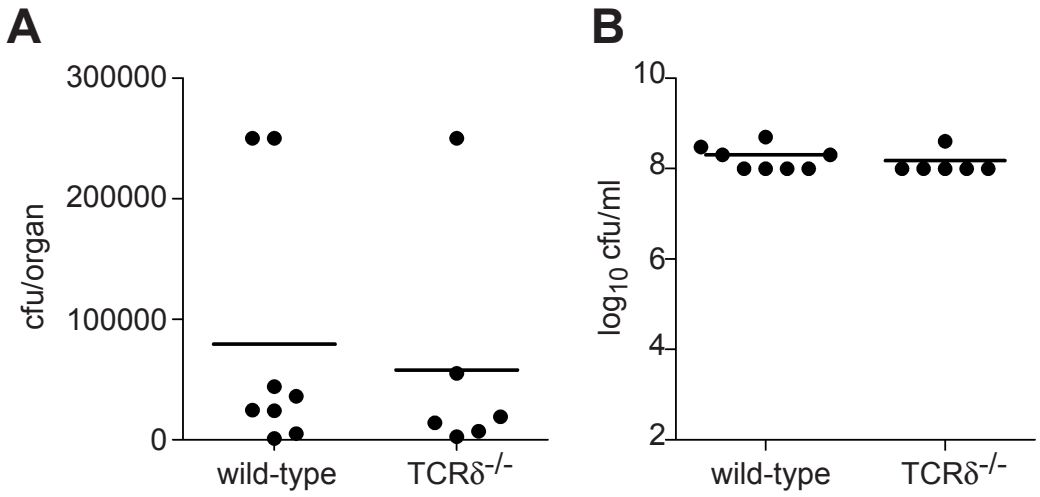

Figure 18: Bacterial quantitation in mice treated for 5 days with DSS followed by 3 days' recovery. Mice were treated for 5 days with 2% DSS and then were returned to normal drinking water for 3 days. Numbers of **(A)** mesenteric lymph nodes (MLN) bacteria or **(B)** luminal bacteria were quantitated in wild-type and TCRδ$^{-/-}$mice. Error bars represent SEM, and results are pooled from two independent experiments.

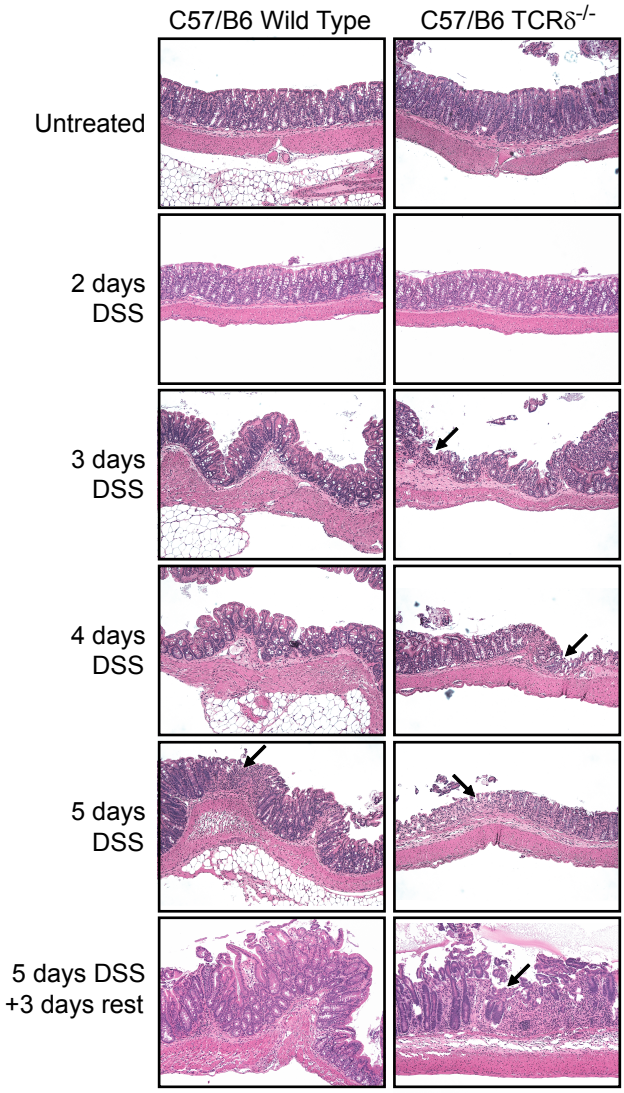

Figure 19: γδ IEL are necessary for wound healing in the colon. Representative H&E images of colon tissue in adult C57BL/6 wild type and TCRδ$^{-/-}$ mice treated with 2% DSS in drinking water for indicated time periods. Sample areas of normal and ulcerated mucosa after 3, 4, and 5 days of DSS treatment are shown. Mucosal erosion, crypt shortening, and ulceration of the colon are apparent 3 days after initiation of DSS treatment. Examination of the histopathology confirmed heightened susceptibility of TCRδ$^{-/-}$ to DSS-induced colonic damage, as evidenced by enhanced mucosal erosion three days after DSS treatment. Mucosal regeneration in wild-type and TCRδ$^{-/-}$ mice was evaluated by returning mice to regular drinking water for 3 days after DSS treatment. While wild-type colons displayed evidence of epithelial regeneration (hyperplastic crypts and surface reepithelialization) after a 3 day repair period,repair is impaired inTCRδ$^{-/-}$ mice. Ulcerated areas indicated by arrows. (Original magnification: 200X)

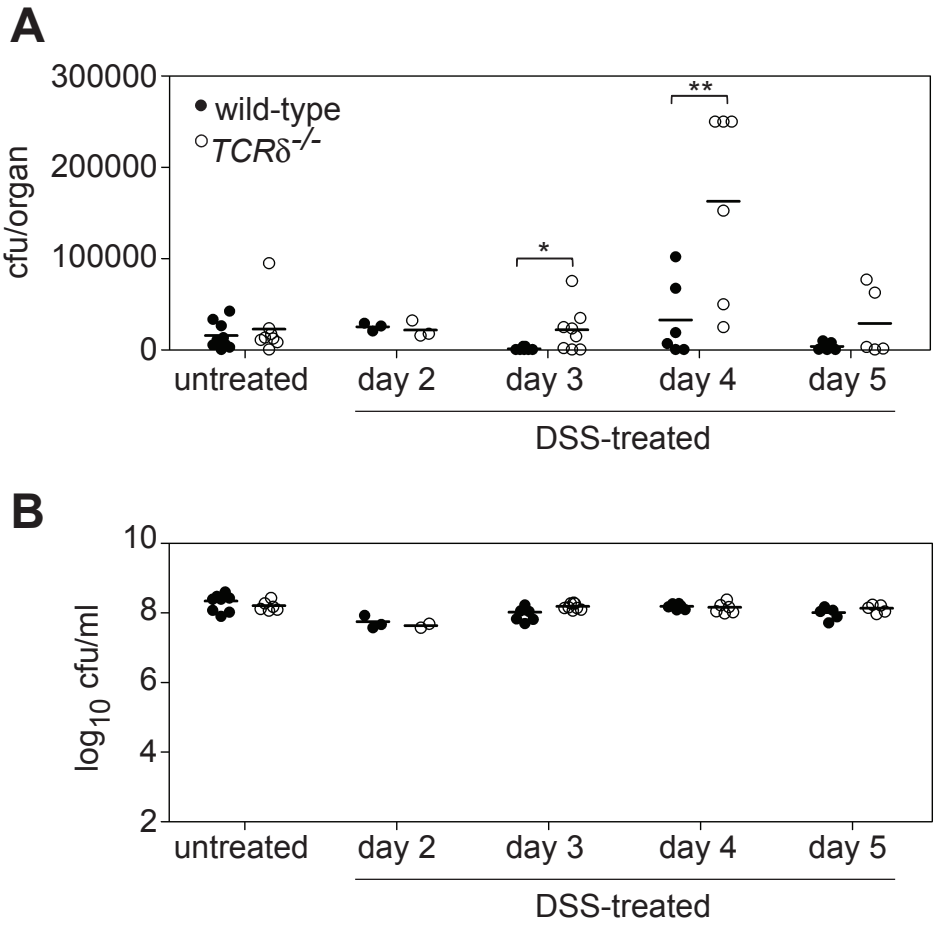

Figure 20: γδ **T cells limit opportunistic penetration of commensal bacteria following mucosal injury**. **(A,B)**Wild-type and TCRδ$^{-/-}$ mice were treated with 2% DSS over a time course of 5 days. Numbers of **(A)** mesenteric lymph nodes (MLN) bacteria or **(B)** luminal bacteria were quantitated in treated and untreated wild-type and TCRδ$^{-/-}$ mice. Error bars represent SEM, and results are pooled from three independent experiments.*,P<0.05;**,P<0.01.

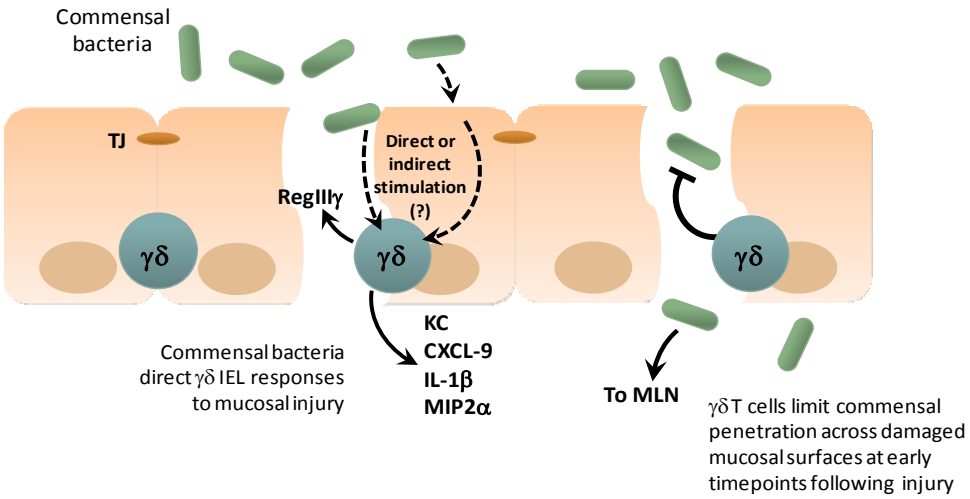

Figure 21: Interactions between commensal bacteria and γδ IEL during colonic mucosal injury. γδ IEL are situated between epithelial cells and are located on the basolateral side of tight junctions which restrict paracellular penetration of luminal bacteria. Upon injury, commensal bacteria stimulate γδ IEL expression of antimicrobial factors, such as RegIIIγ, and of chemotactic cytokines (KC, CXCL-9, IL-1β, MIP2α). It is not yet clear whether this involves direct stimulation of γδ IEL by bacteria or bacterial products, or whether bacteria act indirectly through other cells (e.g., epithelial cells). γδ T cells function to limit bacterial penetration of mucosal surfaces specifically during the early stages following epithelial injury. I propose that bacteria-induced expression of directly antimicrobial proteins and chemotactic cytokines may account for the protective function of γδ T cells during injury. In combination with prior studies showing that γδ IEL stimulate healing of damaged intestinal epithelia [4], my findings suggest a multifaceted role for γδ IEL in restoring homeostasis following mucosal damage. γδ, γδ IEL; TJ, tight junction; MLN, mesenteric lymph nodes.

Table 1:
γδIEL yields

colonization	genotype	treatment	% γδ IEL
conventional	wild-type	-DSS	5.7
conventional	wild-type	+DSS	4.5
germ-free	wild-type	-DSS	5.2
germ-free	wild-type	+DSS	5.6
conventional	$MyD88^{-/-}$	-DSS	5.8
conventional	$MyD88^{-/-}$	+DSS	5.8

Table 1: γδ IELs were isolated from pooled colons by flow cytometry as described in Materials and Methods. Yields of γδ IELs were calculated as a percentage of total intraepithelial lymphocytes.

