The roles of IL-12 and IL-23 in the Innate and Adaptive Immune Responses against Salmonella

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Abstract

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Non-typhoidal *Salmonella* is a major health concern worldwide typically causing gastroenteritis in people of all ages and severe invasive disease in immune-compromised persons in particular in individuals with genetic defects in innate immune signaling molecules Interleukin (IL)-12/IL-23. The innate immune system is pivotal for early control of *Salmonella* and proper induction of the adaptive immune system responsible for eradication of and future protection from this pathogen. IL-12 and IL-23 are important innate signaling molecules that typically work through the promotion of their downstream target Interferon (IFN)-γ and IL-17/-22, respectively. Our overall goal is to better define the roles of these cytokines using of the streptomycin pretreatment model of *Salmonella*-induced enterocolitis in order to develop/modify different modalities and therapies for this disease.

In the acute phase, we found that IL-23 alone was dispensable for protection against systemic spread of bacteria, but synergized with IL-12 for optimal protection. We also found that IL-12 promoted the production of IFN-γ by NK cells, which as mentioned above, is required for resistance against *Salmonella* and also for induction of intestinal inflammation and epithelial injury. In contrast, IL-23 controlled the severity of inflammation by inhibiting IL-12A expression, thereby reducing IFN-γ and prevented excessive mucosal injury.

In late phase studies our data showed that the combined loss of IL-12/-23 led to defective control of oral attenuated *Salmonella* infection, diminished the generation of antigen-specific CD4 T cells, but robust antigen specific antibody responses. IFN-γ -/- mice had a survival disadvantage compared to IL-12/-23 DKO mice. This differential survival may reflect the unexpected production of IFN- γ by IL-12/23 DKO T-cells. Surprisingly, antigen-specific Th1 cells were readily detected upon rechallenge of immunized IL-12/23 deficient mice, indicating that these cytokines are not required for the generation of Th1 cells. Nevertheless, the IL-12/23-independent, antigen-specific Th1 and antibody responses were insufficient for optimal control of late phase *Salmonella* infection. Together these data demonstrate the critical and complex relationship of IL-12 and IL-23 that are essential for early and late phase immunity against *Salmonella*.

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Chapter 1: Innate Immune Response to *Salmonella* Infection

Introduction:

Emerging infectious agents and the ever-increasing number of antibiotic resistant microbes are challenging our ability to treat infectious diseases. Historically, mass vaccination has proven to be an effective means to control infection, and is credited with the eradication of both small pox and polio. In 1974, Leighton Cluff commented, "Immunology has extended our understanding of many diseases, but its applicability to prevention of control of infection still is its greatest contribution to our health. Prevention of infection by immunization has reduced fear, prolonged life, improved productivity, and enhanced cultural and social improvements for everyone. It becomes almost unimaginable what the impact would be if some of the infectious diseases now so prevalent were similarly controlled [1]." This success in the eradication of small pox and polio has not been readily translated to other infections such as malaria and HIV that are endemic and continue to represent major global health problems.

The innate immune system also is critical for host defense against infections. Innate immunity helps control acute infection and is also necessary to program adaptive immune memory needed to sense and effectively eradicate infectious agents. By the 1940s, fractionation and chemical analyses of microbes were beginning to reveal the molecular components that elicited inflammatory responses in mammals. The first microbial component to be studied in detail was "endotoxin" (lipopolysaccharide, LPS). The inflammatory character of other microbial components (for example, dsRNA, DNA, peptidoglycan, and lipopeptides) was also established. We now know that these "pathogen-associated molecular patterns" are recognized by families of innate immune receptors, and are critical to inform us of infection and establish protective immunity to pathogens. A better understanding of innate immunity may let us modulate inflammation as suits us best, produce even better vaccines, and treat autoimmune diseases in a more rational manner than we do at present.

Innate immune detection of pathogens leads to the production of cytokines and other factors that contribute to this defense response. The IL-12 family of cytokines are central to the defense against multiple pathogens, including mycobacteria and *Salmonella*, and act both as regulators of innate defenses and adaptive immunity. My overriding hypothesis is that IL-12 and IL-23 act in concert to establish host immunity to *Salmonella* infection. In this thesis, I will define the role of IL-12 and IL-23 in host immune responses to *Salmonella* infection. *Salmonella*

first infects the gut mucosa, and IL-12 family members have been recently implicated as key mediators of mucosal immunity. IL-23 is important for protection of the gut mucosa against extracellular bacteria, the generation of antigen-specific T-cells, termed Th17 cells, and regulating the activity of a recently identified innate immune population called innate lymphoid cells (ILCs) [2] that are present much earlier than Th17 cells. IL-12 is critical for protection against systemic infections, the generation of antigen-specific Th1 cells, and regulation of the activity of innate lymphoid cells, in particular natural killer (NK) cells. In this thesis, I will explore the role of IL-12 and IL-23 in regulating host inflammatory and immune responses during mucosal infection with *Salmonella*.

Salmonella Epidemiology

Non-Typhoidal *Salmonella* (NTS) contamination of food or fresh produce is responsible for several recent outbreaks of gastroenteritis in the US [3] [4]. Aside from the health problems experienced by affected individuals, these outbreaks cause economic hardship for food manufacturers and erode public confidence in the safety of the US food supply [5], [6]. Certain *Salmonella* serovars can also cause a systemic infection of the reticuloendothelial system, resulting in a disease known as typhoid or paratyphoid fever [7]. Although the incidence in industrialized countries has declined, NTS remains a common cause of bloodstream infections in sub-Saharan Africa particularly in adults with HIV infection and children with HIV, malaria, and malnutrition [8]. Death rates are 20%–25% among adults and children [9]. In sub-Saharan Africa, perhaps surprisingly, typhoid fever is not associated with HIV among adults [10].

As with other enteric diseases, typhoid and NTS are typically transmitted via the fecal-oral route and concentrated in communities without access to clean water or basic sanitation [11]. At present, one in six individuals (1.1 billion people) has no access to clean water and 40% of the world's population (2.6 billion people) lack primitive sanitary facilities [12]. The cost of improving this infrastructure is prohibitive and these figures are predicted to increase by the year 2025 (to 2.9 billion and 4.2 billion respectively) [12]. The most recent estimates indicate that typhoid and paratyphoid fever affect approximately 27.1 million people and cause 217,000 deaths annually, with most of these cases localized to sub-Saharan Africa and southeast Asia [13]. Therefore, *Salmonella* infections are a health concern in developing nations lacking basic societal infrastructure, but are also an important cause of gastrointestinal infection in developed nations where contaminated food and produce are rapidly and widely distributed.

The generation of safe and effective vaccines that could be used routinely and safely in endemic areas would have a significant impact on both typhoidal and non-typhoidal *Salmonella*. Currently three licensed typhoid vaccines (parenteral inactivated whole-cell vaccine, oral attenuated Salmonella enterica serovar Typhi Ty21a, and parenteral Vi polysaccharide) have moderate efficacy against typhoid (3-year cumulative efficacy of 50–55%); however, none of these vaccines are widely used in endemic areas, nor do they protect young children [14]. To help cover children a recent vaccine was developed by improving immunologic properties of Vi polysaccharide (Vi) by conjugating it to recombinant *Pseudomonas aeruginosa* exoprotein A (rEPA). The cumulative efficacy of the new Vi-rEPA vaccine at 3.8 years was higher, 89% (95%CI 76%, 97%) [14], [15], but this vaccine has not yet been licensed for use and was evaluated in only one trial.

Unlike typhoid, there is currently no vaccine against NTS, and greater understanding of host immunity to disseminated infection is required. Mouse *S*. Typhimurium infection mimics typhoidal infection, and aided the development of the initial typhoidal vaccines. A revised mouse oral infection strategy has been developed that models human NTS disease [16]. Greater study of the immune parameters associated with host protection in this mouse model is therefore likely to inform the generation of new vaccines to protect against disseminated NTS infections in humans.

Salmonella Pathogenesis

Salmonellae are Gram-negative bacterial pathogens that infect a wide variety of animal hosts through the ingestion of contaminated water or food [17]. Non-typhoidal *Salmonellae* (NTS) are broadly host adapted and cause natural infections in a wide variety of animals. NTS species typically cause enterocolitis/diarrhea, bacteremia in a subset of individuals, and a prolonged asymptomatic carrier state that can last several weeks after primary infection. NTS species have been further subdivided into serovars that are differentiated by the antigenic make-up of their flagella and LPS. The disease manifestations depend on both host susceptibility and the infectious *S. enterica* serovar [18, 19]. These serovars cause disease in various hosts, but there does appear to be some degree of host adaptation, and some serovars are more virulent in specific hosts. Several serovars including Choleraesuis and Dublin are more commonly associated with bacteremia in humans [18]. Serovars Dublin, Typhimurium and Choleraesuis cause disease in both humans and animals, but cause distinct syndromes in different hosts. Serovar Dublin causes intestinal inflammatory disease, bacteremia and abortion in cows; serovars Typhimurium and Enteritidis cause a typhoid-like systemic illness in mice; and serovar Choleraesuis causes

septicemia in pigs[20, 21]. The virulence factors employed by *Salmonella* species interact with host defense mechanisms in a variety of tissues and cells at different stages of infection resulting in significant host immunopathology, morbidity and mortality. Unlike in industrialized countries, NTS in sub-Saharan Africa may be transmitted person to person, and a unique sub-species has emerged, *Salmonella* Typhimurium ST313, which is associated with invasive infections, and shows evidence of genomic degradation that has been postulated to resemble genomic changes in the human-restricted S. Typhi [10]. Additional factors, such as malaria and HIV co-infection, and the emergence of potentially host-adapted regional NTS variants[10] are probably contributing to the emergence of invasive NTS.

Typhoidal *Salmonella*

Typhoidal strains (*S*. Typhi and *S*. Paratyphi*)* are host range restricted to humans, and cause enteric fever. Human typhoid occurs following the ingestion of *S. enterica* serovar Typhi, usually from contaminated water or animal products or close contact with an infected individual or carrier. [22]. Much of our knowledge and understanding of *S. typhi* pathogenesis has been derived from the study of infection of susceptible mice to *S*. Typhimurium. This knowledge is incomplete since *S*. Typhimurium lacks several host-restricted virulence mechanisms utilized by *S. typhi*. Typhoidal strains rapidly disseminate from the intestinal lumen via M-cell to Peyer's patches (PP) to systemic sites such liver, spleen and bone marrow, where they persist in phagosomal compartments of macrophages. This systemic illness is chronic and characterized by a high fever, abdominal pain, transient diarrhea or constipation, and occasional maculopapular rash [23]. In absence of antibiotic therapy 10-15% of cases are fatal and in some regions fatality rates are as high as 5-7% despite antibiotic treatment. Rarely, asymptomatic carriage and prolonged shedding of bacteria in feces occurs, usually as a result of colonization of the gallbladder [23].

Non-Typhoidal *Salmonella*

As indicated above, NTS serovars cause an infection in immunocompetent patients that manifests as diarrhea, nausea, vomiting, fever and abdominal pain [24]. The organisms most frequently associated with this diarrheal disease are *S*. Typhimurium and *S*. Enteritidis [25].The intestinal infection is typically concentrated in the lower small bowel (enteritis) and colon (colitis). Gastroenteritis resulting from infection with NTS presents approximately 8-48hrs after ingestion with nausea, vomiting, diarrhea abdominal pain and fever [26]. The disease is normally

self-limiting within a week, but shedding of *Salmonella* in the stool can persist for weeks to months. Infection of immunocompromised individuals, either due to age (very young and old) or debilitations in host immunity, can result in severe and even life threatening disease [27].

The intestinal pathology during human enterocolitis is characterized by a massive neutrophil influx in the infected intestinal tissues [28] and stool samples of patients contain a leukocyte population that is dominated by neutrophils [28]. NTS is the most common cause of bacterial-related deaths in the United States [6] and *S. enterica* serovar Typhimurium is the most frequent isolate. NTS bacteria are normally limited to the intestine and associated tissues which include Peyer's patches and mesenteric lymph nodes (MLN) [29]. NTS can also cause disseminated infections that lack gastrointestinal symptoms and may be transmitted by human-to-human contact rather than through an animal reservoir [8,9]. These unusual features are likely to be caused by an ongoing host adaptation of NTS strains in HIV endemic areas in Asia and Africa [9,9,30].

Salmonella Infection Models

We are interested in understanding host innate immune factors that contribute to resistance against *Salmonella* infection and control the quality and quantity of the inflammatory response to this pathogen. Despite the varying host range and disease manifestations caused by typhoidal and non-typhoidal *Salmonella*, many pathogenic strategies and virulence genes are conserved. *In vitro* models of *S.* Typhimurium have allowed genetic, cell biological, and biochemical analysis of the infection process. Although these models are consistent, reliable, and relatively easy to use, they also have their limitations. Because cells are typically studied in isolation, defining the role of complex immune responses that involve dynamic changes in multiple cell types is not easily mimicked *in vitro*. Cultured or transformed mammalian cells also often lack important characteristics, and do not faithfully replicate mature phenotypes of epithelial cells *in vivo*. NTS induced enterocolitis has also been modeled in large animals. One *in vivo* method for studying *Salmonella* enterocolitis is the calf ileal loop model. This model is limited to studies that can last up to several hours but typically less than one day, and thus particularly advantageous for analyzing pathological changes and the virulence factor requirements during the early phase of infection. The calf model of *S.* Typhimurium infection has many similarities to human disease that make it a good model for studying enteritis. However, the feasibility, cost, and lack of diverse set of genetically manipulated animals makes this a less than ideal host to study the host immune response to *Salmonella*.