Table 2:
Assessment of γδ IEL purity

	Cell type:	γδ T cell		αβ T cell	
	Affy ID:	1422188_s_at		1427653_at	
	transcript:	TCRγ		TCRα	
		signal	detection	signal	detection
γδ IEL prep	untreated 1	52644	Present	18	Absent
	untreated 2	68924	Present	31	Absent
	DSS-treated 1	35199	Present	11	Absent
	DSS-treated 2	34807	Present	23	Absent

	Cell type:	macrophage		epithelial cell	
	Affy ID:	1420393_at		1418368_at	
	transcript:	NOS2		RELMβ	
		signal	detection	signal	detection
γδ IEL prep	untreated 1	51	Absent	9	Absent
	untreated 2	18	Absent	11	Absent
	DSS-treated 1	13	Absent	162	Absent
	DSS-treated 2	22	Absent	48	Absent

Table 2: Affymetrix array datasets were queried for transcripts representative of γδ T cells, αβ T cells, macrophages, and epithelial cells. The signal intensity and absolute detection call given by the GeneChip software are shown.

TCRγ = T cell receptor γ; TCRα = T cell receptor α; NOS2 = nitric oxide synthase 2; RELMβ = resistin-like molecule β

CHAPTER FIVE
Results

ANTIBACTERIAL DEFENSE OF THE SMALL INTESTINE BY γδ INTRAEPITHELIAL LYMPHOCYTES

Introduction

The mammalian intestine has coevolved with a large and complex population of enteric bacteria. For the most part, mammals and their intestinal bacteria maintain a mutually beneficial relationship. However, the host is continuously charged with managing the threat of microbial invasion from the large bacterial communities at the intestinal mucosal interface. Despite this continuous threat, the host very rarely mounts strong inflammatory responses to intestinal bacteria, suggesting the evolution of strategies to limit bacterial incursions across the intestinal boundary.

γδ IEL comprise a major T cell population within the intraepithelial compartment of the intestine and are thought to maintain immunological competence in tissues where they are heavily represented. Although rare in the circulation, γδ T cells are prominent at intestinal surfaces where they display a number of characteristics that distinguish them from conventional T cells. These include the ability to secrete epithelial growth factors and to produce innate cytokines and chemokines in the recruitment of inflammatory cells. However, in general, their functions within the intestinal milieu are only starting to become better understood. γδ IELs are intercalated between epithelial cells underneath

the tight junctions, and as such, are in uniquely positioned to act as sentinels at the host-bacterial interface.

In previous studies, I showed that γδ IEL elaborate a complex response during mucosal damage [85]. In addition to increased expression of proinflammatory, cytoprotective, and antibacterial factors, γδ IEL provided early protection of the intestinal mucosa by limiting the numbers of opportunistically invading bacteria through damaged tissues. I reasoned that these γδ IEL damage responses were activated when commensal bacteria penetrated through damaged colonic epithelia, thus providing the first evidence of crosstalk between γδ IEL and commensal bacteria in driving wound healing responses. Surprisingly, these studies further revealed that γδ IEL were also responsive to intestinal microbiota in the absence of mucosal damage (Fig. 22). This suggested a novel role for γδ IEL in maintenance of host-microbial homeostasis in healthy, undamaged tissues.

While regulation of γδ IEL injury responses is now better understood, the mechanism driving γδ IEL responses to commensal microbiota in uninjured tissues remains unclear. Furthermore, the biological functions of γδ IEL in normal, healthy intestines are unknown. Previous work that addressed the regulation of γδ IEL damage responses was restricted to colonic epithelia because DSS exerts its damage exclusively in colonic mucosa [23]. However, given the experimental difficulties of working with colon γδ IEL, including the small numbers that could be isolated, I chose to elucidate the role of γδ IEL in uninjured epithelia of the small intestine. Previous studies have shown that while the numbers of γδ IEL from the colon and small intestine differ [65, 66], they retain similar phenotypes [86]. Therefore, the increased numbers of small intestinal γδ

IEL made this a more tractable system in which to study the mechanism and regulation of γδ IEL microflora responses in uninjured tissues.

In this chapter, I show that γδ IEL can respond to commensal bacteria in uninjured small intestinal tissues, and this response is exaggerated in a model of mucosal penetrance using *Salmonella typhimurium*. I show that γδ IEL responses to intestinal microflora are dynamic and reversible, disclosing a previously unappreciated role for γδ IEL in microbial surveillance at the mucosal surface. I additionally show that activation of γδ IEL antimicrobial responses by enteric bacteria occurs through activation of MyD88 in another intestinal cell population, revealing an indirect pathway of bacterial-γδ IEL cross-talk. Using mice that lack γδ T cells, I further reveal that γδ IEL are essential for controlling bacterial penetration of the intestinal barrier. These findings elucidate an important *in vivo* function of γδ IEL and yield important new insight into how the intestinal mucosal surface maintains homeostasis with intestinal microbiota.

Enteric bacteria activate dynamic and reversible expression of RegIIIγ in γδ IEL

As described in Chapter 3, I previously performed a genome-wide expression analysis to assess bacterial regulation of γδ IEL damage responses. This analysis revealed that bacteria orchestrate expression of a complex and multifaceted damage response in γδ IEL and suggests that these unconventional T cells are acutely responsive to bacterial signals during damage to the colonic mucosa. These studies further revealed, for the first time, that a component of the γδ IEL response was driven solely by

microflora, without detectable damage to the intestinal epithelium. γδ IEL from the colons of undamaged conventionalized mice displayed significantly enhanced expression of RegIIIγ, a potent antibacterial lectin that is expressed throughout the intestinal epithelium (Fig. 22). Consistent with the idea that bacteria modulate host responses in the absence of detectable epithelial damage, previous studies have shown that Paneth cells directly detect enteric bacteria to limit bacterial access to host tissues [22]. Since I observed increased microflora-dependent RegIIIγ in γδ IEL from undamaged colon tissues, I sought to assess whether intestinal microbiota activated a similar response in small intestinal γδ IEL.

RegIIIγ expression was assessed in populations of γδ IEL from germ-free and conventional mice, revealing significantly elevated transcript levels from uninjured conventional mice. γδ IEL isolated from the small intestines of conventional mice showed a 13-fold increase in RegIIIγ expression over their germ-free counterparts (Fig 23A). As previously noted, there were no differences in the numbers of γδ IEL that were recovered from the small intestines of germ-free and conventional mice [26] (Table 3). Intracellular flow cytometry further confirmed that γδ IEL from conventional mice produce increased quantities of RegIIIγ protein as compared to germ-free mice. The number of γδ IEL producing RegIIIγ increased 10-fold in conventional mice in agreement with the Q-PCR analysis (Fig 23B).

The observation that antimicrobial protein expression was induced in a limited subset of the cell population suggested that γδ IEL are likely transiently, and not constitutively, activated by intestinal microbiota. Therefore, I next assessed the dynamics

of RegIIIγ expression in γδ IEL. Treatment of mice with broad spectrum antibiotics revealed that RegIIIγ in γδ IEL was reversible after partial depletion of intestinal microbiota (Fig 24). Conversely, restitution of a complex microbiota in adult-germ free mice resulted in a 40-fold increase in RegIIIγ mRNA expression in γδ IEL (Fig 24). These results suggest that γδ IEL responses to enteric bacteria are dynamic and reversible. Furthermore, they indicate that the RegIIIγ defect in γδ IEL from germ-free mice does not arise from irreversible developmental defects, but is instead limited by the lack of microbial signals.

This microflora-driven response of γδ IEL is not isolated to RegIIIγ expression, but was instead representative of a global transcriptional response (Fig. 25A). Comparison of the transcriptional profiles from γδ IEL isolated from conventional and germ-free mice revealed that intestinal microbiota elicit complex changes in γδ IEL gene expression that includes 192 transcripts that were altered 2-fold or more. Unsupervised hierarchical gene clustering of these transcripts disclosed increased expression of a diverse group of factors involved in cytoprotection, bacterial recognition and antibacterial defense, as well as factors involved in immune activation (Fig. 25A). These included cytoprotective transcripts such as claudin-7 and Trefoil Factor-3 which support epithelial tight junctions and provide mucosal protection [87, 88]. Interestingly, expression of the RegIII C-type lectin family member, PAP, was also upregulated in γδ IEL from conventional mice (Fig. 25B). In good agreement with our prior studies, RegIIIγ was among the best represented transcripts in γδ IEL from conventional mice. These findings were substantiated by real-time quantitative PCR (Q-PCR) analysis of mRNAs from

independently isolated cell populations, suggesting that microflora provide necessary signals to γδ IEL, not only for maintenance of epithelial integrity, but also for activation of other protective immune responses (Fig. 25B).

These data suggest that γδ IEL are responsive to signals from the intestinal microbiota in healthy, uninjured tissues. They are further acutely responsive to shifts in the population of microbiota that would necessitate, or conversely, alleviate the need for RegIIIγ. Taken together, these data suggest a role for γδ IEL in monitoring shifts in intestinal ecology that might leave the intestinal interface vulnerable to the commensal microbiota of the intestine.

γδ IEL respond preferentially to mucosa-penetrant bacteria

I next investigated the nature of the bacterial signals detected by γδ IEL. I used gnotobiotic mice to address this question, as they allow us to define *in vivo* host responses to single bacterial species without interference from the diverse microbial societies that are normally present in the intestine. I first assessed the γδ IEL response to *Bacteroides thetaiotaomicron*, a normal member of the microbiota of mice and humans that remains exclusively within the lumen of the intestine and colonizes to very high levels [21]. 48 hours following oral inoculation of germ-free wild-type C57b/6 mice with 10^8 cfu of *B. thetaiotaomicron*, the small intestines were colonized to ~10^9 cfu/ml. Despite the high level to which *B. thetaiotaomicron* colonized the intestines of germ-free mice, γδ IEL from *B. thetaiotaomicron* monoassociated mice did not show elevated

expression of RegIIIγ compared to γδ IEL from germ-free mice (Fig. 26). This suggested that *B. thetaiotaomicron* alone is not sufficient to activate RegIIIγ expression in γδ IEL.

Since my findings showed that a complex microbiota elicited a dynamic and reversible response in γδ IEL, I next sought an explanation for why there were differences in the induction of RegIIIγ in γδ IEL between mice colonized with a full microbiota and those monoassociated with a symbiont that colonizes to high numbers. One hypothesis that might explain the relative inability of *B. thetaiotaoicron* to induce RegIIIγ expression in γδ IEL might stem from understanding that *B. thetaiotaomicron* predominantly remains sequestered within the lumen of the small intestine and does not closely associate with the intestinal epithelium in healthy mice [89]. Therefore, I addressed the possibility that γδ IEL preferentially respond to bacterial species that become closely associated with the intestinal mucosal surface.

Given the complexity of the normal intestinal microbiota, I reasoned that γδ IEL responses to the larger population of bacteria may be induced by members of the microbiota that gain direct access to host tissue. These enteric bacteria might provide an additional stimulatory signal to γδ IEL that *B. thetaiotaomicron*, which remains sequestered away from the mucosal lining of the intestine, might not deliver. The idea that members of the enteric microbiota possess adherent and invasive properties is plausible and has been postulated as one reason for chronic inflammation observed in IBD [90, 91].

Therefore, I next asked whether direct bacterial interaction with the intestinal epithelium would trigger enhanced γδ IEL antimicrobial expression. *Salmonella*

typhimurium interacts directly with gut epithelia as a first step in barrier translocation and systemic dissemination [92, 93], thus providing a tool to assess host responses after direct bacterial interaction with the intestinal epithelium. I compared γδ IEL cell responses to wild-type *S. typhimurium* and an isogenic, non-invasive mutant ΔSPI-1, which lacks the pathogenicity island SPI-1. The SPI-1 mutant is defective in its ability to invade epithelial tissues as it lacks genes that are required for attachment to and invasion of epithelial cells [93]. 25-fold fewer mutant than wild-type *Salmonella* were recovered from the spleens of infected mice, consistent with the epithelial interaction defect of SPI-1 [94]. Luminal colonization levels did not account for these differences, as total numbers of bacteria recovered from the small intestine were similar between the two strains (Fig. 27B). Wild-type *Salmonella* induced higher RegIIIγ mRNA levels in γδ IEL than did the invasion-defective ΔSPI-1 mutant, paralleling the increased mucosal invasiveness of this strain (Fig. 27A). Together with the *B. thetaiotaomicron* colonization results, these findings suggest that γδ IEL may respond preferentially to bacteria that invade the mucosal surface.

γδ IEL limit Salmonella penetration at early time-points following oral challenge

My previous findings have revealed that γδ IEL provide critical protection against opportunistically penetrating commensal bacteria immediately following intestinal epithelial damage. While studies have elucidated a role for γδ IEL in

maintaining epithelial tight junctions that prevent paracellular crossing of the intestinal epithelium [95, 96], it remains unclear whether γδ IEL provide direct antibacterial protection in uninjured intestinal epithelium. Given the robust RegIIIγ response in γδ IEL following infection with wild-type *S. typhimurium*, I reasoned that γδ IEL might limit *Salmonella* penetration and dissemination. To address this idea, I orally inoculated wild-type and TCRδ$^{-/-}$ mice with wild-type *S. typhimurium*. 48 hours post-challenge. I initially chose this time point as we have previously shown that epithelial cells provide protection against *Salmonella* dissemination after 48 hours [22]. Surprisingly, I detected no *Salmonella* in the spleens of a majority of both wild-type and TCRδ$^{-/-}$ mice, suggesting that the immune response in both strains was sufficient to resolve infection after 48 hours (Fig 28A).

In my previous studies of intestinal damage, I showed that γδ IEL provide protection at very early time-points following intestinal injury. Thus, I next reasoned that γδ IEL may limit bacterial dissemination specifically during early stages of *Salmonella* infection. Consistent with this idea, I recovered 100-fold more *Salmonella* from the spleens of TCRδ$^{-/-}$ mice as compared to wild-type mice 3 hours post-infection (Fig. 28B). The increased splenic *Salmonella* loads were not due to impaired bacterial killing in TCRδ$^{-/-}$ mice, as similar numbers of bacteria were recovered from the spleens of TCRδ$^{-/-}$ mice and wild-type mice following intraperitoneal injection of *Salmonella* (Fig. 28C). These findings indicate that γδ IEL are essential for limiting *Salmonella* penetration across the mucosal barrier and prevent systemic dissemination at very early time-points after oral challenge. These results are consistent with the idea that γδ IEL play a role in

early innate responses against bacteria that directly associate with and penetrate the intestinal epithelium.

γδ IEL detect bacteria through a cell non-autonomous, MyD88-dependent pathway

To delineate the host factors that govern expression of the RegIIIγ program in γδ IEL, I analyzed γδ IEL responses in mice that lack the innate immune adaptor MyD88 and are therefore deficient in the sensing of conserved microbe-associated patterns. As γδ IEL expression of RegIIIγ is acutely responsive to bacterial signals, and since previous work has found MyD88-dependent regulation of RegIIIγ in small intestinal tissues [22, 97], I asked whether small intestinal γδ IEL responses were also regulated through a similar pathway. Numbers of γδ IEL recovered from MyD88-deficient mice were similar to numbers recovered from wild-type mice (Table 3), in agreement with published data [75]. I detected significantly reduced expression of RegIIIγ in γδ IEL from conventionally-raised MyD88-deficient mice in contrast to conventional wild-type mice (Fig. 29A). Thus, RegIIIγ is induced in small intestinal γδ IEL through a MyD88-dependent signaling pathway.

In agreement with Q-PCR analysis, intracellular flow cytometry confirmed that γδ IEL from MyD88-deficient mice produced significantly reduced quantities of RegIIIγ as compared to wild-type γδ IEL (Fig 29B). Of interest, in our previous work, we found that colon γδ IEL do not exhibit a MyD88-dependent RegIIIγ response. In support of these seemingly disparate observations, previous work has shown that RegIIIγ expression

in both intact tissue and in epithelial cells is regulated through a MyD88-dependent pathway in the small intestine [97], but is driven through MyD88-independent IL-22 and IL-23 in the colon [98]. Differences in regulation of RegIIIγ in γδ IEL from the small intestine and the colon support the idea that γδ IEL function is likely dictated by their physical microenvironment [99].

The observation that RegIIIγ in small intestinal γδ IEL is driven through a MyD88-dependent pathway suggested a role for innate pattern recognition in γδ IEL responses to intestinal microbiota. In order to delineate the mechanism of bacterial sensing by γδ IEL, I asked whether γδ IEL responses to intestinal microbiota are elicited by direct interactions between bacteria and γδ IEL, or whether other cells detect bacteria and then shape γδ IEL responses through indirect mechanisms of cell-cell communication. Several studies support both models of γδ T cell activation. On one hand, there are studies showing that circulating γδ T cells can respond directly to bacterial products [81]. On the other hand, separate studies have revealed that epidermal γδ T cells respond to antigen expressed by stressed keratinocytes [51], suggesting an indirect detection mechanism.

To address whether small intestinal γδ IEL directly detect bacteria through cell-autonomous MyD88 signaling, I performed bone marrow transplantation studies and took advantage of the observation that the intestine is partially radioresistant and retains populations of residual recipient cells after lethal irradiation [100] (Fig. 30A, Table 4). Consistent with this report, I noted incomplete intestinal reconstitution after transfer of MyD88-deficient bone marrow into lethally irradiated wild-type hosts. This could not be

explained by experimental error as I observed complete immune reconstitution of the periphery (Table 4). As a result, lethally irradiated wild-type hosts fostered a population of transplanted MyD88$^{-/-}$ γδ IEL but retained a population of residual wild-type γδ IEL.

These chimeric mice thus provided a unique tool to assess the role of cell-intrinsic MyD88 in bacterial detection by γδ IEL, since RegIIIγ expression could be assessed in populations of co-housed MyD88$^{-/-}$ and wild-type γδ IEL. One possibility was that γδ IEL directly detect bacteria through cell-intrinsic MyD88 signaling. In this case, donor MyD88$^{-/-}$ γδ IEL would be expected to display significantly reduced levels of RegIIIγ as compared to wild-type recipient γδ IEL. Alternatively, γδ IEL might indirectly detect bacterial signals through cell-cell communication with other intestinal cells. In such a scenario, γδ IEL cell-intrinsic MyD88 would be dispensable for bacterial detection, and thus γδ IEL would be expected to express RegIIIγ irrespective of MyD88.

Consistent with the model of indirect bacterial detection, I found that adoptive transfer of MyD88$^{-/-}$ bone marrow into lethally irradiated wild-type mice restored expression of RegIIIγ in MyD88$^{-/-}$ γδ IEL. Donor and residual recipient γδ IEL were separated based on Ly5.1/5.2 lineage markers and Q-PCR analysis of mRNA revealed a 30-50 fold increase in RegIIIγ expression in γδ IEL from MyD88$^{-/-}$ donor mice when housed in a conventional (i.e., specific pathogen-free) environment (Fig. 30). Unfortunately, the complement chimeras assessing RegIIIγ in wild-type γδ IEL transplanted into MyD88$^{-/-}$ recipients could not be assessed because γ-irradiation resulted in death of γδ IEL from MyD88$^{-/-}$ recipient mice. Despite this technical limitation, my results demonstrate that direct MyD88 signaling in γδ IEL does not drive RegIIIγ,

suggesting that γδ IEL instead respond to intestinal microflora through indirect signals received from other intestinal cells.

An important question that this study leaves unanswered is whether these signals are derived from non-hematopoeitic cell lineages such as epithelial cells, or from residual hematopoetic cells such as macrophages or dendritic cells [23, 101], which directly sense bacteria through MyD88. Most importantly, our results reveal an intimate collaboration between γδ IEL and other intestinal populations which engage in a dialog with the intestinal microflora. This collaboration adds another critical level of regulation that likely minimizes inappropriate immune activation while promoting host-microbial homeostasis at the intestinal interface.

Discussion

The intestinal epithelium is the primary barrier between the vast enteric microbial community and internal host tissues. It is now clear that specialized epithelial cells regulate intestinal homeostasis through direct recognition of bacteria and through induction of complex antibacterial programs [22]. However, little is known about how members of the intraepithelial niche regulate interactions with intestinal microflora. In this study I gained new insight into this question by examining γδ IEL, which constitute a major intestinal T cell population. While γδ IEL have previously been shown to play a multifaceted role in restoring homeostasis after epithelial damage [4, 85], their role in protection of undamaged tissues has remained unclear.

By analyzing γδ IEL in germ-free mice, I have discovered that commensal bacteria provide critical regulatory input to γδ IEL, even in the absence of damage to the host-bacterial interface. In these studies, I followed RegIIIγ expression in γδ IEL, and have used this response to gain insight into how γδ IEL respond to intestinal microbiota. My results reveal that γδ IEL respond to commensal microbes in a dynamic and reversible manner and can be restored in adult germ-free mice reconstituted with a conventional microbiota or reversed in microflora-depleted conventional mice. These results demonstrate that in addition to triggering expression of antibacterial factors in epithelial cells [20-22, 102], commensal bacteria also elicit antibacterial responses in a mucosal T cell population.

While γδ IEL are responsive to the larger complex population of enteric bacteria, they do not appear to be activated by bacterial species such as *Bacteroides thetaiotaomicron* that are non-invasive [89]. However, by using *Salmonella* infections as a model of intestinal penetration [93, 94], I show that γδ IEL responses are preferentially triggered by invasive bacteria (fig. 31). Additionally, γδ T cells are essential for limiting translocation and dissemination of *Salmonella* across the mucosal barrier during the first few hours after oral challenge. This establishes their role in early immunity against bacterial invasion of the intestinal interface. Despite robust RegIIIγ expression in γδ IEL after oral infection with invasive bacteria, it remains to be determined experimentally whether the regulated production of RegIIIγ could account for the antibacterial function of γδ IEL. Given the difficulties in experimentally manipulating γδ IEL *in vitro*, such

studies will require development of animal models that allow cell-specific manipulation of RegIIIγ in γδ IEL.

While the *Salmonella* oral infection studies provided a model suggesting that γδ IEL preferentially respond to mucosa-adherent and -invasive bacteria, these studies did not exclude the possibility that the *Salmonella* infections altered the sensitivity of γδ IEL to microbial stimulation. Therefore, it will be important to determine whether mucosa-penetrating enteric bacteria similarly trigger enhanced responses in γδ IEL. In support of this idea, the responsiveness of γδ IEL to the complex population of enteric microbiota suggests that there are members of this population which opportunistically cross the mucosal barrier [90, 103], even in the absence of overt intestinal damage. In combination with my previous finding that γδ IEL limit opportunistic penetration of commensal bacteria through damaged mucosa [85], my findings are consistent with a model in which γδ IEL promote intestinal homeostasis by specifically targeting bacteria that cross the mucosal barrier.

Having determined that γδ IEL responses can be induced by indigenous microbiota in healthy intestinal tissues, I next delineated a mechanism for how these interactions were mediated. Bacterial signaling through the Toll-like receptor adaptor protein MyD88 is critical for maintaining mucosal homeostasis and limiting penetration of commensal bacteria and pathogens across the epithelial barrier [22]. Likewise, here I have shown that a component of the γδ IEL response to intestinal microbiota is also governed by bacterial signaling through a MyD88-dependent pathway. However, cell-autonomous expression of MyD88 on γδ IEL was not required for elaboration of these

responses, suggesting that interactions between γδ IEL and other intestinal cells govern γδ IEL responses to intestinal microbiota (fig. 31). This supports previous studies revealing that innate recognition of intestinal microbes is a complex process involving interaction and communication between multiple cell types [23]. More importantly, it suggests a role for innate pattern recognition in activating γδ IEL responses to intestinal microbes.

A key question that remains from these studies is the cellular origin of the MyD88-dependent responses driving γδ IEL responses to bacteria (fig. 31). A plausible source of these indirect conversations might be enterocytes or other specialized lineages, such as the Paneth cell, which directly detect bacteria *in vivo* [22]. Alternatively, these signals could also be derived from residual hematopoetic cells such as macrophages and dendritic cells, which directly sense bacteria through MyD88. There are studies that provide evidence for both models. First, bone marrow chimera experiments show that epithelial expression of RegIIIγ does not require MyD88 expression in hematopoietic lineages [97], supporting the idea that non-hematopoietic cells provide the signals necessary for γδ IEL RegIIIγ production. On the other hand, other studies have provided evidence of intestinal cell crosstalk after hematopoietic immune cell detection of bacteria through TLR-dependent MyD88 signaling [23].