Direct study of human restricted pathogens, such as *S. typhi*, is limited by the lack of practical animal infection models. Although primates exhibit symptoms of *Salmonella* infections observed in humans, high cost, scarcity of animals, and ethical considerations have prevented the widespread use of this model. Much of the understanding of typhoid pathogenesis has arisen from the study of infection of mice with *S.* Typhimurium, which more closely resembles typhoid than enterocolitis. Mice are a convenient animal model for many infections due to their economy of size and the availability of numerous immunological reagents and mutant strains. The mouse model for typhoid varies from that of human typhoid fever. For example, in the case of *S.* Typhimurium infection of mice, a few genes, such as the *spv* operon, are essential in mice, but not required by Typhi to infect humans. Hence it remains a challenge to correlate the pathogenesis of Typhimurium infection in mice with Typhi in humans. Recent derivation of humanized mice has generated some hope. One such example, namely the humanized immune system (HIS) mouse model, reconstitutes human immune cells into a severely immunodeficient mouse [31]. Unfortunately, this technology is also costly to maintain and limited by its inability to develop adaptive immune responses. Thus this technology needs additional development to be useful as a model system for studying the full range of human immune responses to *Salmonella* infections.

Salmonella Oral Mucosal Infection Model

As discussed above the pathogenesis of NTS immunity is largely contained by the mucosal immune system leading to enterocolitis in human, primates and other large animals [24, 32]. However infection can become blood borne in immunocompromised hosts. Mice are intrinsically resistant to *S.* Typhimurium enterocolitis [24]. Mouse studies of *in vivo* immunity to *Salmonella* have been largely focused on systemic infection models due to the inefficiency of murine enteric colonization and lack of enterocolitis. Thus much less is known about the mechanisms of enteric salmonellosis.

For many years, people have observed that gut microbiota prevent colonization of pathogenic enteric species, termed colonization resistance. Colonization resistance in mice might be the result of a complex interplay between *S.* Typhimurium, the resident gut microbiota, the gut mucosa and the gut associated immune system. Germ-free mice are highly susceptible to enterocolitis from various bacteria, including *Salmonella* [33, 34]. Similarly, the decimation of gut commensals by antibiotics eliminates colonization resistance, and permits enteric infection in mice by *S.* Typhimurium. Antibiotic pretreated mice develop acute intestinal inflammation in

response to oral *S*. Typhimurium infection, which resembles human disease. Upon oral pretreatment of mice with a single high dose of streptomycin (20mg), *S*. Typhimurium efficiently colonizes the large intestine (10^8-10^{10}CFU/g) and triggers severe acute colitis as early as 8h post-infection [35], [16], [36], [37], [17]. Although both germ free (GF) and streptomycin pretreated mouse models of *S*. Typhimurium infection demonstrated efficient colonization of the large intestine and comparable cecal and colonic inflammation, GF mice exhibited more pronounced damage and reduced regenerative response of the cecal epithelium [34]. Thus gut microbiota may contribute to tissue homeostasis, in addition to colonization resistance.

Nramp1 (Slc11a1) is a divalent cation transporter that is critical for the control of *Salmonella* growth inside of phagocytes, and is defective in some mouse strains, notably the C57BL/6 and Balb/c strains, resulting in marked susceptibility to *Salmonella* infection. In Nramp1 deficient hosts oral *Salmonella* infection proceeds rapidly to systemic disease leading to death. When exploring the differences with streptomycin pretreatment between Nramp1 competent and deficient mouse strains, two points were concluded [38]: 1) acute *S*. Typhimurium colitis is similar between the two strains and 2) long-term infections in the Nramp1+ strains are feasible and display characteristics of chronic, crypt-destructive colitis. These long-term features are very similar to those seen in inflammatory bowel disease. The streptomycin pretreatment model is a cost and time efficient model for human enterocolitis. Nramp1 deficient strains are appropriate for studying acute infections with virulent pathogens and longer term infections with attenuated strains of *Salmonella*, and Nramp1 sufficient strains are useful for studying short and long term infections. The GF and antibiotic pre-treatment mouse models for examining gastrointestinal inflammation and immunity have their own limitations as they require the manipulation of the microbiota. However, the antibiotic pre-treatment model is highly reproducible and easy to employ, making it a robust system for modeling human NTS enterocolitis.

Molecular pathogenesis of *Salmonella* Infection

Salmonella is an intracellular pathogen, residing in a membrane bound vacuole within infected cells. Successful colonization of murine hosts by *S*. Typhimurium depends on the functions of a large number of virulence factors that afford adaptation to nutritionally limited sites, serum resistance, anti-microbial peptide resistance (i.e. lectins and cathelicidins) and the ability to repair damage inflicted by host defense mechanisms. During infection, *Salmonella* utilizes type III secretory systems (TTSS)-1 and -2 encoded within *Salmonella* pathogenicity islands 1 and 2

(SPI-1 and -2), respectively, to introduce bacterial effector proteins into host cells. TTSS-1 and -2 contribute to acute inflammatory responses [32], which result in enterocolitis and diarrhea [28]. *Salmonella* penetrates the epithelial lining of the small intestine either by invasion of M cells, epithelial cells or via alternative pathways that directly access lamina propria dendritic cells [39],[29]. Genes required for invasion of nonphagocytic cells by *S. enterica serovar* Typhimurium are clustered in SPI-1 [16,40]. Effector proteins translocated via SPI-1 induce dramatic rearrangements in the actin cytoskeleton, resulting in the protrusion of large folds of membranes in a process called macropinocytosis [41]. SPI-1 also secretes factors that help establish a permissive intracellular niche, induce proinflammatory cytokines and chemokines [42], [43], promote the transmigration of polymorphonuclear granulocytes (PMNs or neutrophils) across epithelial cell layers [44], and trigger cell death [45]. In an *in vivo* streptomycin pretreatment model, SPI-1 allows *S.* Typhimurium to infect epithelial cells and lamina propria cells and causes acute inflammation during the first two days of infection [35,36]. Thus SPI-1 is important in establishing early mucosal colonization and inflammation. Mutant strains deficient in SPI-1 function show delayed gut inflammation, and are modestly attenuated after oral infection of mice, but are not defective when the epithelial barrier is bypassed (i.e. intraperitoneal or intravenous infection) [40].

SPI2 encodes TTSS-2 and harbors genes that are required for intracellular replication of *Salmonella* within phagocytes, and to establish systemic infection [46,47]. *Salmonella,* like many intracellular pathogens, infect macrophages and dendritic cells (DCs), within which they replicate and are transported to privileged sites [26]. Survival and replication within host phagocytic cells is essential to *Salmonella* pathogenesis in animals [23]. *Salmonella* form an intracellular vacuole termed as *Salmonella* containing vacuole (SCV) within the phagocytes. The SCV resembles a vesicle that is arrested at a late stage of endolysosomal maturation, which may help *Salmonella* evade host detection and acquire nutrients [26]. The SPI-2 TTSS is expressed after *S.* Typhimurium has entered an epithelial cell or a macrophage. Several SPI-2 effector proteins target the cytoskeleton and vesicular trafficking to enhance intracellular survival [35]. SPI-2-deficient strains are highly attenuated after oral infection, as well as after intraperitoneal or intravenous infection [47]. In an *in vivo* streptomycin pretreatment model, SPI-2 allows *S.* Typhimurium to infect lamina propria phagocytes and promotes delayed inflammation at day 3 post- infection [35,36]. Unlike SPI-1, the delayed SPI-2 mediated inflammatory response is dependent on TLR recognition [36]. Thus, TTSS-1 and -2 contribute to pathogenicity and colitis via different mechanisms

Innate Immunity

Host recognition and control of invading microbes involves two branches of the immune system: the innate and adaptive branches. Chapter 2 will largely focus on innate immunity, whereas Chapter 3 will emphasize adaptive immunity. Physical, chemical and cellular barriers comprise the innate immune system. Breach of the skin and/or mucosal membranes activates a rapid innate immune response. Phagocytic cells (such as monocytes, macrophages, neutrophils and DCs), innate lymphoid cells (iLC) and Natural Killer (NK) cells act as the first cellular barrier upon microbial breach of the epithelium. These innate immune cells express germline-encoded receptors (pattern recognition receptors, PRRs) that detect conserved motifs commonly associated with many pathogens (pathogen-associated molecular patterns, PAMPS), such as sugars, proteins, lipid-bearing molecules or nucleic acids [48]. Engagement of PRRs elicits a multifaceted and robust response: activation of the complement system, production of cytokines, chemokines, and antimicrobial peptides, which enhance containment and killing of pathogens, and processing and presentation of antigens on mature dendritic cells (DCs) [49].

Macrophages

Macrophages reside in the tissues and ingest cells and other material through a process termed phagocytosis. Upon phagocytosis of microbes, the microbe-containing phagosome acidifies and fuses with lysosomes, which is sufficient to destroy most microbes. However, many pathogens, such as *Salmonella* modify the phagolysosome, and evade host cell killing. Compared with the classical phagolysosomes, SCVs have reduced antibacterial activity and less acidity, but are still limiting for certain nutrients such as aromatic amino acids and purine bases [50, 51].

In order to kill pathogens, macrophages produce a multitude of antimicrobial molecules, including NADPH phagocyte oxidase [52, 53] and inducible nitric oxidase synthase (iNOS) [54]. The NADPH phagocyte oxidase catalyzes the production of superoxide radicals that can be metabolized to a variety of toxic reactive oxygen species (ROS) [53]. Patients deficient in the NADPH phagocyte oxidase are susceptible to recurrent microbial infections, including salmonellosis [55], a clinical condition known as chronic granulomatous disease (CGD). The gp91phox gene encoding an essential component of the NADPH phagocyte oxidase has been targeted in mice, resulting in the elimination of oxidative burst in neutrophils and macrophages and an increased susceptibility to multiple microorganisms including *Salmonella* Typhimurium [53, 56, 57].

iNOS [54] is an enzyme that generates nitric oxide, which has potent antimicrobial activity. iNOS(-/-) mice are susceptible to *Salmonella* infection, and have increased bacterial burden within reticuloendothelial organs after the first week of infection indicating the importance of iNOS during later stages of *Salmonella* infection. This late defect was attributed to the inability iNOS-deficient granulomas to limit bacterial replication [54].

Macrophages also produce cytokines, such as IL-1, TNF and IL-6, that promote inflammatory responses [58]. Macrophages use compartmentalized detection systems to respond to bacterial infection. Toll-like receptors (TLRs) respond to extracellular or vacuolar stimuli, whereas Nod-like receptors (NLRs) respond to cytosolic perturbations. In general, TLRs orchestrate downstream immune responses by activation and production of specific cytokines and chemokines. TLRs also induce expression of the precursor forms of some cytokines (pro-IL-1β and pro-IL-18). Some NLRs regulate the activation of caspase-1, which controls the proteolytic processing and release of IL-1 and IL-18, and a unique form of cell death, termed pyroptosis [59]. Other NLRs regulate additional signaling pathways, such as NF-kB activation [60], [61], [62].

Macrophages often function in a primarily protective capacity, and can act as antigen presenting cells (APC), which activate adaptive immune cells. The dominance of macrophages as an APC is more prominent during secondary *Salmonella* infection [63]. Several studies in humans as well as in murine models demonstrated that protective immunity against *Salmonella* critically depends on IL-12 and might be complemented by IL-23 [58,64, 65,66]. After infection of macrophages, *S*. Typhimurium induces IL-12 and IL-23 in a TLR4-dependent manner [67].

Neutrophils:

Neutrophils are the most abundant leukocytes in peripheral blood, with an important role in the early stages of the inflammatory response. These cells also undergo chemotaxis and/or degranulation in response to activation by various surface receptors, which include IgG-Fc, complement, Toll-like receptors (TLRs), cytokines (such as IFN-γ) and chemokines (like IL-8) [68]. The short lifetime of neutrophils minimizes propagation of those pathogens[69]. Neutrophils play a key role in the front-line defense against invading pathogens. Neutrophils have three strategies for directly attacking micro-organisms: phagocytosis (ingestion), release of soluble anti-microbials (including granule proteins), and generation of neutrophil extracellular traps (NETs). Neutrophils release various classes of granules in the extracellular environment: 1) azurophilic granules, containing myeloperoxidase (MPO) and defensins; secondary granules such as lactoferrin; and tertiary granules with cathepsins and gelatinases as main proteins [68]. Recent studies have shown that neutrophils can also generate the so-called neutrophil extracellular traps (NETs) formed by

granule substances and nuclear components capable of culling off the virulence factors and destroying extracellular bacteria. NETs are abundant in inflammatory sites, act directly on microorganisms and serve as a physical barrier to prevent spread [70].