In addition to its role in TLR signaling, MyD88 also plays a critical role in the IL-1 receptor (IL-1R), and the IL-18 Receptor (IL-18R) signaling pathways [104]. Therefore, it will be important to distinguish whether the non-cell autonomous MyD88 signaling that drives small intestinal γδ IEL RegIIIγ arises from direct recognition of

bacteria through TLRs, or through IL-1/IL-18 signaling. First, the possibility that IL-1R or IL-18R might regulate RegIIIγ in γδ IEL will be addressed by analyzing Caspase-$1^{-/-}$ mice, which are unable to produce active forms of IL-1β and IL-18 [105]. Alternatively, analysis of RegIIIγ in γδ IEL from TLR-deficient mice will address whether TLRs might be involved in regulating the indirect conversations that activate γδ IEL. In support of this possibility, recent findings have suggested that TLRs, rather than IL-1R or IL-18R, direct expression of the MyD88-dependent antimicrobial response [22]. While the exact identity of these TLRs has yet to be identified, various TLRs including TLR2, 4, 5, and 9 are detected in intestinal tissues [106].

Another question that remains is the molecular nature of the signal(s) that alerts γδ IEL about the presence of intestinal bacteria. While data presented here effectively rule out the possibility that γδ IEL directly sense bacteria through cell-autonomous TLR/MyD88 signaling, they do not address how γδ IEL receive this information. One logical possibility is that γδ IEL receive information about microbial threats through a mechanism involving the T cell receptor. This would be consistent with the model showing a keratinocyte-responsive γδ TCR [51, 54]. Another possibility is that γδ IEL are activated through other unconventional activation pathways that bypass the TCR. Consistent with this idea, previous studies have shown that γδ IEL show preferential and robust expansion in response to soluble factors such as IL-15 and IL-2 [107-109]. Whether γδ IEL are directly activated through their TCR, or whether they require activation by supplemental soluble factors, these studies support the idea that γδ IEL

participate in critical collaborations with neighboring cells to provide antibacterial protection to the host.

In summary, these findings suggest a model in which γδ IEL defend epithelial surfaces against mucosal penetration of bacteria. In principle, such a role makes sense since they would be well-equipped to mount an immediate response to invading bacteria while recruiting other immune cells. Additionally, these findings further highlight the elaborate and critical collaborative network between cell lineages of the intestine in maintaining beneficial relationships with luminal microbes. These relationships are essential for a healthy intestinal environment, as recent work suggests that inflammatory disorders of the intestine likely arise through dysregulated responses to the microbial milieu of the intestine[59, 110]. The fact that indigenous gut microbiota induce alterations in γδ IEL gene expression suggests that bacteria-lymphocyte cross-talk plays a critical role in shaping mucosal biology.

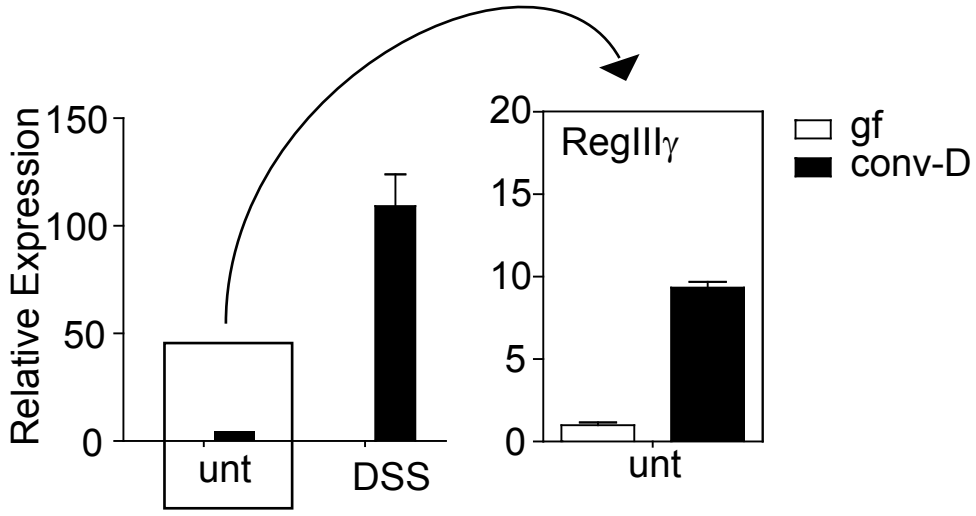

Figure 22: Commensal bacteria direct RegIIIγ in γδ IEL from undamaged colon. Expression of the antibacterial lectin RegIIIγ was quantitated by Q-PCR of amplified γδ IEL mRNAs from untreated and DSS-treated mice. Q-PCR analysis revealed increased expression of RegIIIγ in γδ IEL after conventionalization of germ-free mice, even in the absence of epithelial damage. All Q-PCR assays were performed on γδ IEL isolated from pooled colons (n>5 mice), run in triplicate, and are shown as mean values normalized to GAPDH. Relative expression levels were calculated in relation to untreated gf samples. Results are representative of two independent experiments. Error bars, ±SEM. conv-D, conventionalized for 72 hours; gf, germ-free. Unt, untreated; conv-D, conventionalized for 72 hours; gf, germ-free. From Ismail et al, 2009 [85].

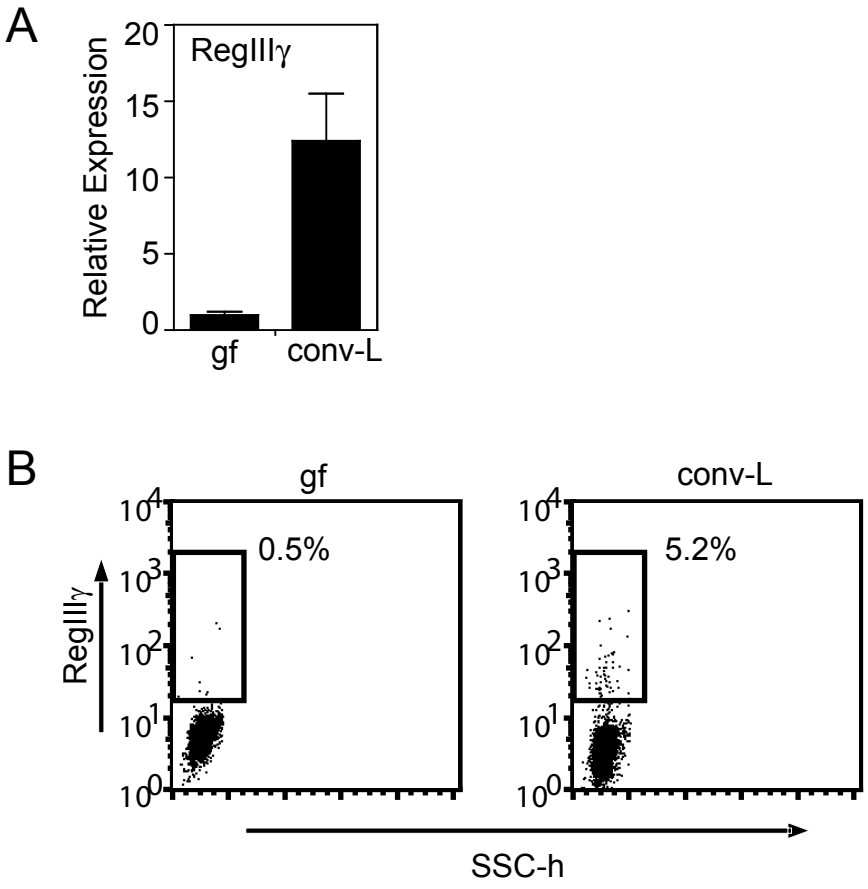

Figure 23: RegIIIγ protein production in γδ IEL is regulated by the microbiota. RNA was isolated from sorted γδ IEL, and Q-PCR analysis was performed to quantitate expression of RegIIIγ. Assays were performed on small intestinal γδ IEL (n=5 mice), run in triplicate, and are shown as mean values normalized to 18S rRNA. Relative expression levels were calculated in relation to gf samples. Error bars, ±SEM. Flow cytometry was performed on total IEL populations germ-free and conventional mice. Intracellular staining was carried out with antibodies directed against RegIIIγ, as well as a control rabbit polyclonal antibody. Gated γδ IEL populations are shown, and percentages of the gated populations are given. SSC, side scatter, gf, germ-free; conv-L, conventional.

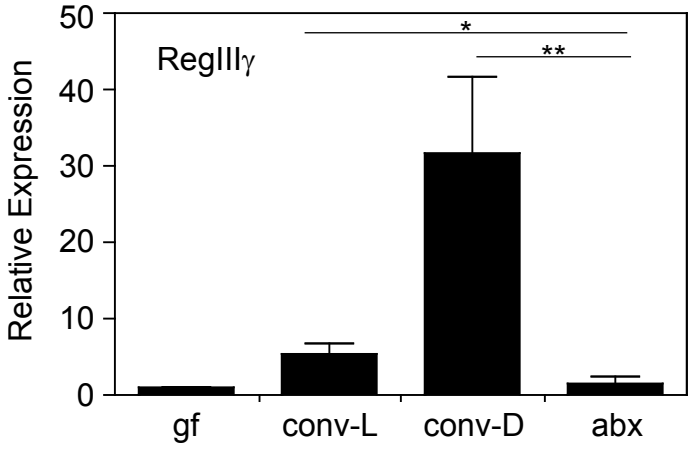

Figure 24: γδ IEL exhibit a dynamic and reversible response to enteric microbiota. Q-PCR analysis was performed to quantitate expression of RegIIIγ in γδ IEL as described in Figure 22A. Assays were performed in triplicate, and are shown as mean values normalized to 18S rRNA. Relative expression levels were calculated in relation to germ-free samples. Error bars, ±SEM. Gf, germ-free; conv-L, conventional; conv-D, conventionalized for 72 hours; abx, antibiotic-treated. * p=0.05, ** p=0.01.

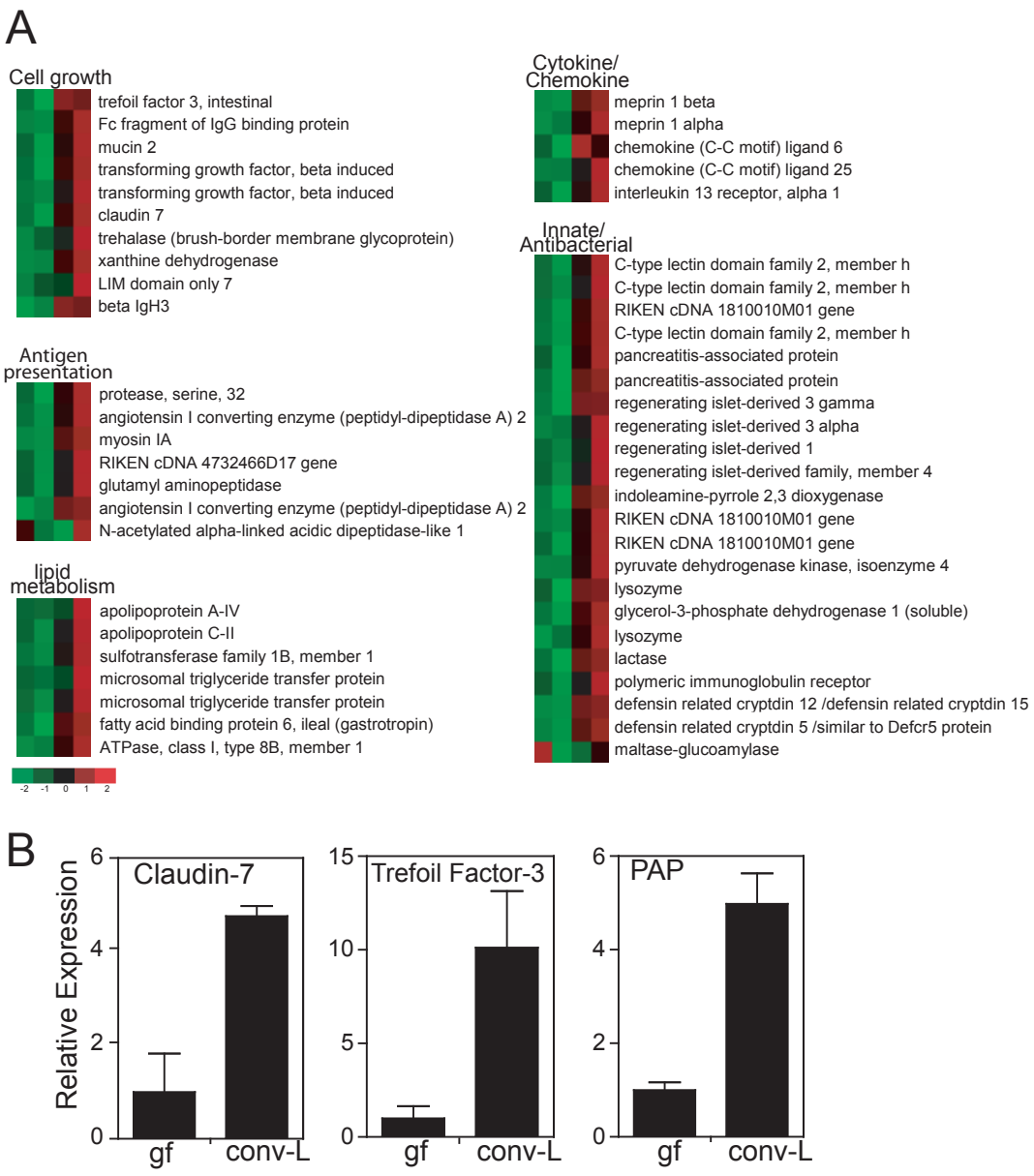

Figure 25: Intestinal microbiota elicit a complex γδ IEL response. (A) Affymetrix Mouse Genome 430 2.0 arrays were used to compare transcript abundance between γδ IEL from germ-free and conventional small intestines. Differentially expressed transcripts were identified as outlined in Materials and Methods, revealing 192 transcriptional changes between the germ-free and conventional groups. Key functional groups were delineated using Gene Ontology (GO) terminology, and are displayed as heatmaps in which expression level is defined by Z-score (defined in Materials and Methods). **(B)** Quantitative Real-Time PCR validation of cytoprotective/antibacterial responses in separate populations of γδ IEL as described previously in Fig. 23.

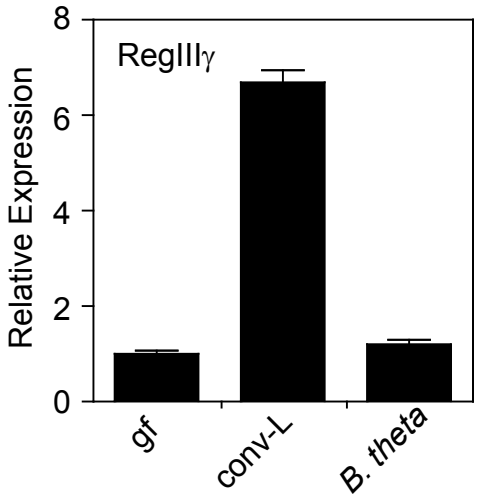

Figure 26: *Bacteroides thetaiotaomicron* does not elicit RegIIIγ expression in small intestinal γδ IEL. Germ-free C57BL/6 wild-type (wt) mice were orally inoculated with 10^8cfu of *B. thetaiotaomicron* (*B. theta*; n=4 mice) and sacrificed after 48 hours. (A) Q-PCR for RegIIIγ in γδ IEL isolated from *B. thetaiotaomicron* monocolonized, germ-free, and conventional mice. Assays were performed as described in Figure 23. Relative expression levels were calculated in relation to germ-free samples. Error bars, ±SEM.

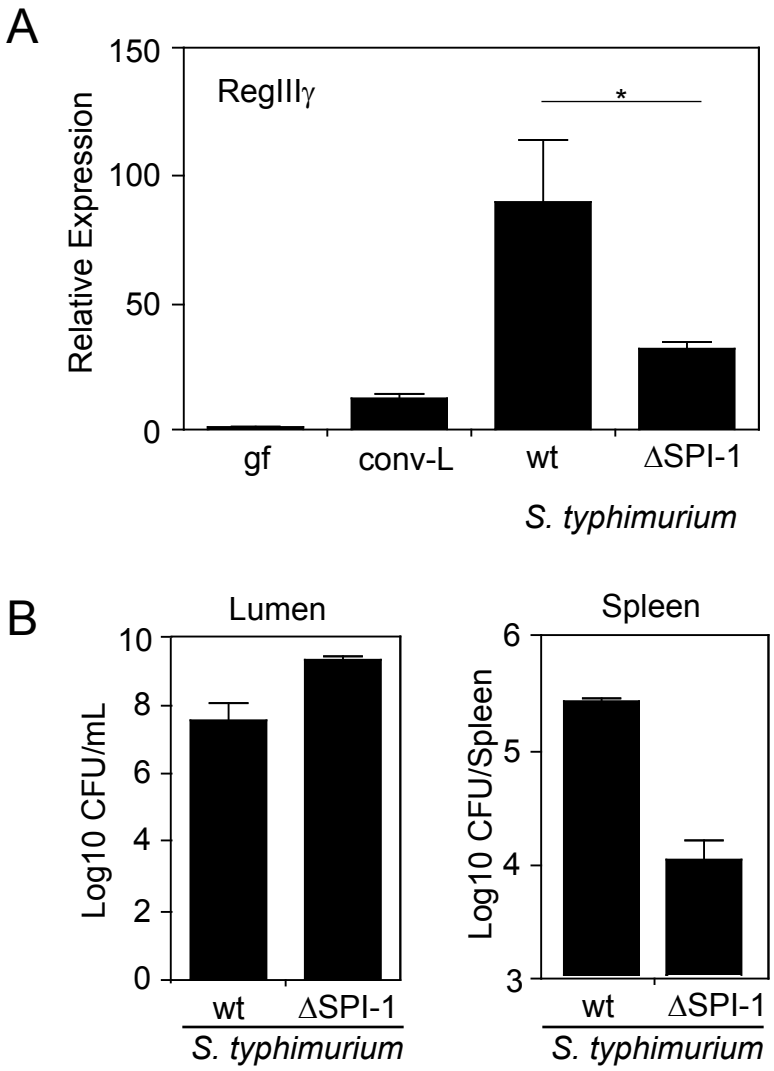

Figure 27: Invasive *Salmonella typhimurium* elicits RegIIIγ expression in small intestinal γδ IEL. 10^8 cfu of wild-type *S. typhimurium* (wt *St*) or the isogenic mutant ΔSPI-1 were introduced orally into germ-free wild-type C57BL/6 mice for 48 hours. **(A)** Q-PCR for RegIIIγ in γδ IEL isolated from wt *St*, ΔSPI-1, germ-free, and conventional mice. Assays were performed as described in Figure 23. Relative expression levels were calculated in relation to germ-free samples. **(B)** Quantitation of bacterial numbers in the lumen and spleens of infected mice n=3-4 mice/group, error bars, ±SEM. *, $P<0.05$.

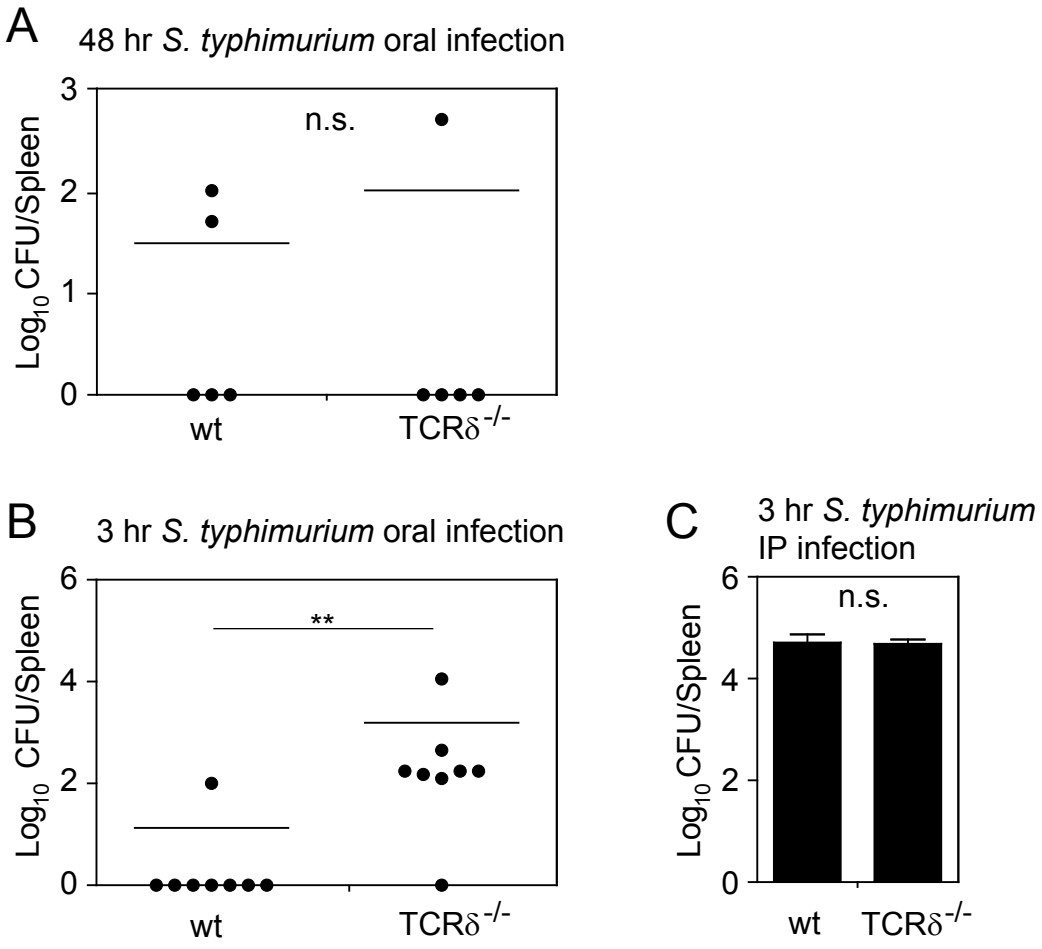

Figure 28: γδ IEL limit *Salmonella* translocation and dissemination. 10^8 CFU of wild-type *S. typhimurium* (wt *St*) were introduced orally into conventional wild-type and TCRδ$^{-/-}$ C57BL/6 mice. **(A)** Quantitation of bacterial numbers disseminated to the spleen after a 48 hour oral infection **(B)** 3 hour oral infection **(C)** 3 hour intraperitoneal infection was performed. n=3-8 mice/group, error bars, ±SEM. **, P<0.01. n.s., no significance.

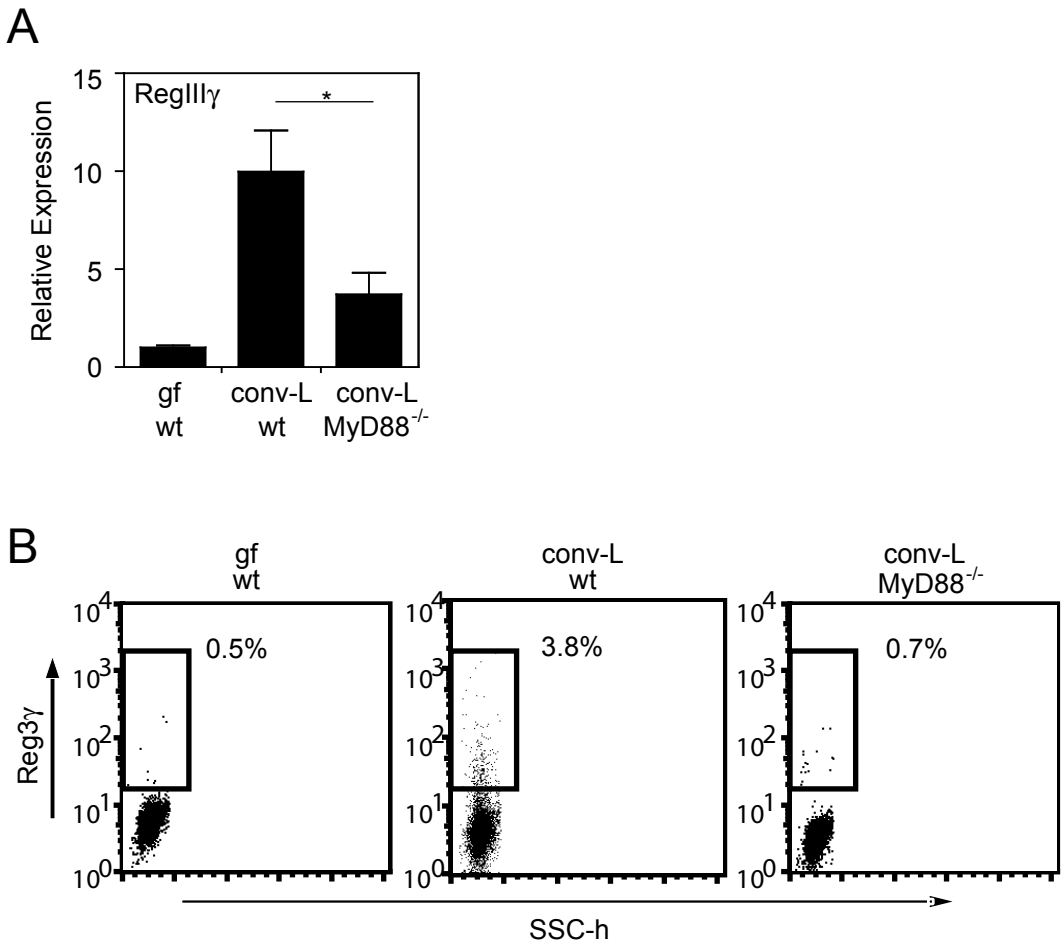

Figure 29: Expression of RegIIIγ in small intestinal γδ IEL is regulated by MyD88. RegIIIγ was quantitated by **(A)** Quantitative real-time quantitative PCR analysis (Q-PCR) of sorted small intestinal γδ IEL and **(B) f**low cytometry performed on total IEL populations. Intracellular staining was carried out with antibodies directed against RegIIIγ, as well as a control rabbit polyclonal antibody. Gated γδ IEL populations are shown, and percentages of the gated populations are given. SSC, side scatter, gf wt, germ-free wild-type; conv-L wt, conventional wild-type; conv-L MyD88$^{-/-}$, conventional MyD88$^{-/-}$