Previous studies highlight the different role of neutrophils in systemic and gastrointestinal infection models with *Salmonella* [35] [16]. In the streptomycin enterocolitis models infections are characterized by rapid recruitment of neutrophils to mucosal sites [16]. Whereas macrophages play a dominant role in control of intracellular *Salmonella* replication, neutrophils help kill extra-cellular bacteria that are released by macrophages and other cells [59].

Dendritic Cells (DCs)

DCs play a key role in eliciting an inflammatory response against invading microbes. DCs display a number of PRR on their surface and within endosomes, including TLRs. DCs reside in tissues, such as skin, liver, and intestine, where they capture antigens and become activated and migrate to regional lymph nodes, where they process and present antigens to T cells. Immature DCs are highly efficient in capturing antigens. Upon encounter with PAMPs, DCs undergo a maturation process that induces up-regulation of the co-stimulatory molecules CD80 and CD86 and major histocompatibility complex (MHC) I and MHC II, and migrate into lymphoid tissues [71]. Mature DCs are very efficient in presenting intracellular and exogenous antigens on MHC I or II molecules, respectively, and delivering strong co-stimulatory signals to naïve T cells [72], acting as a bridge between the innate and adaptive immune responses.

Salmonella passing through M cells are met by DCs in the dome area [73]. *In vitro* studies using phagocytes expressing the DC marker CD11c have shown that *Salmonella* survives inside these cells and induces IL-1, IL-6 and IL-12 secretion. CD18+ DC are also vehicles for rapid dissemination for *Salmonella* [73]. In another study it was found that in response to *Salmonella* DCs are more potent producers of the cytokines IL-12, IL-23 and IL-27p28 than macrophages[74]. Because subepithelial DC express high levels of major histocompatibility class II complex and are very efficient at presenting antigen to naïve T-helper cells, they likely play a crucial role in inducing mucosal immunity to enteropathogens.

Natural Killer (NK) cells

Natural killer cells (NK) originate in bone marrow, constituting 5% to 20% of blood mononuclear cells. They are an important line of innate defense, recognizing and lysing cells infected by viruses, bacteria and protozoa, as well as tumor cells. Furthermore, NK cells recruit neutrophils and macrophages, and activate DCs and T and B lymphocytes [75]. IL-15 drives the

expansion of NK cells, and IL-12 and IL-18 are potent inducers of NK cell IFN-γ production and cytolytic activity. Activated NK cells lyse infected and transformed cells, and secrete pro-inflammatory cytokines (especially IFN-γ) [75], [76].

In contrast to wild-type mice, *S*. Typhimurium extensively colonized the gut of IL-15-/- (NK cell deficient) mice and wild-type mice depleted of NK cells, speaking to the importance of NK cells in protection against *Salmonella* gut colonization [77]. In an acute murine enterocolitis model, *Salmonella* infection induces intestinal expression of NK cell recruiting chemokines and a corresponding increase in NK cell numbers in the gut [78]. NK cells are also required for IFN-γ production and intestinal inflammation during acute *Salmonella* infection in T and B cell-deficient mice [78]. T and B-cells do not appear to play an appreciable role during the acute phase of *Salmonella* infection [79]. These findings suggest that NK cells play an important role in the intestinal innate immune response to acute *Salmonella* infection by rapid production of IFN-γ[69], [80], [81].

Other lymphoid populations exist that produce important mucosal cytokines and participate in mucosal pathogenic responses. Invariant NK T cells (NKT), which have a role in antitumor immune responses and antiviral immunity [82], have also been recently reported to produce IL-17 in the context of inflammatory diseases [83], [84]. Innate lymphoid cells (iLCs) are immune cells that lack a specific antigen receptor yet can produce an array of effector cytokines that match T helper cell subsets [85-87]. IL-22-producing iLCs express the NK cell–associated receptors (natural cytotoxicity receptors, NCRs) NKp44 in humans and NKp46 in mice [86]. They function in lymphoid organogenesis, tissue remodeling, antimicrobial immunity, and inflammation at barrier surfaces.

Importance of IL-12 and IL-23 innate immunity and beyond

During the initial phase of the *Salmonella* infection cells in the intestinal mucosa directly contact the pathogen. The initial signals emanating from these infected cells are probably not easily detected by gene expression analysis or other methods that average host responses over the entire tissue. Such selective responses are best appreciated once the signals are amplified and the entire mucosa has switched to defense. Several cytokines and factors that are induced during this amplification phase include IL-12, IL-23, IFN-γ, TNFα, IL-17 and IL-22 [88], [89] [16]. The next section focuses on these cytokines and their functional significance to the control of *Salmonella* replication, inflammation and tissue homeostasis during the acute phase of oral *Salmonella* infection.

IL-12

IL-12 is a heterodimer composed of subunits p40 and p35 (**Figure 1**). *In vivo*, IL-12 acts on multiple aspects of innate and adaptive immune responses to *Salmonella*. IL-12 is produced early after infection by macrophages and dendritic cells (DC) to promote IFN-γ production by NK and Th1 T cells. IFN- γ in turn contributes to macrophage activation by enhancing bactericidal effector mechanisms, antigen presentation and pro-inflammatory cytokine production. IL-12 also has autocrine effects on antigen presenting cells (APC) and up-regulates IL-12R expression [90], [91]. Thus, IL-12 represents a functional bridge between early non-specific innate resistance and subsequent antigen-specific, Th1 type immune responses to *Salmonella*.

IL-23

IL-23 is a heterodimer composed of p40, which it shares with IL-12, and a unique second chain, p19 [92] (**Figure 1).** IL-23 supports the differentiation of T-helper (Th)-17 cells, which produce IL-17 and IL-22, and have been linked to immunity against extracellular pathogens and autoimmune diseases [91]. The adaptive Th17 cell response develops after the initial exposure to a pathogen, and as a first line of defense innate IL-17 producers contribute to the innate defense against pathogens that require IL-17-mediated responses. Among these, iLCs [86], [83] and γδ T cells [93] have been shown to be potent sources of IL-17. IL-23 also promotes NK cell activity and IFN-γ production by synergizing with IL-12 and IL-18 [94]. IL-23 synergizes with IL-12 for maximal memory IFN-γ producing Th1 induction, but IL-23 is not required for naïve Th1 differentiation [95] [96] [97]. Therefore, IL-23 and IL-12 are important molecules that appear to have distinct but overlapping roles in regulating cellular components of innate and adaptive immunity.

IL-12 and IL-23 impact Salmonellosis in man and mouse

Individuals with deficiencies in both IL-12 and IL-23 signaling, due to defects in the *IL12Rβ1* or *IL12p40* genes (Fig. 1) have increased risk of mycobacterial and *Salmonella* infections [98], [99]. There is also a higher prevalence of *Salmonella* disease in patients with deficiencies in IFN-γ signaling. This suggests that at least part but possibly not all of the genetic susceptibility to *Salmonella* infection is due to defective IL-12/23-dependent IFN-γ production [99], [27], [90].

In addition, mice deficient in p35 and p40 are more susceptible to attenuated *Salmonella* Enteritidis i.p. infection and have markedly decreased production of IFN-γ [100]. In mice IL-23

alone is dispensable for resistance to systemic *Salmonella* infection, but a role for IL-23 in this resistance becomes evident when IL-12 is absent [66]. Indirectly the potential downstream target of IL-23 was suggested to be IL-22 and not IL-17 [66]. In a separate report, IL-17A was found to make a small contribution to systemic *Salmonella* infection resistance [101]. These data support the current dogma that loss of p35 (no IL-12) dramatically increases susceptibility to systemic *Salmonella* infection and loss of p40 (no IL-12 or IL-23) further augments this susceptibility [100], [102]. These data, along with the human studies, suggests that IL-12 and IL-23 contribute to host resistance against *Salmonella* infection through the regulation of IFN-γ, IL-17 and IL-22.

Much less is known about the cellular targets for IL-12 and IL-23 during acute *Salmonella* infection in the gut. The role of IL-23 was recently analyzed in acute *Salmonella* colitis in mice. In this study IL-23 was suggested to promote gut mucosal inflammation after acute *Salmonella* infection [88]. The primary cellular target for IL-23 during acute *Salmonella*-induced colitis was γδ-T cells, which had increased IL-23R expression and were a major source of IL-17; however, γδ-T cell knockout mice had no defect in inflammation [88]. Additional potential cellular targets include iLCs, a newly defined class of innate lymphoid cells, which have been shown to be critical for IL-17 and IL-22 production in other models of mouse colitis [83]. Because the role of IL-23 during mucosal infection by *Salmonella* is still undefined we will explore its role in controlling bacterial replication and mucosal inflammation during the acute phase of *Salmonella* infection in Chapter 2.

Adaptive Immunity to Salmonella and the roles of IL-12 and IL-23

The innate immune system reacts early in infection and often clears the infection on its own. When innate immunity is overmatched, the adaptive immune response is required to establish effective host defense against the pathogen. B and T cells comprise the adaptive immune branch, and these cells recognize the invading pathogen with highly antigen specific receptors, and a diverse array of effector functions that are influenced by the innate immune response. B cells produce antibodies that bind pathogen structures, and in some instances neutralize critical pathogen activities. Antibodies also have distinct classes of effector domains (Fc regions) that permit binding by Fc receptors on different cells and by serum components, promoting phagocytosis, cellular activation, and complement activation [103,104]. CD8 T cells, or cytotoxic T cells, detect peptide fragments of pathogens presented by MHC class I molecules, destroy infected cells, and secrete cytokines to limit replication of intracellular pathogens. CD4 T

cells, or helper T cells, detect peptide fragments of pathogens presented by MHC class II molecules, and aid in the activation of B cells, cytotoxic T cells, macrophages and other immune cells to promote a wide variety of effector immune responses [105]. The CD4 T cell response is diverse and several different subtypes have been defined (such as Th1, Th2, Th17 and Treg), which appear to be programmed by their environment including infectious agents in order to tune the immune response, provide the most effective means to control infections and establish homeostatic relationships with commensal microorganisms. This section will review what is known about the adaptive system's role in resistance to *Salmonella* infection, and also examine the specific contribution of IL-12 and IL-23 in establishing T and B cell immunity to *Salmonella*.

CD4 T cells mediate adaptive immune resistance to *Salmonella*

The immune response to *Salmonella* can be divided into two phases. During the early stage of *Salmonella* infection, resistance is largely mediated by the innate immune system, including NK cells, macrophages and neutrophils [77], [63], [106]. These cells suppress bacterial growth during the first weeks of infection [107]. The effect of T-cells is not apparent in the murine system until about 2-3 weeks following *Salmonella* infection [79]. For example, athymic (non-functional T cells) and euthymic (functional T cells) animals have little difference in bacterial burden during the early phase of infection. In contrast, 2-3 weeks post infection, bacterial counts decrease in euthymic mice but increase in athymic mice [79]. The defect in athymic mice appears to be largely due to defective CD4 T cell mediated immunity [108]. *Salmonella* is also one of the most frequent pathogens in humans with Bare Lymphocyte Syndrome, a hereditary condition with defects in MHC class II, resulting in loss of CD4 activation and function [109]. Thus CD4 T cells are critical for protection against primary infection and the development of effective immunity to *Salmonella* infection.

CD4 T cells have the capacity to differentiate into multiple effector phenotypes. At present, five distinct CD4 T-cell lineages have been described: Th1, Th2, Th17, regulatory T-cells (Tregs) and follicular T helper (Tfh) cells [110], [111], [112], [113]. *Salmonella* infection induces predominantly Th1 cells, although Th17 cells have also been detected. IFN-αβ, IFN-γ, IL-12, IL-18, IL-6 and IL-1β stimulate the development of Th1 cells [111], [112]. To combat intracellular infection Th1 cells produce cytokines, such as IFN-γ and IL-2. IL-6, TGF-β, and IL-23 drive the development of Th17 cells in both humans and mice [113]. This subset of CD4 T cells produces the lineage-defining cytokines IL-17A and IL-17F, as well as IL-21, and IL-22 [114], [115]. These cytokines help to protect against extracellular bacteria, fungi, and play roles in autoimmunity [114].

IFN-γ dominant immunity to *Salmonella*

IFN-γ is highly induced in the *S*. Typhimurium-infected mucosa [116]. T-cells and NK cells are prime sources of IFN-γ production. Evidence from knockout mice and cell depletion experiments suggests that IFN-γ contributes to full-blown enteritis at days 2–4 after infection and that IFN-γ signaling is required for controlling pathogen loads in the mucosal tissue [78], [88], [77], [117]. These findings are in line with the established role of IFN-γ signaling at the interface between innate immunity (e.g. the enhanced macrophage killing) and adaptive immune responses (e.g. enhanced Th1 T-cell responses). IFN-γ has been shown to be very important in resistance to *Salmonella*. IFN-γ knockout mice infected with attenuated *Salmonella* are approximately 1000X more susceptible to infection than wild-type mice after intravenous infection [118].

IFN-γ also contributes to enteric inflammation at very early time points, where IFN-γ-deficient mice have a delayed onset of overt mucosal inflammation [119], [120] [78]. In particular, interferon-related genes (e.g. *cxcl9*, *igp1*, *cxcl10*, *gbp2*) are expressed with delayed kinetics in these mice. IFN-γ seems to coordinate not only T-cell responses and the anti-microbial activity of phagocytes but also goblet cell functions. Knockout mice lacking IFN-γR signaling in stromal cells were defective in triggering the release of mucin from goblet cells, a hallmark mucosal response in the streptomycin mouse model [121]. Enhanced mucin secretion is thought to limit pathogen access to the epithelial surface and thereby restrict mucosal pathogen invasion [80]. This is in line with the functional importance of IFN-γ in the early phase of *Salmonella* infection.

Salmonella-specific Th1 cells express the transcription factor T-bet, which defines the Th1 lineage and is required for IFN-γ production by these cells. IFN-γ is required for bacterial clearance during the late phase of *Salmonella* infection [122]. Using gene-targeted immunodeficient mice, Hess et al found that mice lacking CD4+ TCR-αβ cells and mice unable to respond to IFN-γ (deficient in the IFN-γ receptor [IFN-γR]) were highly susceptible to infection with an attenuated live vaccine strain of *Salmonella* (aroA-) [118]. Oral infection of 129Sv mice (Nramp1+) with WT *Salmonella* establishes a low chronic persistent infection that requires IFN-γ to limit bacterial replication and control the infection [46]. These same mechanisms are also important for protection against *Salmonella* infections in humans; humans with deficiencies in IL-12 or IFN-γR signaling have increased susceptibility to *Salmonella* infection [123]. Th1 requirement has been shown necessary in studies of chronic *Salmonella* infection. *Salmonella*-specific Th17 cells represent a minor population of CD4 T cells elicited by the infection, and their contribution to *Salmonella* immunity is undefined.

Role of IL-23 downstream mediators: IL-17/-22

The IL-17 cytokines IL-17A and IL-17F are strongly induced in the *S*. Typhimurium-infected mucosa, a phenotype apparently requiring Nod1/Nod2 and MyD88 signaling [124], [125], [117], [126]. Based on the increased susceptibility of IL-17-deficient mice in different mucosal bacterial infection models and DSS colitis [127], [128], [129], it had been suggested that IL-17 might be a key cytokine amplifying mucosal defense [88], [125]. However, the importance of IL-17 may be pathogen-specific. In humans, IL-17 signaling (IL-17RA, IL-17F) is important for defense against mucocutaneous candidiasis but not against *Salmonella* infection [130]. Similarly, in the streptomycin mouse model of *Salmonella* infection, antibody-mediated neutralization of IL-17A and IL-17F or IL-17RA deficiency did not affect mucosal inflammation. Moreover, the induction of more than 40 key cytokines and host defense genes typically triggered in response to mucosal *S*. Typhimurium infection were unaffected in the IL-17RA knockout mice [117]. Therefore, IL-17 does not seem to influence the pathogen–host interaction during the early phase of the mucosal response against *Salmonella,* although it appears to be important for defense against other microbes such as Citrobacter [126] and Candida [131]. To clarify the role of IL-17 in host defense against *Salmonella* we will look at the role of the IL-17 and its major signaling receptor IL-17RC, which is highly expressed in the gut epithelial cells, during acute phase *Salmonella* infection.

The cytokine IL-22 was first described as an IL-10-related T cell-derived factor that induces acute phase proteins in hepatocytes and produce large quantities of IL-22, which binds to IL-22 receptors on epithelial cells to modulate epithelial cell function [66]. iLCs from germ-free mice produce low levels of IL-22 [133], suggesting that intestinal bacteria drive IL-22 expression by these cells. Interestingly, iLC-derived IL-22 seems to be required for epithelial cell expression of Reg3γ [133], an antimicrobial protein that specifically targets Gram-positive bacteria, including segmented filamentous bacteria (SFB) [134]. Colonization of mice with SFB contributes to the expansion of IL-17 producing cells [135]. Reg3γ expression is dependent on both epithelial cell-intrinsic TLR signaling through MyD88 and IL-22 produced by iLCs. IL-22 was identified as a possible downstream target of IL-23 during the late phase of systemic *Salmonella* infection [66]. The role of IL-22 during acute *Salmonella* infection is unclear, and will be investigated herein.

Auxiliary Role of CD8 T cells and B cells in immunity to *Salmonella*

As for CD8+ T cells, their role in immune responses directed against *Salmonella* infection appears to be a minor one. Mice treated with anti-CD4 were unable to control late-phase *Salmonella* replication, whereas non-treated or anti-CD8 treated mice were able to clear *Salmonella*. This suggests that CD4 and not CD8 T-cells are responsible for acquired resistance, which occurs during the late phase of primary infection [79]. The most compelling evidence of the large role of CD4 T-cells versus CD8 T cells was shown in mice that deficient in MHC class I were resistant to *Salmonella* infection; however, mice deficient in αβ T cells were more susceptible than MHC class II deficient mice [118]. Another group found that mice lacking CD8+ T cells showed only a minor reduction in their ability to resist primary infection [136]. These studies support a limited ancillary role for CD8 T cells in immunity to *Salmonella*.

In addition to Th1 responses being essential for immunity to typhoid, B cells have been to shown to be important. B cell-deficient mice on the C57BL/6 background fail to develop robust protective immunity to *Salmonella* [54], [137]. Antibody may promote presentation of *Salmonella* antigens and augment CD4+ T cell immunity [138] [139]. Human vaccines against *Salmonella* also generate protective antibody [140]

Conclusion

The main goal of my thesis is to provide an enhanced understanding of host innate immune factors that contribute to resistance against *Salmonella* and that control the quality and quantity of the inflammatory response to this pathogen in a model that better mimics human disease in the hopes of creating improved therapies.

In this study, we focus on the role of IL-12 and IL-23, two critical host factors known to be necessary for protection against invasive NTS. Very little is known about IL-12 and IL-23's role in the gut particularly during the early stages of infection. Key elements of the mucosal immune response to *Salmonella* infection have been identified. However, additional work is required define the sequence of events, assign functional roles to particular cytokines and cells, and decipher redundancies in this system. Most likely, many of these mechanisms will be of general importance for understanding mucosal homeostasis, pathogen handling by the intestinal mucosa and how these early responses influence the context and magnitude of the systemic response. To start to address the overlap between mucosal and systemic contribution to human NTS disease we have utilized the streptomycin mouse model. This is an excellent system for addressing these issues, and in particular the contribution of cytokines IL-12 and IL-23 to early and late phase mucosal immunity.

Although more is known about the role of these cytokines during late-phase adaptive immune responses, all of these studies rely upon systemic or typhoidal infection models, which lack a significant enteric phase. In chapter 2 we use the colitis mouse model to better address how these molecules interact to protect against early colonization and mucosal inflammation.

In Chapter 3 we turn our attention to the role of IL-12 and IL-23 during the late phase of *Salmonella* infection. In chapter 3, we will evaluate the late phase responses in the context of an active enteric infection using the streptomycin pretreatment model. Because this model more closely mimics human NTS disease, it may provide additional insight to the role of IL-12 and in particular, IL-23, in promoting host defenses against *Salmonella* infection in humans during the adaptive phase of immunity.

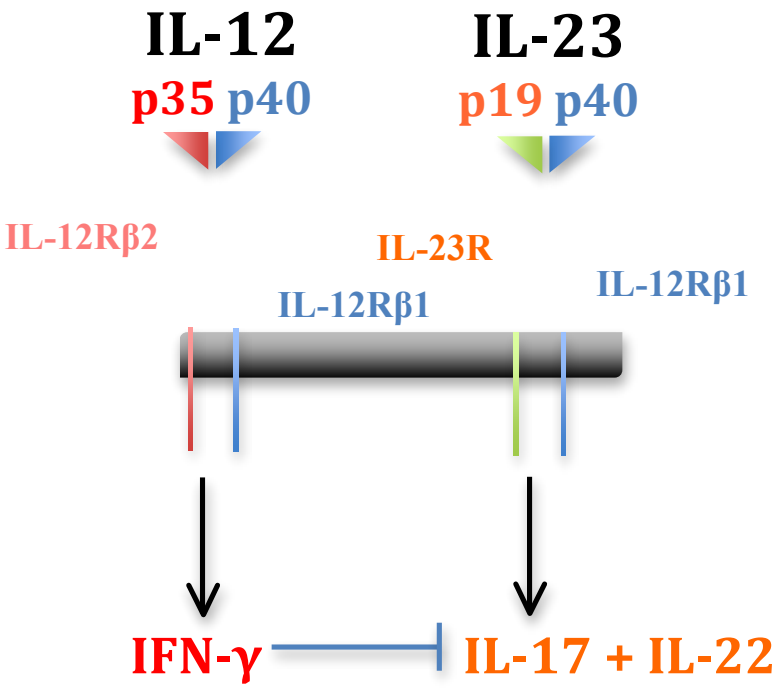


Figure 1: IL-12 and IL-23 cytokines, receptors and downstream cytokines

IL-12 is a covalently linked heterodimer composed of a light chain (IL-12p35) and a heavy chain (IL-12p40). The IL-12 receptor comprises IL-12Rβ1 and IL-12R β2, both of which have homology to gp130. IL-12–stimulated Th1 cells produce IFN-γ and suppress the differentiation of Th17 cells. The IL-12p40 component of IL-12 can also dimerize with IL-23p19 to form IL-23. The association of IL-12R β1 and IL-23R forms the receptor for this heterodimer. Upon activation, activated macrophages and dendritic cells at the site of infection rapidly produce IL-23. IL-23 then activates local resident Th17 cells, and other IL-22 or IL-17–producing innate immune cell

Chapter 2: Homeostatic Regulation of *Salmonella*-Induced Mucosal Inflammation and Injury by IL-23

Introduction:

IL-12, a heterodimeric cytokine consisting of p35 and p40 subunits, is produced during *Salmonella* infection by dendritic cells and macrophages, and regulates T-cell and NK cell IFN- γ production [141]. During *Salmonella* infection in mice, IFN-γ activates macrophages, and in the absence of IL-12, IFN-γ production is reduced and susceptibility to infection is increased [142]. Both IL-12 and IFN-γ help provide strong protective immunity against intracellular pathogens, such as non-typhoidal *Salmonella*, Mycobacterium species [143], influenza virus, *Francisella tularensis*, and *Yersinia pestis* [144]. IL-23 is a key cytokine for the development and function of Th17 cells, and contributes to the production of IL-17 and IL-22 by T-cells and lymphoid tissue inducer-like cells [145, 146]. IL-23 through downstream cytokines IL-22 and IL-17, help control infections by extracellular pathogens, such as *Candida albicans, Citrobacter rodentium, Pseudomonas aeuginosa, and Mycoplasma pneumonia*, and more recently data have also implicated these Th17 cytokines in inflammatory responses elicited by intracellular pathogens, such as *Salmonella enteritidis, Chlamydia muridarum, and Mycobacterium bovis* [125], [147], [148], [149], [128], [150]. Thus the nature of the pathogen (extracellular vs. intracellular) may not be the sole determinant for IL-23 mediated protective immunity, and other factors, such as site of infection (systemic vs. mucosal) may also determine the relative significance of IL-12 and IL-23.

Systemic and oral typhoidal mouse models of *Salmonella* infection indicate that IL-23 function is largely masked by IL-12 [66] and that IL-23 enhances protection against systemic *Salmonella* infection primarily through IL-22 [145] and to a lesser extent IL-17A [101]. Because mice are highly resistant to intestinal colonization by *Salmonella* and the murine typhoidal infection model does not evoke prominent intestinal inflammation as is typically present in humans and larger animals, it is possible that the role of IL-23 is less important in mice [101]. In order to address this possibility, the role of IL-23 was analyzed in an acute colitis model in mice, which demonstrated that IL-23 was required for cecal inflammation, IL-17A production and neutrophil recruitment[125]. The same group also showed that γδ-T cells were the primary producers of IL-17A and cellular target for IL-23 [125]. The roles of IL-12 and its interactions with IL-23 during

acute *Salmonella* induced enterocolitis have not been investigated.

In this study, we use the acute streptomycin pretreatment colitis model of *Salmonella*-induced acute enterocolitis[16] to define the roles IL-12 and IL-23 in regulating host resistance and intestinal inflammation during acute *Salmonella* infection. Our data show that IL-12 promotes the production of IFN-γ, which is largely produced by NK cells during acute infection[78]. IFN-γ is required for resistance against *Salmonella* and also for induction of intestinal inflammation and epithelial cell injury. In contrast, IL-23 limits inflammation and mucosal injury. In conclusion, interactions between IL-12 and IL-23 help protect against mucosal *Salmonella* infection, limit IFN-γ-mediated intestinal injury, and maintain epithelial homeostasis.

Results:

IL-12 and IL-23 control gut colonization and systemic spread during oral S. Typhimurium infection.