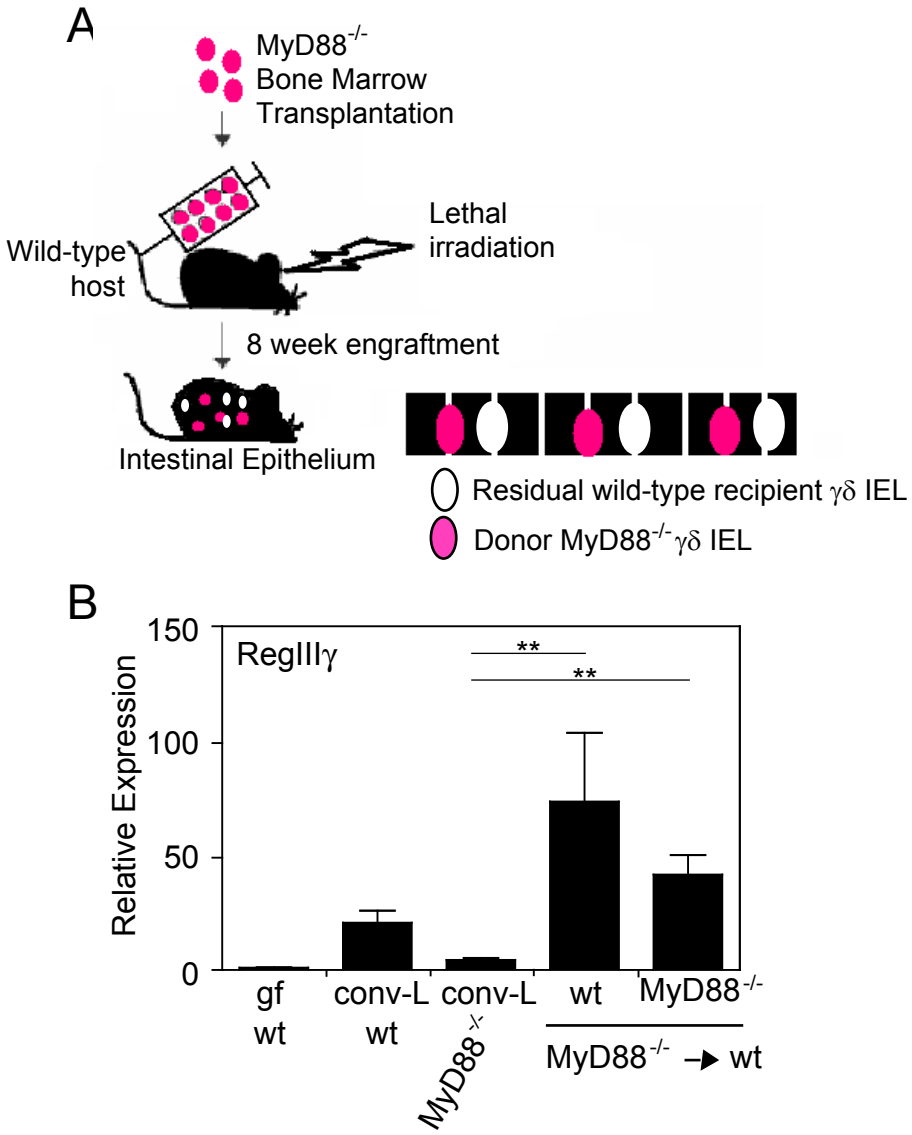

Figure 30: Cell-autonomous MyD88 signaling is not required for γδ IEL RegIIIγ expression. **(A)** Schematic of experimental strategy. Ly5.1 wild-type recipient mice were γ-irradiated 9 Gy and reconstituted with $5x10^6$ bone marrow cells from Ly5.2 donor $Myd88^{-/-}$ mice (6-8 weeks of age). Analysis of bone marrow chimeras confirmed that the intestine retains populations of residual recipient cells while also fostering transplanted cells (Table 4). **(B)** At 8 weeks after reconstitution, mice were sacrificed and Ly5.1 and Ly5.2 γδ IEL were isolated by FACS sorting. These separate populations were analyzed for expression of RegIIIγ by Q-PCR. Relative expression levels were calculated in relation to germ-free samples as described previously. Gf, germ-free; conv-L wt, conventional wild-type. Error bars, ±SEM.

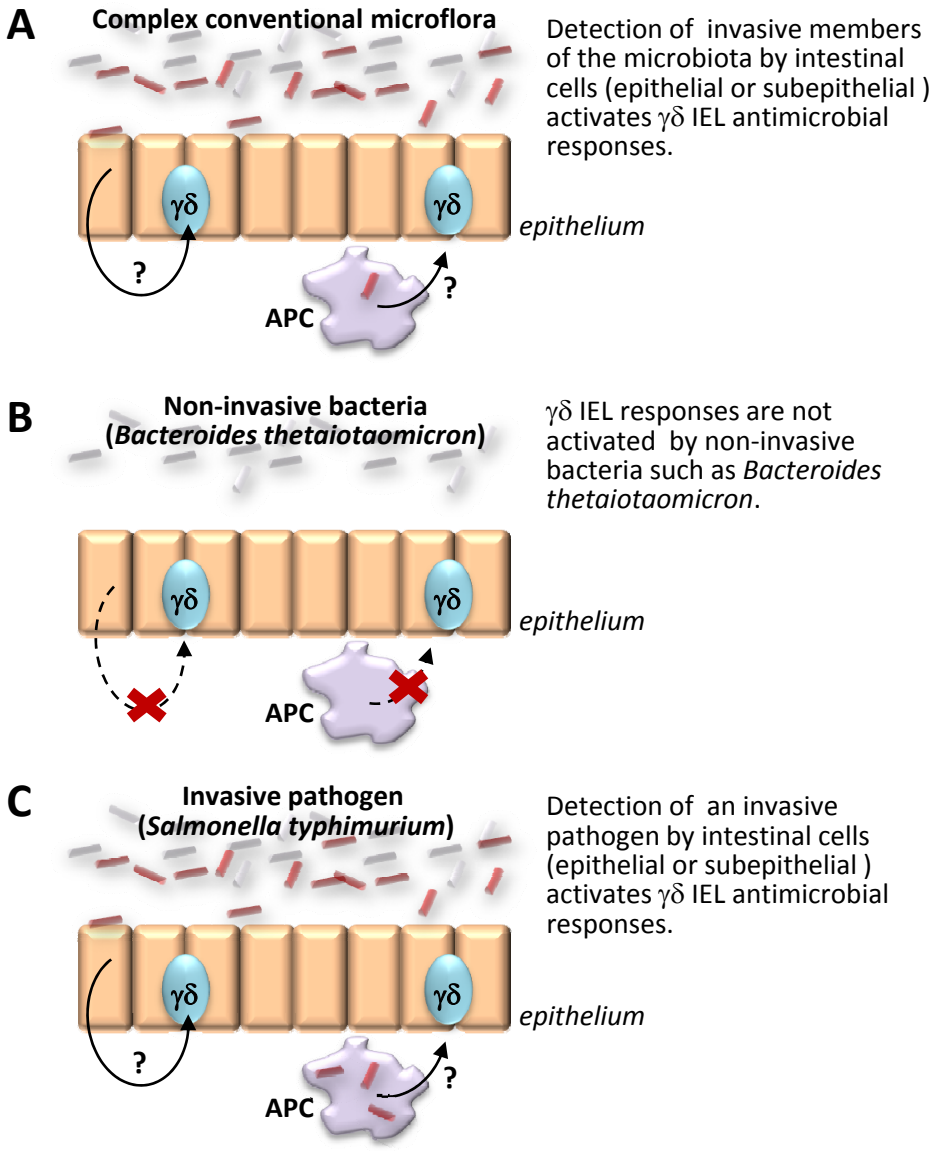

Figure 31: Indirect interactions between enteric bacteria and γδ IEL.
γδ IEL are situated between epithelial cells on the basolateral side of tight junctions which restrict paracellular penetration of luminal bacteria.
A) Exposure to a complex intestinal microbiota activates γδ IEL RegIIIγ expression. These responses were **B)** absent when mice were colonized with a non-invasive bacterial species, *Bacteroides thetaiotaomicron* and were **C)** triggered when exposed to an invasive species such as *Salmonella typhimurium.* It is not yet clear whether epithelial cells or subepithelial immune cells such as dendritic cells, macrophages, B cells, or other T cells stimulate γδ IEL responses. I propose that bacteria-induced expression of directly antimicrobial proteins may account for the protective function of γδ T cells against invasive enteric bacteria. γδ, γδ IEL.

γδ IEL recovery from small intestinal tissues

colonization	genotype	% γδ IEL
conventional	wild-type	3.6
germ-free	wild-type	3.8
conventionally-raised	$MyD88^{-/-}$	3.4
conventionalized	wild-type	4.0

Table 3: γδ IELs were isolated from small intestines by flow cytometry as described in Materials and Methods. Yields of γδ IELs were calculated as a percentage of total intraepithelial lymphocytes.

% Bone Marrow Transplantation Efficiency

Source	Intestine	Periphery
Transplanted donor MyD88$^{-/-}$ Bone Marrow	40.4 +/- 10.3	86.2 +/- 6.9
Residual recipient Wild-type Bone Marrow	59.6 +/- 10.3	13.8 +/- 6.9

Table 4: Transplantation efficiency in the periphery and intestines.

Determination of bone marrow transplantation efficiency in lethally irradiated wild-type C57/b6 mice transplanted with $5x10^6$ MyD88$^{-/-}$ bone marrow cells. Peripheral chimerism was determined 4 weeks post-transplantation FACS analysis of blood leukocytes with antibodies against ly5.1/ly5.2 markers. Intestinal chimerism was determined 8 weeks post-transplant upon sacrifice of mice for γδ IEL acquisition.

TABLE 5: QUANTITATIVE PCR PRIMER SEQUENCES

gene	forward primer sequence	reverse primer sequence
GAPDH	5'-TGGCAAAGTGGAGATTGTTGCC	5'-AAGATGGTGATGGGCTTCCCG
βig-h3	5'-CGAAACCGACATCATGGCCACAAA	5'-TGGAATACGCTGACGCCTGTTTGA
PAP	5'-TACTGCCTTAGACCGTGCTTTCTG	5'-GACATAGGGCAACTTCACCTCACA
RegIIIγ	5'-TTCCTGTCCTCCATGATCAAAA	5'-CATCCACCTCTGTTGGGTTCA
lysozyme	5'-ATGCCTGTGGGATCAATTGCAGTG	5'-TCTCTCACCACCCTCTTTGCACAT
IL-1β	5'-TGGTACATCAGCACCTCACAAGCA	5'-AGGCATTAGAAACAGTCCAGCCCA
CXCL-9	5'-TCAGATCTGGGCAAGTGTCCCTTT	5'-TGAGGTCTATCTAGCTCACCAGCA
MIP2α	5'-GCAGTATTCCTTGGCTGGCCATTT	5'-ATTCTTCCTACACCGGCATGACCT
KC	5'-TGTGTGGGAGGCTGTGTTTGTATG	5'-AATGTCCAAGGGAAGCGTCAACAC
18S	5'-CATTCGAACGTCTGCCCTATC	5'-CCTGCTGCCTTCCTTGGA
Trefoil Factor-3	5'-TGGCCTGTCTCCAAGCCAATGTAT	5'-TGCATTCTGTCTCCTGCAGAGGTT
Claudin-7	5'-TGCCTTGGTAGCATGTTCCTGGAT	5'-AGCCGATAAAGATGGCAGGTCCAA

CHAPTER SIX
Conclusion

Recent work has focused on resolving how the host maintains beneficial relations with its microbial neighbors. A question that has driven the field of mucosal immunology has been how mammals can maintain vast consortia of intestinal microbes without becoming diseased. Key evidence suggests that epithelial cells provide critical protection of the host by detecting bacteria and limiting microbial access to host tissues through strict compartmentalization of microbes within the lumen of the gut [80, 111]. These studies have provided insight into the mechanisms of bacterial detection and host responses in limiting opportunistic penetration of commensal bacteria into host tissues.

While γδ IEL constitute a major intestinal T cell population, their exact biological functions have remained unclear. γδ IEL exhibit a number of unique characteristics that suggest that they play a role in innate immune defense. These include a rapid response that is not restricted by MHC [112], the ability to present foreign antigens to cognate αβ T cells [35, 36], and the ability to directly kill infected host cells [113]. In combination with the fact that they are heavily represented in bacteria-colonized tissues, these characteristics suggested that γδ T cells might be well-adapted to provide antibacterial defense at the intestinal epithelial surface.

The underlying goal of this thesis was to understand the role of γδ IEL in maintaining homeostasis with symbiotic intestinal microbes and in protecting against bacterial pathogens. Having co-evolved with resident bacteria, it seemed likely that γδ

IEL might depend on interactions with the intestinal microbiota for many aspects of their development and function. Nevertheless, little was known about the extent to which γδ IEL biology would require microbial signals. In addition to identifying γδ IEL responses requiring microbial input, my *in vivo* work reveals a novel role for γδ IEL in direct antibacterial defense of the intestinal epithelium. Further investigations into how mucosal T cell populations of the gut respond to luminal microbes would, therefore, lend insights into the defenses employed by the host in maintaining homeostasis in the intestine.

γδ IEL responses to microbial signals

The study of intestinal γδ IEL has been slow due to experimental challenges that do not exist for other T cell populations [64]. Though insights into the characteristics of γδ IEL relative to other T cell populations have been obtained through functional genomic studies of γδ IEL isolated from the small intestine [30, 31], the functional relevance of these responses is still not well understood. Given their predominance in mucosal tissues, the idea that γδ T cells might provide protection against bacteria has previously been proposed. A number of studies have revealed that circulating γδ T cells are responsive to microbial patterns as reflected by enhanced chemokine expression [81, 114]. Additionally, some studies disclosed that γδ T cells play a role in immunity against parasitic infections [95, 115], yet other challenge experiments suggested that pathogenic bacteria do not trigger significant changes in γδ IEL gene expression [30]. As a result,

the role of γδ IEL in antibacterial defense at mucosal surfaces has remained unclear. My work directly addresses the role of γδ IEL at the host-mucosal interface, revealing that γδ IEL play essential roles during and after mucosal damage to limit opportunistically penetrant bacteria. My work also shows that γδ IEL prevent dissemination of invasive bacteria such as the oral pathogen, *Salmonella typhimurium*, in otherwise healthy tissues. Therefore, I show the first clear evidence that γδ IEL promote host-microbial homeostasis by providing critical antibacterial defense of the intestine.

By combining studies in germ-free mice with functional genomics screens, I have now obtained new insights into the biology of γδ IEL at the intestinal interface [85]. These studies have clarified the role of γδ IEL at the intestinal interface and have revealed that commensal bacteria provide critical regulatory input to γδ IEL biology. This is the first report of such a dialog between a T cell and a microbe population. Because of their unconventional characteristics, γδ T cells are often considered to be part of both the innate and adaptive immune systems. The functional genomics studies undertaken here provide the first comprehensive insight into the extent to which γδ IEL biology is directed by microbial stimuli and how these responses could, indeed, bridge innate and adaptive responses.

Though other genomics screens have revealed that γδ T cells mount a proinflammatory response after *ex vivo* stimulation with MAMPs [114], the studies described in my work further disclose a tight and regulated relationship between γδ IEL and intestinal microflora *in vivo*. These responses include innate immune responses that are not typically associated with other T cell populations, establishing the unconventional

properties of γδ IELs. Taken together, my studies reveal for the first time that γδ IEL express a multifaceted response to intestinal microbiota in order to maintain intestinal epithelial integrity and provide critical protection during periods of increased intestinal vulnerability.

The role of γδ IEL in during mucosal damage

Prior studies established that γδ IEL play an essential role in restoring homeostasis following acute epithelial injury [4, 51, 116]. In my thesis, I set up a model of colonic damage using Dextran Sulfate Sodium (DSS) to gain a global molecular insight into the γδ IEL injury response. These studies revealed that DSS-induced colonic injury activates complex γδ IEL responses that taken together, orchestrate various aspects of antibacterial defense.

By producing proteins that directly target invading bacteria, while simultaneously initiating a secondary line of defense through recruitment of additional immune cells, γδ IEL play an essential role in limiting bacterial penetration through injured host tissues. As this occurs concomitantly with expression of factors that stimulate epithelial repair [4], my findings suggest that γδ IEL function to coordinate multiple responses that restore epithelial integrity, provide antibacterial defense, and restore host-microbial homeostasis following intestinal damage. While γδ IEL in healthy, intact epithelia have limited contact with commensal populations that are normally confined to the gut lumen, they are strategically situated to detect penetration of bacteria through damaged epithelia.

Supporting the idea that γδ IEL play a sentinel antibacterial role at injured epithelia, I found increased opportunistic penetration of commensals through injured tissues in DSS-treated mice lacking γδ T cells.

The transcriptional profile of γδ IEL in response to intestinal microbiota suggested that γδ IEL play a role in antibacterial defense of the intestine. However, the direct role of microbe-induced factors in γδ IEL still remains unclear. My transcriptional studies have clearly linked proinflammatory, antibacterial, and cytoprotective responses in γδ IEL with the presence of an indigenous microflora. However, the exact role that these factors play in γδ IEL regulation of intestinal homeostasis at the host-microbial interface is not well-characterized. For example, while *in vitro* studies have revealed a clear role for RegIIIγ in antibacterial defense against gram-positive organisms [20, 117], ongoing studies in mice lacking RegIIIγ expression will delineate the exact *in vivo* role of this protein in promoting intestinal homeostasis. Though previous studies have shown that γδ T cells recruit macrophages to areas of skin damage through an indirect signals [84], the role of the microbe-directed chemotactic program in γδ IEL following intestinal damage has not been directly addressed. Therefore, gene-targeted mouse models will be essential in delineating the role of these factors specifically in the γδ IEL compartment. Nevertheless, my studies highlight the plasticity of the γδ IEL response to intestinal microbiota and reveal the potentially complex conversations in which γδ IEL might be involved to mediate antibacterial defenses at the intestinal interface.

γδ IEL contributions to homeostasis in healthy intestinal tissues

While previous studies established the role for γδ IEL in damaged intestinal epithelia, I hypothesized that they might also play a critical role in the biology of a healthy, undamaged, intestinal epithelium. The intestinal interface is under constant stress from microbial insult, and given the vulnerable nature of the intestinal epithelium, it could be reasonably expected that γδ IEL might play a similar role in promoting integrity of the epithelium. Therefore, I tested the hypothesis that γδ IEL function to protect undamaged intestinal tissues.

While direct damage to the intestine provides a convenient route of entry for enteric bacteria, it is not the only means by which commensal bacteria can gain access to deeper host tissues. It is well-understood that members of the microbiota that cross the epithelial barrier elicit exaggerated immune responses, even in otherwise healthy tissues [90, 103]. Bacteria that localize near the apical surface of epithelial cells are sampled by dendritic cells (DCs) and are translocated to mesenteric lymph nodes (MLN) [79, 111, 118]. Therefore, bactericidal function at the intestinal interface is thought to limit the numbers of bacteria that can closely associate with host cells at the mucosal interface. Supporting this idea, γδ IEL responded to the complex intestinal microbiota even in the absence of distinguishable damage to the intestinal epithelium. Interestingly, while γδ IEL were responsive to the larger complex population of enteric bacteria, they did not respond to single non-invasive bacterial species such as *Bacteroides thetaiotaomicron* [89]. However, by using *Salmonella* infections to model direct host-bacterial interactions [93, 94], I showed that γδ IEL responses were instead preferentially triggered by this

model invasive bacterial species. These results suggest a model in which γδ IEL participate in surveillance of the intestinal epithelium, specifically detecting bacteria that invade across gut epithelia.

The observation that γδ IEL prevent early invasion of *Salmonella typhimurium* immediately after oral infection reiterates their role in protecting mucosal integrity and in maintaining host-bacterial homeostasis. This protective role is evident within hours of infection of *Salmonella typhimurium*. Interestingly, I found that γδ IEL may not play a direct role in limiting *Salmonella* dissemination during later stages of infection, suggesting that secondary lines of immune defense take over after initial bacterial breach of the intestine. This is not surprising since their location directly underneath epithelial tight junctions thus puts γδ IEL in a unique position to provide immediate antimicrobial defense after the first signs of bacterial penetration of the mucosal barrier. Therefore, as members of the intestinal microenvironment, γδ IEL have adapted to the unique environmental pressures from luminal microbiota, and elaborate very early immune responses to maintain and protect the integrity of the epithelium.

In light of the fact that γδ IEL are found in intestinal tissues very early after birth, it would be interesting to determine their role during the weaning transition, a key developmental transition in the gut. During this time, the host undergoes dramatic shifts in microflora composition, accompanied by increased expression of antibacterial factors [20]. One view of this interaction is that it represents an active host effort to maintain epithelial barrier integrity and limit bacterial penetration of mucosal surfaces despite the withdrawal of maternal antibodies during this period. Their pivotal role in protecting the

mucosa against opportunistically penetrating intestinal bacteria suggests that γδ IEL are very well-equipped to protect the intestinal mucosa during the large shifts in the gut ecosystem occurring during the weaning transition. Therefore, it is possible that γδ IEL might play a critical role in early host defense before the development of a full adaptive immune response.

Mechanisms of microbial detection by γδ IEL

Controversy surrounding γδ IEL detection of bacteria has been fueled by the unconventional microbial recognition properties of γδ T cells relative to other T cell populations. Prior studies have shown that γδ T cells, unlike conventional αβ T cells, can directly detect bacteria and bacterial products through various innate pattern recognition receptors [81, 114]. Given the critical role of epithelial MyD88 in driving key antibacterial programs in the intestine, I first assessed the role of this innate adaptor in driving γδ IEL responses to intestinal microbiota. Interestingly, my studies revealed that bacteria direct γδ IEL responses through MyD88-dependent and –independent pathways. For example, in agreement with previous studies on total colonic tissue [98], I found that RegIIIγ expression in colon γδ IEL after mucosal damage was driven through a MyD88-independent pathway. On the other hand, studies in small intestinal γδ IEL revealed MyD88-dependent regulation of this bactericidal protein, in support of previous observations in intact small intestinal tissue [22, 97]. These results suggest the existence of a complex process of bacterial detection that is tissue specific. However, taken

together my results suggested that innate recognition of bacteria drives a subset of γδ IEL responses in both tissues.