Streptomycin pretreated C57BL/6, $p19^{-/-}$, $p35^{-/-}$ and $p40^{-/-}$ mice were infected orally with a low dose of *S*. Typhimurium (10^3 CFU) and bacterial burden was measured in the cecum and liver 3 days later (Fig 2-1 A and B). IL-12 deficient mice ($p35^{-/-}$) had elevated CFU in the liver relative to WT and had elevated counts in both the cecum and liver relative to IL-23 deficient mice. In contrast, mice deficient in both IL-12 and IL-23 ($p40^{-/-}$) had elevated CFU in the cecum and liver when compared to WT or $p19^{-/-}$ mice (Fig. 2-1 A and B), and elevated liver CFU when compared to $p35^{-/-}$ mice. These results suggest that loss of IL-12 alone results in increased early systemic dissemination, and that the additional loss of IL-23 increases cecal colonization, and further increases systemic spread, as seen in previous studies [66]. The loss of IL-23 alone did not affect gut colonization or systemic spread, as bacterial burden in $p19^{-/-}$ mice was not significantly different from WT mice. Similar findings were observed for the $p19^{-/-}$, $p35^{-/-}$ and wild-type C57BL/6 mice that were congenic for the $Slc11a1^{G169/G169}$ resistant allele, although the overall bacterial burden in the liver was lower in these mice, consistent with the protective role of $Slc11a1^{G169}$ (Fig. S1).

IL-12 family members differentially regulate cecal inflammation and injury.

Gross examination of the ceca on day 3 following *S*. Typhimurium infection revealed that all ceca were small, thick and pale compared to mock treated controls (Fig. 2-1 C). Infected ceca from $p35^{-/-}$ and $p40^{-/-}$ mice were slightly larger and had more visible cecal contents relative to

WT and p19$^{-/-}$ ceca suggesting less inflammation in the former groups. Histological analysis revealed similar levels of inflammation amongst the various groups (Fig 2-1 F, with representative sections shown in 2D and 2E). Although not part of the inflammatory score, we noted that some mice had prominent numbers of apoptotic bodies in the lamina propria usually near the base of the epithelial crypts (arrows in Fig. 2-1 E). Quantification of the histologic cell death revealed that p19$^{-/-}$ mice had significantly more dead cells within the lamina propria compared to the other mice (Fig. 2-1 G). To confirm our histological observations, we performed TUNEL staining, a common method for detecting DNA fragmentation that occurs during apoptotic cell death. Compared to *S*. Typhimurium infected WT mice, p19$^{-/-}$ mice had more, and p35$^{-/-}$ and p40$^{-/-}$ mice had fewer TUNEL-positive cells (Fig. 2-2 A and B). Conversely, the number of goblet cells, an indicator of epithelial cell differentiation, was decreased in the p19$^{-/-}$ and increased in p35$^{-/-}$ and p40$^{-/-}$ mice relative to WT and p19$^{-/-}$ mice, uncovering a novel role for IL-12 and IL-23 in regulating gut epithelial responses to *Salmonella* infection (Fig. 2-2 C and D). Taken together, our data indicate that loss of IL-23 resulted in increased *Salmonella* induced epithelial cell death that required functional IL-12.

Differential contribution of IL-17A and IL-22 to Salmonella induced inflammation and injury.

We next hypothesized that downstream targets of IL-23 may protect the epithelium from apoptosis. We therefore examined gene expression in cecal tissue for IL-17A, IL-22, and Reg3γ. IL-17A expression correlated strongly with IL-23 genotype; IL-17A was induced to similar levels in WT and p35–/– mice, and not induced in p19$^{-/-}$ and p40$^{-/-}$ mice (Fig. 2-3 A). In contrast, induction of IL-22 was only absent in p40$^{-/-}$ mice (Fig. 2-3 B), while Reg3γ, an antimicrobial peptide that is regulated by IL-22 [148], was reduced in both p19$^{-/-}$ and p40$^{-/-}$ mice (Fig. 2-3 B and C).

To test whether IL-17 or IL-22 may have a cytoprotective role during *S*. Typhimurium infection in the cecum, we infected WT and IL-17RC$^{-/-}$ mice (C57BL/6 *Slc11a1*$^{G169/G169}$ background) and found that similar to p19$^{-/-}$ mice (Fig 2-1 A and B), the loss of IL-17RC did not affect cecal or liver CFU (Fig. 2-3 D and E). The IL-17RC gene is expressed highly in the small intestine and colon in both mice and humans, and in particular by murine colonic epithelial cells [151], [152], [131]. IL-17RC appears to be required for both IL-17A and IL-17F signaling [131]. The ceca from infected mice showed comparable levels of inflammation (Fig. 2-3 F). However, cell death was increased in IL-17RC$^{-/-}$ mice (Fig 2-3G), which was confirmed by TUNEL staining (Fig. 2-3

H and I), and suggests that IL-17 may have a cytoprotective effect on cecal epithelium similar to that observed for IL-23. We also infected IL-22$^{-/-}$ C57BL/6 mice and found that IL-22 did not affect cecal colonization or systemic spread of *S*. Typhimurium (Fig. S2A–B). However, IL-22$^{-/-}$ mice had significantly less inflammation and fewer TUNEL-positive cells than C57BL/6 controls (Fig. S2C–D), suggesting that IL-22 contributes to acute cecal inflammation. Decreased cell death in IL-22$^{-/-}$ mice correlated with the decreased inflammation.

Preservation of TNF, IL-6, IL-1B and Nos2 expression correlates with protection against *Salmonella* infection

We determined whether the expression of selected genes correlated with protection against *Salmonella* infection (Fig. 2-4 A–D). TNF and Nos2 were decreased in the p40$^{-/-}$ relative to all other mice (Fig. 2-4 A and B); basal levels of TNF were increased in mock-infected p19$^{-/-}$ mice although levels were substantially lower than infected mice. In addition, p19$^{-/-}$ expressed more IL-6 than each of the other mice (Fig. 2-4 C), and more IL-1B than the p35$^{-/-}$ and p40$^{-/-}$mice (Fig. 2-4 D). Anti-inflammatory cytokines, IL-10 and TGF-β (Fig 2-4 E and F) showed similar patterns of expression as the pro-inflammatory cytokines, suggesting that decreased anti-inflammatory cytokine expression did not explain the increased inflammation and injury.

TNF and Nos2 expression appear to be critical for limiting *Salmonella* growth in mice, as evidenced by the marked susceptibility of p40$^{-/-}$mice to infection. TNF and Nos2 producing dendritic cells are critical mediators of host defense against the intracellular bacterium *Listeria monocytogenes* [153]. To identify cells that were producing TNF, we isolated and examined lamina propria cells from mock and *Salmonella* infected C57BL/6 mice. *Salmonella* infection resulted in a marked expansion of CD11b^{hi}, CD11c^{+} cells (Fig. 2-4G). During *S*. Typhimurium infection, these cells upregulated class II MHC and were the primary producers of TNF; neutrophils (CD11b^{hi}, Ly6g^{hi} CD11c^{null} MHC classII^{null} cells) also contributed to a lesser extent (data not shown). Within the CD11b^{hi}, CD11c^{+} cell populations, the proportion of MHC class II positive and the TNF/MHC class II double positive populations were significantly increased in p19$^{-/-}$ and decreased in p40$^{-/-}$ relative to the other mice (Fig. 2-4 I and J). These data indicate that loss of IL-23 promotes the accumulation of TNF producing cells when functional IL-12 is present, and limits their accumulation when IL-12 is absent.

IL-23 regulates IFN-γ mediated enterocolitis in an IL-12 dependent manner.

Increased expression of IL-6 and IL-10 in the ceca of infected p19$^{-/-}$ mice was dependent on functional IL-12 (Fig. 2-4 C and E). Because some of the consequences of IL-23 deficiency were dependent on the functional status of IL-12, we examined the expression of IFN-γ, a major downstream target of IL-12 (Fig. 1), in cecal tissue of infected mice. Unexpectedly, the loss of IL-23 also led to significantly elevated IFN-γ message and as expected p35$^{-/-}$ and p40$^{-/-}$ mice had decreased levels of IFN-γ relative to WT mice (Fig. 2-5 A). To better characterize the mechanism for IL-23 inhibition of IFN- γ, we analyzed IL-12p35 expression (Fig 2-5 B). IL-12p35 expression was increased in p19$^{-/-}$ relative to WT mice and directly correlated with IFN-γ levels. This suggests that IL-23 inhibits IFN-γ in an IL-12p35 dependent manner. To further explore the role of IFN-γ in cecal inflammation and injury, we infected IFN-γ$^{-/-}$ mice with *S*. Typhimurium. IFN-γ $^{-/-}$ mice had higher levels of cecal colonization, and as anticipated, had increased liver CFU at day 3 post-infection (Fig. 2-5 C and D) similar to IL-12p35-deficient mice (Fig 2-1 A and B). Remarkably, IFN-γ$^{-/-}$ mice had substantially reduced cecal inflammation (Fig. 2-5 E, Fig. S3A–D), and even less than p35$^{-/-}$ or p40$^{-/-}$ mice (Fig. 2-2). The IFN-γ$^{-/-}$ mice also had less injury to the cecal mucosa, as evidenced by fewer TUNEL-positive cells and better preservation of Alcian blue positive cells (Fig. 2-5 F and G, and Fig. S3E). The decreased inflammation in IFN-γ$^{-/-}$ mice was associated with fewer CD11b^{hi}, CD11c^{+} TNF/MHC class II double positive inflammatory monocytes in the lamina propria, and lower levels of TNF, IL-6 and IL-1β message in the cecal tissue (Fig. S4). There was no difference in IL-17A expression in the cecum (Fig. S4F). These data suggest that IFN-γ is not only important for protection against *Salmonella*, but is also a key regulator of gut inflammation and injury, which is modulated by the activities of IL-23 and IL-12.

NK1.1+ cells are the primary IFN-γ producers during acute S. Typhimurium infection.

Due to IFN-γ's critical role in acute cecal inflammation, we sought to identify the cell types that produce IFN-γ during *Salmonella* infection. In mock-infected mice, IFN-γ production is essentially undetectable, and stimulation with PMA and ionomycin induces IFN-γ in CD4, CD8, γδ and NK T cells, as well conventional NK cells (Fig. S5A). Upon *S*. Typhimurium infection, all of these subsets demonstrate spontaneous increases in IFN-γ production and NK cells have the highest proportion of lamina propria IFN-γ producing cells (Fig. S5B). Amongst the various mock treated mouse groups, there were no differences in the percentage of NK cells (data not shown). After infection, the percentage of NK cells in the lamina propria decreased in all backgrounds, but p35$^{-/-}$ and p40$^{-/-}$ animals decreased to a lesser degree (Fig. 2-6 A), possibly

reflecting an IL-12 dependent dilution of NK cells by recruitment of other cells or loss of NK cells during infection. Both $p35^{-/-}$ and $p40^{-/-}$ NK cells failed to produce IFN-γ *ex vivo* (Fig. 2-6 B and C). The loss of IL-23 had no effect on the percentage of NK cells in LP relative to WT mice (Fig 2-6 A), but resulted in a significant increase in IFN-γ production (Fig. 2-6 B and C). Thus in the lamina propria during *Salmonella* infection, IL-12 and IL-23 have opposing roles in regulating IFN-γ by NK cells. Because previous reports have concluded that alveolar and splenic myeloid cells, especially neutrophils, are the primary producers of IFN-γ during acute *S.* Typhimurium infection [69], [154] we examined IFN-γ production by lamina propria neutrophils ($Ly6G^{hi}$ $CDllb^{hi}$) or monocyte/macrophages ($Ly6g^{int}$ $CDllb^{int}$ $CDllc^{+}$) in mock or *S.* Typhimurium infected mice. Lamina propria monocytes and neutrophils showed no specific staining for IFN-γ (Fig. S6A–B). Once again, IFN-γ was produced predominantly by the $CDllb^{int}Ly6g^{null}CDllc^{null}$ cell population, consistent with NK cells (Fig. S6A–B). These data support the role of NK cells as key producers of IFN-γ in the gut following acute *Salmonella* infection.

Discussion

The mucosal immune system is continuously exposed to trillions of microbes and faced with the daunting task of differentiating benign commensal microbes from detrimental pathogens. This distinction is essential in order to avoid excessive inflammatory responses to commensal organisms and to mount appropriate inflammatory responses against pathogens. Because commensals and pathogens share many if not all of the molecular patterns that are recognized by the innate immune system, additional factors that define pathogenicity, such as invasion and toxicity, must be interpreted by the host at the mucosal surface. Thus benign or mutually beneficial interactions with microbes are non-inflammatory or even immunosuppressive [155], whereas microbes that alter this homeostatic balance trigger inflammation. Innate immune receptors are critical for sensing microbes and regulating inflammatory responses through the production of immunomodulatory cytokines. Several cytokines are essential to maintain tolerance to the gut microbiota, as evidenced by the association of IL-10, FoxP3 or TGFβ1 function in mice or humans with inflammatory bowel disease (IBD) or autoinflammatory disorders [156], [157]. Other cytokines are critical for protection against pathogens that infect hosts through mucosal surfaces. Notably, IL-12/23p40 is essential for proper control of mycobacterial and *Salmonella* infections in humans and mice [99], [100], [66]. IL-12/23p40 and IL-23R have also been associated with mouse and human IBD, respectively, suggesting that these cytokines not only contribute to the proper control of infectious pathogens, but also contribute to excessive and harmful inflammation.