Despite microarray identification of several factors involved in direct immune recognition of bacteria, including transcripts that encompassed factors such as TLR1, TLR2, and CD14, my studies instead suggested that γδ IEL do not directly sense bacteria. While small intestinal γδ IEL from MyD88$^{-/-}$ mice had significantly reduced levels of RegIIIγ, bone marrow transplantation studies revealed that γδ IEL cell-intrinsic MyD88 was not required for these responses. Instead, a likely scenario is that other intestinal cells detect bacteria through MyD88-dependent signaling and then relay this information to γδ IEL. However, given the fact that MyD88 plays also plays a critical role in the IL-1 receptor (IL-1R), and the IL-18 Receptor (IL-18R) pathways [104], it will be necessary to distinguish whether the non-cell autonomous MyD88 signaling arises from direct recognition of bacteria through TLRs, or through IL-1/IL-18 signaling. Nevertheless, my results clearly reveal that conversations between γδ IEL and other intestinal cells drive the protective functions of these lymphocytes.

Crosstalk between γδ IEL and other intestinal cells.

An interesting aspect to γδ IEL biology is their role in directing the responses of other cellular populations found in the gut. Such a function is likely to be unique to γδ IEL, given that these cells are constitutively present in the epithelia of germ-free mice [26] (Fig. 32). Although αβ IEL occupy a similar niche in the epithelial layer, they are

unlikely to fulfill the same role, since they are recruited into the epithelial layer under the direction of bacterial signals [26] (Fig. 32). Supporting the idea that γδ IEL direct the responses of other immune cells, I found that γδ IEL isolated from DSS-treated conventional tissues (and not germ-free tissues) had enhanced expression of chemotactic factors that recruit neutrophils, macrophages, and $CD4^+$ T cells to areas of infection or immune activation.

Another interesting aspect of γδ IEL biology suggested by my work is that γδ IEL must collaborate with other intestinal cells to respond to intestinal microflora. In the small intestine, this may occur through communication with other intestinal cells which directly detect bacteria through MyD88-dependent pathways. However, my experimental findings so far do not identify specifically which cell population is responsible for MyD88-dependent capture of bacterial signals. One possibility is that these MyD88-dependent signals could come from enterocytes, the most abundant epithelial cell lineage in the intestine. Recent reports demonstrate that IEL constitutively express junctional molecules similar to those expressed by epithelial cells, and strongly suggest that IEL bind to and directly interact with epithelial cells of the intestine [95]. Additionally, previous studies revealed an intimate crosstalk between γδ IEL and their epithelial neighbors. These studies disclosed a tight and highly inducible γδ IEL proliferative response to soluble epithelial factors, supporting the idea that epithelial cells might directly detect bacteria from the gut and relay these signals to activate γδ IEL responses [63]. Alternatively, these signals could also be derived from subepithelial cells such as macrophages and dendritic cells, which directly sense bacteria through MyD88 [23, 101].

Regardless of how γδ IEL receive microbial input, my studies highlight the efficiency of the γδ IEL express microbe-responsive programs and reveal a previously unidentified role for γδ IEL in antibacterial defense of the intestine very early after pathogen insult or mucosal damage. For example, γδ IEL play a critical role in defending the host against *Salmonella typhimurium* as early as 3 hours after oral infection. Additionally, their role in preventing opportunistic penetration of commensal bacteria through damaged tissues is most evident during the earliest stages of mucosal injury. However, the fact that I detected bacterial regulation of γδ IEL transcriptional responses later during recovery suggests that commensals impact γδ IEL function even after bacterial penetration has been contained. Therefore, it will be interesting to delineate the sequence of γδ IEL responses in orchestration of their antibacterial defense.

A recurring idea in mucosal immunology is that of a balance between pro-inflammatory and anti-inflammatory immune responses. Such a balance is critical in mediating immediate responses against microbial threats while regulating exaggerated immune responses that could unnecessarily endanger the host. Therefore, it is possible that γδ IEL play a secondary regulatory role in dampening immune responses after initiating the proinflammatory responses characterized in my studies. In support of this regulatory role, one study revealed exaggerated inflammatory responses to intracellular bacteria in γδ T cell-deficient mice [119]. Therefore, the complex and dynamic transcriptional responses of γδ IEL to microflora suggest that these cells might be essential mediators in actively, or negatively, regulating intestinal immune responses.

Nevertheless, my studies suggest that the acute antibacterial intervention by γδ IEL may be required to maintain host-microbial homeostasis at the intestinal interface.

Taken together, my work provides further evidence for the intimate cross-talk that occurs at intestinal tissues between cells of various cell lineages. In support of this idea are other studies revealing that crosstalk between γδ T cells and monocytes drives inflammation in early immune responses to bacterial infections [34]. Furthermore, γδ T cells have been described to promote dendritic cell maturation early after microbial invasion [120]. These studies further strengthen the idea that γδ T cells achieve crosstalk with various cellular compartments through distinct and unconventional mechanisms.

The role of γδ IEL in antibacterial defense of the intestine

Despite their prevalence in mucosal tissues, the biological functions of γδ IEL have remained obscure. *In vivo* studies described in this thesis have revealed a novel role for γδ IEL in antibacterial defense of the small and large intestine, disclosing that γδ IEL protect the mucosal barrier against opportunistically invading commensal bacteria or by directly invasive oral pathogens. Taken together, my findings have yielded critical insight into how γδ IEL may promote beneficial host-microbial relationships in the intestine.

I first described a role for γδ IEL in protection of damaged mucosa against opportunistically invading commensals. This protective role was most evident immediately after mucosal damage and subsided during advanced stages of damage,

perhaps coinciding with recruitment of other immune cells. Next, studies in the small intestine revealed that in addition to preventing opportunistic invasion of commensals through damaged mucosa, γδ IEL also limited dissemination of a model invasive oral pathogen, *Salmonella typhimurium*, in otherwise healthy tissues. Strikingly, γδ IEL limited *Salmonella* dissemination within a few hours of oral challenge, suggesting that γδ IEL elaborate immediate responses early after pathogenic insult. A protective role for γδ IEL at the intestinal interface makes sense given that these cells are well-situated to mount an immediate response designed to limit penetration of bacteria through the epithelium. This is likely to be especially important in healthy individuals where there may be frequent transient exposures to damage-inducing environmental factors or intrinsically invasive oral pathogens.

The observation that γδ IEL provide critical early protection against invading bacteria is a unique feature of γδ IEL consistent with their niche underneath the epithelial tight junctions. This immediate antibacterial response is unlikely to be seen in other immune cells, which are recruited during later stages of intestinal damage or infection. Furthermore, these studies provide an explanation for why this protective effect may not have previously been defined, since the γδ IEL antibacterial responses are not observed during later stages of infection when microbes have significantly invaded host tissues, and when secondary immune responses may play a dominant role in intestinal defense. Thus, γδ IEL play an active role during the earliest stages of mucosal damage or infection to limit both commensals and pathogens, alike.

γδ IEL in Inflammatory Bowel Disease (IBD)

My studies also suggest the possibility for new therapeutic targets for inflammatory bowel disease (IBD). This disorder affects more than 1 in 1000 people in the United States [121]. While there is no cure for IBD, growing evidence implicates dysregulated immune responses to commensal in this group of chronic disorders [59]. IBD is characterized by focal intestinal lesions [122] and increased bacterial penetration through mucosal layers [123]. Additionally, other studies have shown that IBD patients exhibit increased numbers of intestinal γδ T cells [124]. Previous studies have reported amelioration of epithelial damage after therapeutic administration of factors induced during the γδ T cell response to damage [51, 125]. Therefore, my investigations of the molecular basis for γδ IEL responses to epithelial injury and intestinal microbes might provide novel targets to facilitate therapeutic manipulation of gut epithelial repair in IBD patients.

The unique ability of γδ IEL to recruit adaptive immune mediators, while interacting with resident innate cells of the intestine, likely contributes to critical immune cross-talk during intestinal immune responses. The specific niche of γδ IEL is likely the driving factor in the rapid responses to intestinal bacteria that I have discovered. The observation that γδ IEL direct a complex chemokine/cytokine profile to opportunistically invading commensal bacteria suggests that their responses are specifically designed for a rapid immune response. Coupled with my discovery of an antibacterial role for γδ IEL at the host-mucosal interface, these studies are consistent with the idea that a major role for intestinal γδ IEL is to provide a first line of defense against invading bacteria. My

findings would suggest that they function both to protect against opportunistic invasion by commensals following epithelial injury, and to limit invasion of pathogenic bacteria. Therefore, IBD might be manifested, in part, from dysregulated γδ IEL responses resulting in break-down of defenses designed to provide early protection against invading bacteria. Therefore, closer evaluation of γδ IEL biology might lead to development of therapies for IBD that take advantage of the multifaceted responses of these cells in maintaining host-microbial homeostasis of the intestine.

Conclusion

The studies presented in this thesis provide critical new insight into the biological functions of intestinal γδ T cells. My findings have revealed that intestinal bacteria provide critical regulatory input to γδ IEL in the small and large intestine, regulating production of antimicrobial and proinflammatory factors. This finding reveals for the first time that cross talk between the intestinal microbiota and the host extends to a mucosal T cell population, suggesting that these microbes profoundly shape host mucosal immune responses. More importantly, I have uncovered a previously unappreciated function for γδ IEL in antibacterial protection of the mucosal interface. This antibacterial role functions both to protect against opportunistically invading commensals and to limit dissemination of intrinsically invasive bacterial pathogens. A striking and unique feature of γδ IEL relative to other intestinal immune cells is that they function specifically to protect against bacterial invasion immediately following challenge. Thus, γδ IEL protect

against opportunistic invasion of commensals directly following injury, and limit pathogen dissemination in the first hours after oral challenge. This function is consistent with their residence at the mucosal surface and their physical niche beneath the tight junctions of epithelial cells. Thus, γδ IEL fulfill a unique physical and functional niche in the mucosal immune system, and are essential for maintaining host-microbial homeostasis in the intestine.

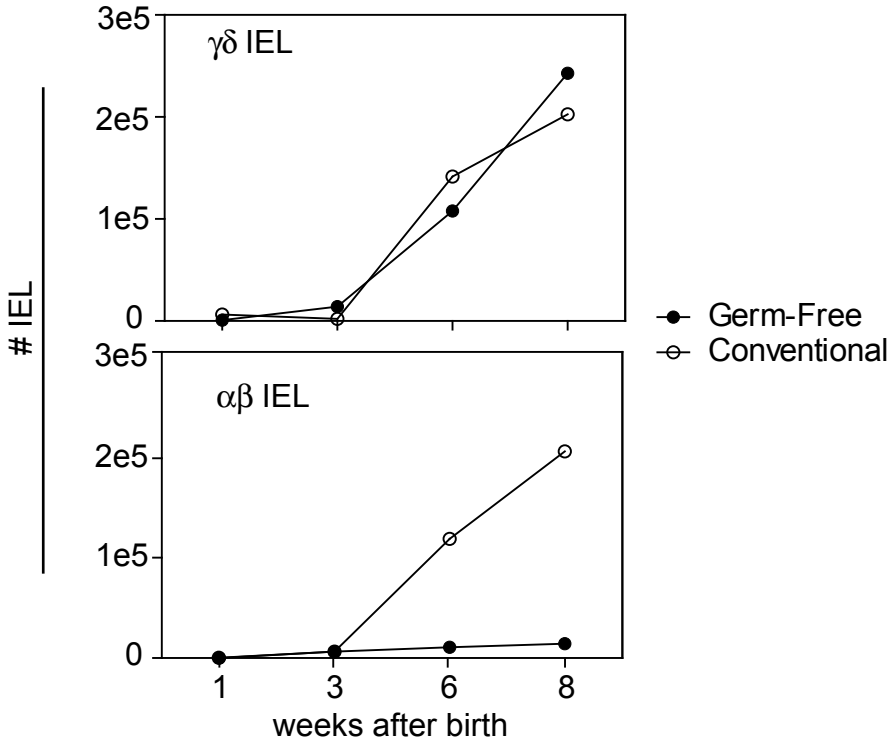

Figure 32: Developmental profile of γδ and αβ IELs. Mice were sacrificed at indicated times after birth and total IEL were isolated from the small intestines of C57/B6 mice as described in Materials and Methods. IEL were stained for FACS analysis using α–TCRδ-PE and α-TCRβ-FITC antibodies (BD Biosciences). A total of $5x10^5$ live cells were analyzed and total number of either γδ IEL or αβ IEL from that population of live cells is displayed.

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