Our studies shed light into the molecular mechanisms that underlie the apparent masking of IL-23 by IL-12 during *Salmonella* infection [100]. Our detailed analyses indicate that IL-23 does not simply have redundant functions that are compensated for by IL-12 during *Salmonella* infection in p19–/– mice. Instead the interactions between IL12 and IL-23 are complex. Loss of IL-23 resulted in decreased expression of some genes, such as IL-17A and Reg3γ, and increased expression of others such as IL-12p35 and IFN-γ. In the presence of functional IL-12, the loss of IL-23 may not affect susceptibility to *Salmonella* infection due to enhanced IFN-γ production and accumulation of TNF and Nos2 producing cells. However, the compensatory gain in IL-12 and IFN-γ mediated protection against *Salmonella* infection does not go without consequence, and the trade-off is enhanced IL-12 and IFN-γ dependent tissue injury. In the absence of functional IL-12, IL-23 preserves the expression of inflammatory mediators, such as IL-17A, IL-22, Reg3γ, TNF and Nos2, and the accumulation of TNF producing cells in the large intestine of *Salmonella* infected mice.

Our study demonstrated that IL-12 and IL-23 regulate several important inflammatory mediators in the cecum during acute *Salmonella* infection. In particular, both IL-12 and IL-23 contributed to the regulation of MHC class II^{+} TNF producing CD11b^{hi}, CD11c^{+} cells. The expression of MHC class II and TNF were also highly dependent on IFN-γ, and our studies indicated that IFN-γ is a major factor for promoting acute *S*. Typhimurium induced intestinal inflammation. Because the intestinal inflammation seen in IFN-γ KO mice was substantially lower than p35$^{-/-}$ and p40$^{-/-}$ mice, IFN-γ appeared to also function independent of these cytokines as a critical inducer of early inflammation. IFN-γ is known to trigger release of several CCR-1,-2 and CXCR3 binding chemokines [158] and it is possible that IFN-γ recruits additional inflammatory cells and helps transform the normally immunosuppressive environment of the cecal mucosa into a pro-inflammatory and Th1-biased environment that helps fight *Salmonella* infection. This hypothesis is consistent with the severe reduction in CD11b^{hi}, CD11c^{+} cells and NK cells in the cecum of IFN-γ KO mice infected with *Salmonella* (see Fig. 2-6).

IL-23 regulation of mucosal injury correlates with IL-12 dependent IFN-γ expression; IL-23 suppresses IL-12 expression during mucosal *Salmonella* infection, and thereby limits IFN-γ production. During *Salmonella* infection IL-23 deficiency resulted in increased IL-12 and IFN-γ production, which is associated with excessive mucosal injury as evidenced by loss of goblet cells and increased cell death within the mucosa. The cell death is most notable in the base of the epithelial crypts, and requires IL-12 and IFN-γ. It is possible that cytokines may be directly toxic to epithelium, or that the cell death may be a manifestation of other IFN-γ regulated factors such as reactive oxygen and nitric oxide. A recent study by Songhet et al. compliments our results and

demonstrates that IFNγR contributes to inflammation and epithelial cell injury during acute *Salmonella* infection, and bone marrow chimera studies indicate that IFNγR expression by stromal cells is required for loss of goblet cells seen during acute infection [121]. IFN-γ can act directly on intestinal epithelial cells to influence the β-catenin signaling pathway and epithelial cell homeostasis [159]. Thus during *Salmonella* infection IFN-γ may act directly on epithelial cells, and excessive IFN-γ production may yield epithelial cell injury and death.

In addition to regulating IL-12 and IFN-γ production, our data indicated that IL-23 also has cytoprotective functions, which were mediated at least in part by IL-17RC. IL-17RC$^{-/-}$ mice had increased basal cell death and TUNEL positive cells in the mucosa similar to that observed in p19$^{-/-}$ mice (Fig. 2-3). IL-17RC is highly expressed on the epithelium of the large intestine [151], [152] and IL-17 has been associated with cytoprotection from inflammation in other models associated with increased IFN-γ production [160], [161]. Administration of α-IL-17A monoclonal antibody had no effect in patients with moderate to severe Crohn's disease, and exacerbated disease in some patients, further suggesting a protective role for IL-17A [162]. Thus IL-17 appeared to have important cytoprotective functions in the mucosa, and may be especially important in limiting IFN-γ mediated host cell injury.

IL-6 and IL-10 gene expression were also significantly increased in infected p19$^{-/-}$ mice, and these cytokines may contribute to host defense and mucosal tissue injury against *Salmonella* infection in p19$^{-/-}$. Upregulation of these cytokines also appears to be dependent on functional IL-12, as seen for IFN-γ. Although we focused our analysis on IFN-γ, IL-6 and IL-10 may also be important mediators of the mucosal responses to *Salmonella* infection. Additional studies will be needed to determine how these factors contribute to host defense, mucosal inflammation and tissue injury during acute *Salmonella* infection.

Our finding that IL-23 cross-regulated IL-12 and modulated IFN-γ is novel to host immune regulation against *Salmonella*, but has been observed in other models. In the TNBS induced colitis model mice deficient in IL-23 produced more IL-12 and were more susceptible to development of colitis than WT mice [97]. In this study, dendritic cells from p19$^{-/-}$ produced elevated levels of IL-12 upon TLR ligation, and treatment of p19$^{-/-}$ mice with an IL-12p40 neutralizing antibody prevented lethal colitis [97]. Similarly, in a tumor initiation and metastasis model, IL-23 suppressed NK cell and IFN-γ dependent anti-tumor activities [163]. Although our data indicate that IL-23 modulates IL-12 expression, IL-23 may also antagonize IL-12 binding to its receptor [164]. Taken together, the data indicate that IL-23 can also negatively impact IFN-γ effector functions and oppose the action of IL-12.

IL-23 also regulates IL-22 production, but during acute *Salmonella* infection, there was only a slight decrease in IL-22 production in $p19^{-/-}$ mice, which was not significant but consistent with prior studies [125]. The IL-22 regulated gene Reg3γ was decreased significantly in the $p19^{-/-}$ mice. There was a significant decrease of IL-22 expression in $p40^{-/-}$ mice but no noticeable difference in $p35^{-/-}$ ceca (Fig. 2-2), suggesting IL-22 dependence on IL-23 is partially masked by IL-12. Surprisingly, IL-22 deficiency resulted in decreased inflammation (Fig. S2). Thus during acute *Salmonella* infection IL-22 promotes intestinal inflammation, and a portion of this response is regulated independently of IL-23. IL-22 is made by multiple cell types, and in other models of acute intestinal infection and inflammation, ILC's appear to be the primary source of IL-22 [146], [165]. In contrast to IFN-γ, we were unable to detect significant levels of IL-22 produced spontaneously in lamina propria cells, suggesting that additional signals mimicked by PMA and ionomycin are needed [166], [167]. It is not known how innate lymphoid cells and NK cells sense infection in the mucosa, and whether they employ innate immune receptors, or respond to changes in cytokines or host cell surface ligands.

In summary we demonstrated that during acute oral *Salmonella* infection, IL-23 suppressed IL-12 and IFN-γ mediated mucosal injury, and, in combination with IL-12, is essential for optimal protection against systemic dissemination. IL-23 contributes to both cytoprotection in the mucosa and host defense, and is critical for fine-tuning the intestinal mucosal inflammatory response in order to limit excessive injury and promote pathogen clearance. Thus cytokines IL-12 and IL-23 work in concert to help respond to infectious pathogens and maintain epithelial homeostasis.

Experimental Procedures

Mice:

C57BL/6 (B6), IL-12p35$^{-/-}$, IFN- γ$^{-/-}$ and IL-12p40$^{-/-}$ mice were from The Jackson Laboratory and bred in-house. IL-23p19$^{-/-}$, IL-17RC$^{-/-}$ and IL-22$^{-/-}$ mice bred onto the C57BL/6 background were kindly provided by Dr. Wenjun Ouyang (Genentech)[168,148]. Information on the generation of C57BL/6 *SLc11a1*$^{G169/G169}$ congenic mice can be found in the Supplemental Methods section. Age-matched male and female mice between the ages of 8 and 12 wk were used in experiments. Mice were bred and housed in a specific pathogen-free facility at the University of Washington. All experiments were performed under Institutional Animal Care and Use Committee-approved protocols.

Bacterial Strains:

Salmonella enterica serovar Typhimurium strain SL1344[169] was grown overnight at 37°C with shaking (200 rpm) in Luria-Bertani (LB) broth. This strain is resistant to streptomycin. Animal experiments: Mice were fasted for 4-6 hr and pretreated with 20mg of Streptomycin (ISC Bioexpress, Solon, Ohio) in 0.1 cc sterile phosphate buffered saline (PBS) by oral gavage. The following day mice were fasted for 4-6 hr and infected with $1x10^3$ *S*. Typhimurium or nothing (mock) in 0.1 cc of sterile PBS by oral gavage. At 72hrs after infection, mice were euthanized. Whole cecum and liver were weighed prior to further analysis. To assess bacterial burden sections of each organ were weighed and homogenized in PBS with 0.025% Triton X-100 (EMD chemicals, Darmstadt, Germany), and serial dilutions were plated on streptomycin (50µg/ml) containing MacConkey agar plates.

Histopathology/Immunohistochemistry:

Samples of cecum were fixed overnight at RT in 10% buffered formalin (Sigma-Aldrich, St. Louis, MO), and then processed. Four-micrometer sections were cut and stained with hematoxylin and eosin (H&E) for morphological analysis. Sections were blinded to mouse genotype and evaluated by a board certified anatomic pathologist (KDS). All scoring categories range from 0-3 with increasing level of severity. Scores were assigned for changes to the cecum as follows: submucosal expansion (S) - 0 = no significant change, 1 = <25% of the wall, 2 = 25-50% of the wall, 3 = >50% of the wall; mucosal neutrophilic infiltrate (M) - 0 = no significant infiltrate, 1 = mild neutrophilic inflammation, 2 = moderate neutrophilic inflammation, 3 = severe neutrophilic inflammation; lymphoplasmacytosis (L) - 0 = no significant infiltrate, 1= focal infiltrates (mild), 2= mutifocal infiltrates (moderate), 3 = extensive infiltrates involving mucosa and submucosa (severe); goblet cells loss (G) - 0 = >28/HPF, 1 = 11-28/HPF, 2 = 1-10/HPF, 3 = <1/HPF. Sections were scored for two other criteria, epithelial integrity and architectural distortion, but these were not affected at 3d timepoint and thus not included in the final scoring. The combined pathological score for each tissue sample was determined as the sum of these individual scores: 0 no signs of inflammation; 1–3 minimal signs of inflammation that are normally found in untreated specific pathogen-free mice and therefore considered as nonspecific; 4-6 slight inflammation; 6–8 moderate inflammation; >9 profound inflammation. Basal cell death was assessed qualitatively by grading cell death within the basal regions of the crypts as follows: 0 = no significant dead cells, 1 = focal dead cells, 2 = multifocal cell death, and 3 = extensive cell

death. TUNEL and Alcian Blue staining material and methods can be found in Supplemental Methods section. From each group cecal sections from a minimum of 3 different mice were analyzed based on morphology and TUNEL (brown) or Alcian blue (blue) positive cells. At least 10 random high-powered fields (40X objective) were counted from each mouse and means calculated.

Quantitative Real-Time PCR (qPCR):

For analysis of changes in gene expression in the mouse cecum after *Salmonella* infection, tissue samples were collected and immediately frozen and stored at -80°C until processing. RNA was then extracted from frozen tissue with TRIzol Reagent (Invitrogen, Carlsbad, CA) according to the instructions of the manufacturer. At least 2 μg of RNA from each sample was reverse transcribed in 20μl reactions using oligo-dT and the Superscript III reverse transcription reagent (Invitrogen). Real-time PCR was performed on cDNA with TaqMan chemistry (ABI) and commercially available, inventoried probe-primer sets (Applied Biosystems (ABI), San Francisco, CA) according to the manufacturers recommended procedures (ABI). The GAPDH probe-primer set (ABI) was used in all reactions as an internal positive control and to normalize the data. Real-time PCR was performed using and ABI 7900 real-time PCR system.

Preparation of Lamina Propria (LP) cells:

To prepare single cell suspensions, large bowel (cecum and colon) were resected, and contents purged with PBS. The isolation of lamina propria cells was adapted from Lugering et al. [170], and is described in detail the Supplemental Methods. LP cells were counted using Trypan Blue exclusion to measure viability (>95% viability) and approximately 1 x10^5 - 10^6 cells/well were distributed in 96 well round bottom plates. LP cell suspensions from mock or infected animals were cultured for approximately 4hrs with 10ug/ml Brefeldin A (Sigma) at 37°C in 96-well round bottom plates prior to staining for flow cytometry. In some experiments, cells were also stimulated with PMA (50ng/ml) and ionomycin (0.7μM) for 4 hours in the presence of BFA.

Flow Cytometry:

All antibodies used for flow cytometric analysis were purchased from BD Biosciences (San Jose, CA) or eBioscience (San Diego, CA). Following incubation, cells were treated with 2mM EDTA in PBS for 10min at 37°C to detach adherent cells. All future steps were incubated on ice. Cells were washed with PBSA (1% w/v BSA in PBS), and then Fc receptors were blocked using α-

CD16/32 for 15 min. Surface and intracellular staining material and methods can be found in Supplemental Methods section. We collected 1-3x10^5 gated events using a BD LSR II or Canto I flow cytometer. The data were analyzed using FlowJo software (Treestar, Inc., Ashland, OR).

Statistics:

We calculated statistical significance with Prism software (GraphPad, La Jolla, CA). Unless otherwise specified, all studies for which data are presented are representative of at least two independent experiments. The D'Agostino & Pearson omnibus normality test confirmed that the log transformed gene expression and CFU data passed the normality test. Statistical analysis of more than two normally distributed groups was performed using ANOVA with Bonferroni post-test; comparison of two normally distributed groups was done using the unpaired Student's T-test with Welch's correction for unequal variance. For multiple comparisons of non-Gaussian distributed data, the groups were analyzed using the Kruskal–Wallis test followed by Dunn's post-test, and comparison of two groups of non-Gaussian distributed data was performed using the Mann–Whitney rank-sum test. Statistical significance was defined as a P value <0.05, and indicated in the figure legends.

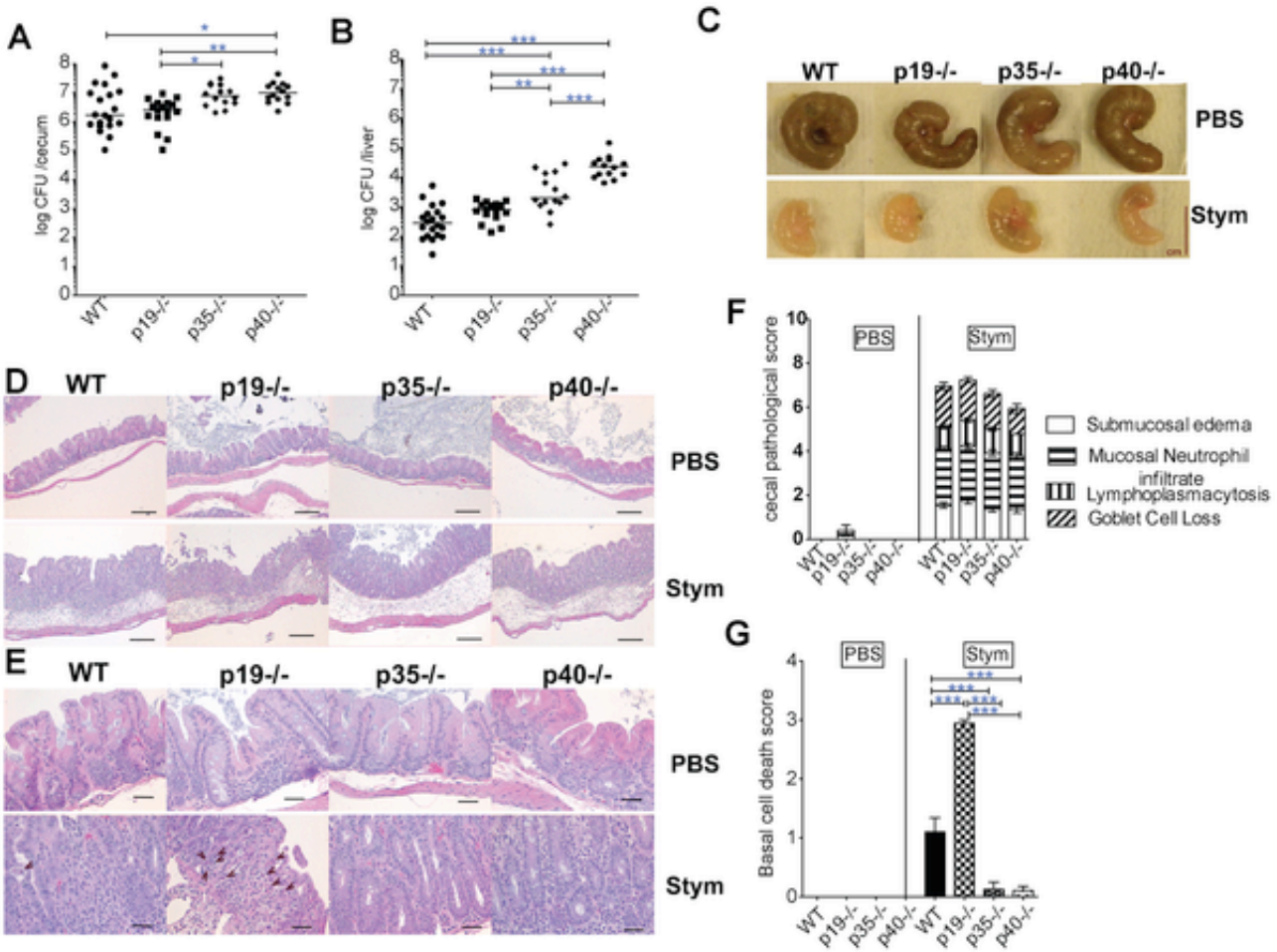


Figure 2-1: IL-12 and IL-23 are required for resistance against *S.* Typhimurium (Stym).

WT, p19$^{-/-}$(no IL-23), p35$^{-/-}$(no IL-12), and p40$^{-/-}$ (no IL-12/-23) mice were orally infected with 1x10^{3} CFU of *S.* Typhimurium 1 day after 20mg streptomycin pretreatment. (A) Cecum and (B) liver were collected from infected mice 3 days post infection. Organs were weighed, homogenized and plated to determine CFU/organ. Bars represent the median bacterial load. Pooled data from 3 separate experiments are shown in A and B. (C) Representative ceca are shown for each group at 3d after oral inoculation with PBS (mock) or Stym. A one centimeter bar indicates the magnification. (D) 10X and (E) 40X magnification of histopathology of H&E stained cecal sections of WT, p19$^{-/-}$, p35$^{-/-}$, and p40$^{-/-}$ mice 3d after infection. Bar in (D) represents 100 micron and (E) 20 micron. Notched arrow-heads in (E) indicate cell death. (F) Blinded scoring was performed on H&E-stained cecal sections. The inflammatory score equals the sum of the separate categories (edema, PMN infiltration, lymphoplasmacytosis, and goblet cell loss). (G) H&E stained cecal sections from WT, p19$^{-/-}$, p35$^{-/-}$, and p40 mice 3d after infection were blinded and qualitatively assessed for cell death: 0 (no significant), 1 (focal), 2 (mild), 3 (severe) in the basal epithelium. At least 3 mock treated animals and at least 13 infected mice from each group from 3 separate experiments were scored in 1F and 1G. Mean values ± SE are shown. Statistical significance was determined using the one way ANOVA test with Bonferroni post test (A, B, F and G). *:p<0.05, **:p<0.01, ***:p<0.001.

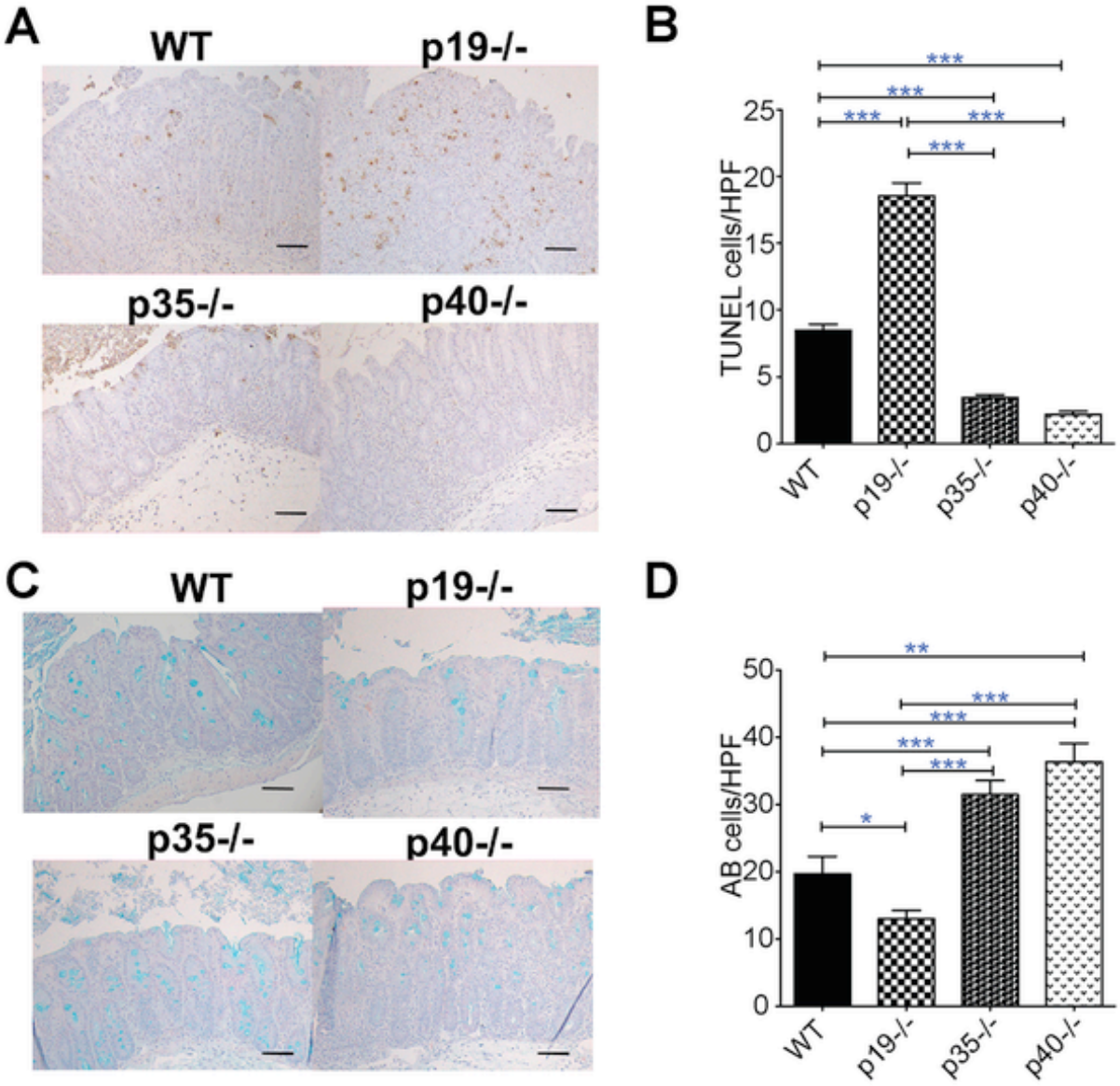


Figure 2-2: Loss of IL-23 in the context of functional IL-12 results in increased epithelial cell death and loss of goblet cells.

(A) 20X magnification of TUNEL-positive (brown) or (C) Alcian Blue (blue) cells of representative infected cecal sections. The bar in (A) and (C) represents 20 micron. The mean ± SE of TUNEL (B) positive cells per high-power field was quantified from cecal sections WT (n=4), $p19^{-/-}$ (n=5), $p35^{-/-}$ (n=6)and $p40^{-/-}$ (n=3) mice. The mean ± SE Alcian Blue positive cells were quantified per high-power field from cecal sections of the same mice (n=3 per group). Statistical significance for (B and D) was determined using the one way ANOVA followed by Bonferroni post test. *:$p<0.05$, **:$p<0.01$, ***:$p<0.001$

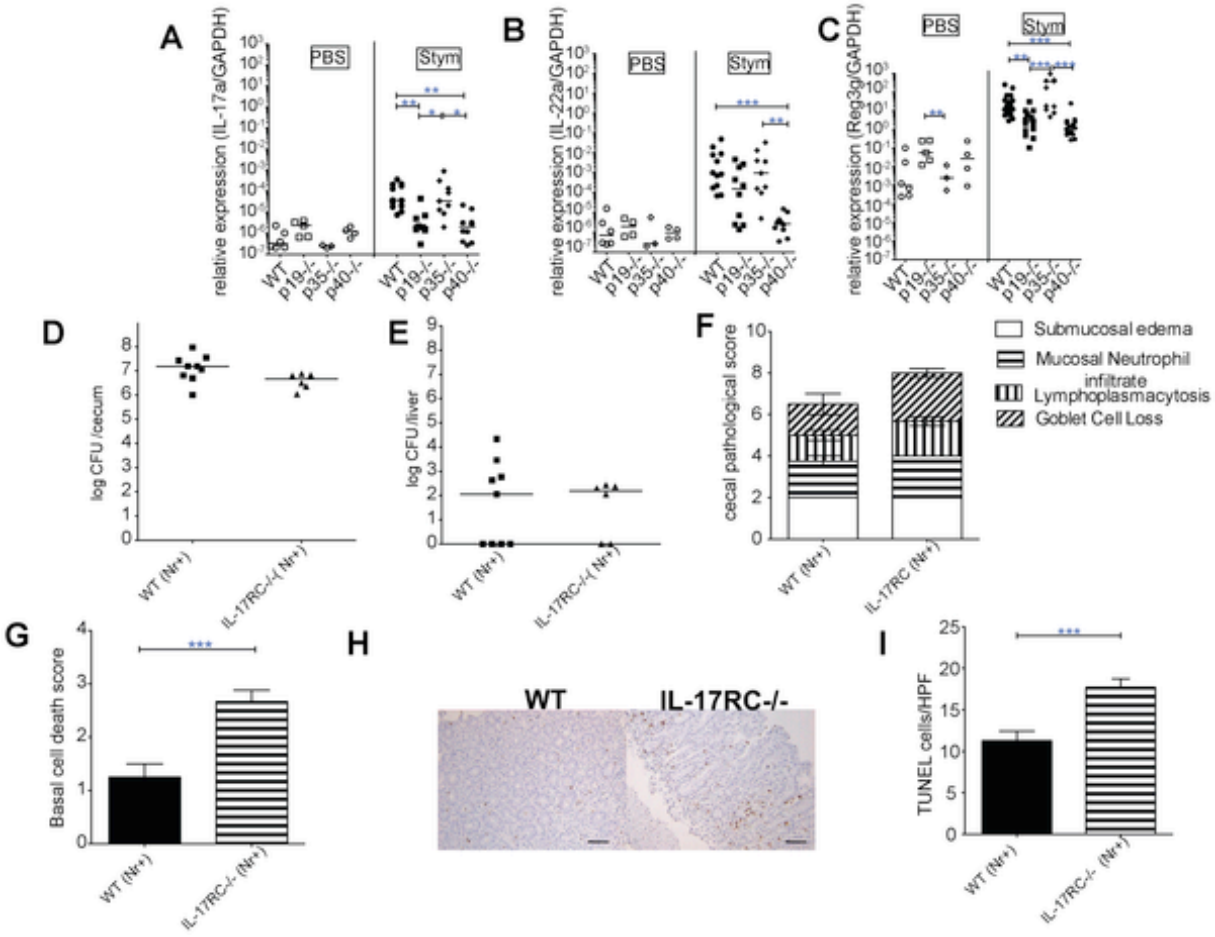


Figure 2-3: IL-17 has cytoprotective activity during *Salmonella* induced injury.

(A) Changes in IL-17A, (B) IL-22 (C) Reg3γ gene expression elicited 3d after oral PBS (mock) or *S*. Typhimurium (Stym) infection in the ceca of WT, $p19^{-/-}$, $p35^{-/-}$, $p40^{-/-}$ mice were measured by qPCR. Data are expressed as the ratio of mRNA levels of the gene of interest divided by GAPDH expression from the same RNA. Pooled data from 3 separate experiments are shown. (D) Cecum and (E) liver were collected from IL-$17RC^{-/-}$ and C57BL/6 mice congenic for *Slc11a1*$^{G169/G169}$ three days post infection. Organ homogenates were diluted and plated to determine CFU/organ. Bars represent the median bacterial load from two experiments. (F) Histological changes and (G) basal cell death at 3d post-infection from infected WT and IL-$17RC^{-/-}$ H&E stained cecal sections were done as described in Fig. 2-1. (H) 20X magnification of TUNEL-positive (brown) cells of representative infected cecal sections. Bar in (H) represents 50 micron. (I) TUNEL positive cells were quantified per high-power field in the cecal sections from a minimum of 3 infected mice and two experiments. Statistical significance was determined using one way ANOVA with Bonferroni post test for A-C, Mann Whitney for D&E, and unpaired Student's t-test for F,G & I. *:$p<0.05$, **:$p<0.01$, ***:$p<0.001$.

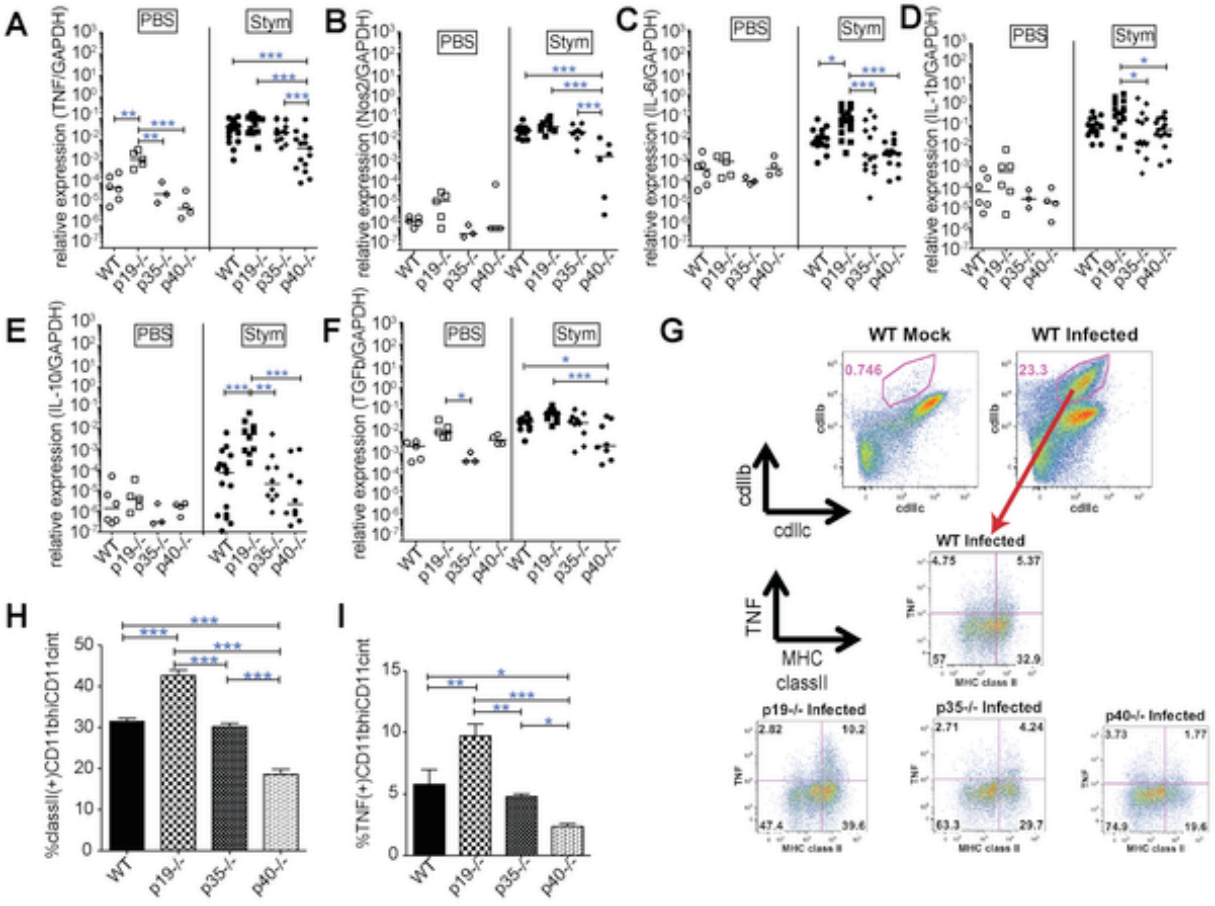


Figure 2-4: IL-23 limits IL-12 dependent pro-inflammatory cytokine production and the activation of gut mononuclear phagocytes.

(A) TNF, (B) Nos2, (C) IL-6, (D) IL-1β, (E) IL-10 and (F) TGF-β cecal gene expression at 3d after oral *S*. Typhimurium or mock infection of WT, $p19^{-/-}$, $p35^{-/-}$, $p40^{-/-}$ mice was determined by qPCR. Data are expressed as the ratio of mRNA levels of the gene of interest divided by GAPDH expression from the same RNA. Bars show median values and the data are pooled from 3 separate infections. (G) Following mock or *Salmonella* infection, lamina propria (LP) cells were isolated from the cecum and colon of C57BL/6 mice as detailed in Materials and Methods section. LP cells were treated for 4hrs *ex vivo* with BFA, followed by surface staining with CD11c, CD11b and class II MHC, followed by intracellular TNF. The $CD11b^{hi}$ $CD11c^{+}$ cell population (red gates) were the primary TNF producers. TNF and class II MHC expression by the $CD11b^{hi}$ $CD11c^{+}$ cell populations is shown for representative animals in the lower panels. Quantification of $CD11b^{hi}$/$CD11c^{+}$ positive cells for (I) MHC $classII^{hi}$ expression and (J) MHC $classII^{hi}$ TNF^{+} populations. Statistical significance was determined using one way ANOVA followed by Bonferroni post test. *:p<0.05, **:p<0.01, ***:p<0.001

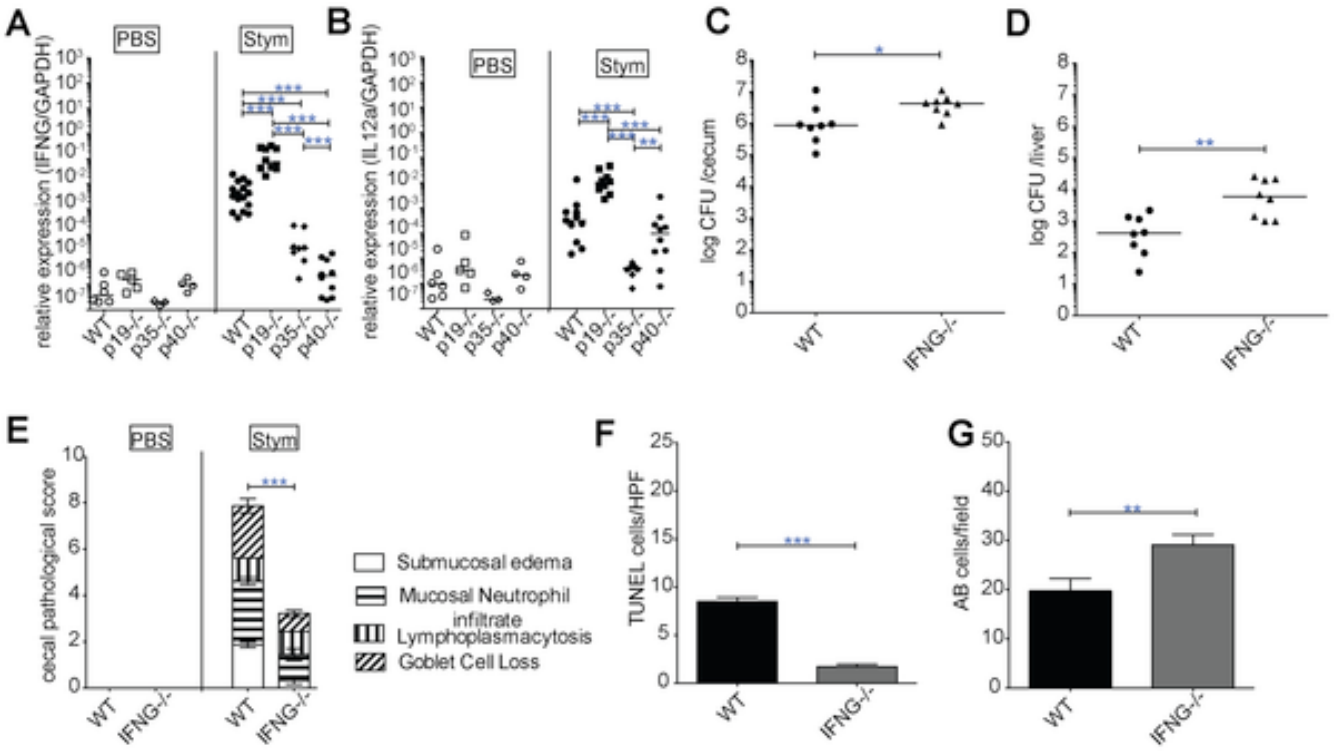


Figure 2-5: **IL-23 inhibits *Salmonella* induced IL-12 dependent IFN-γ mediated inflammation.**

(A) IFN-γ, and (B) IL-12a (IL-12p35) cecal gene expression at 3d after oral WT *Salmonella* infection +/- mock treated mice from WT, $p19^{-/-}$, $p35^{-/-}$, $p40^{-/-}$ mice was determined by qPCR. Bars show the median and the data are pooled from 3 separate infections. (C) Cecum and (D) liver were collected from WT and IFN-$\gamma^{-/-}$ 3 days post infection. Organ homogenates were diluted and plated to determine CFU/organ. Bars represent the median bacterial load and the data are pooled from 2 separate infections. (E) Histologic changes were quantified on H&E-stained cecal sections as described in Fig. 2-1 . (F) TUNEL and (G) AB positive cells were quantified per high-power field in the ceca of at minimum 3 infected mice/group. Results are expressed in mean ± SE. Statistical significance was determined using one way ANOVA followed by Bonferroni post test for A and B, the Mann-Whitney test was used for C and D, and unpaired Student's t-test was used for E-G. *:$p<0.05$, **:$p<0.01$, ***:$p<0.001$.

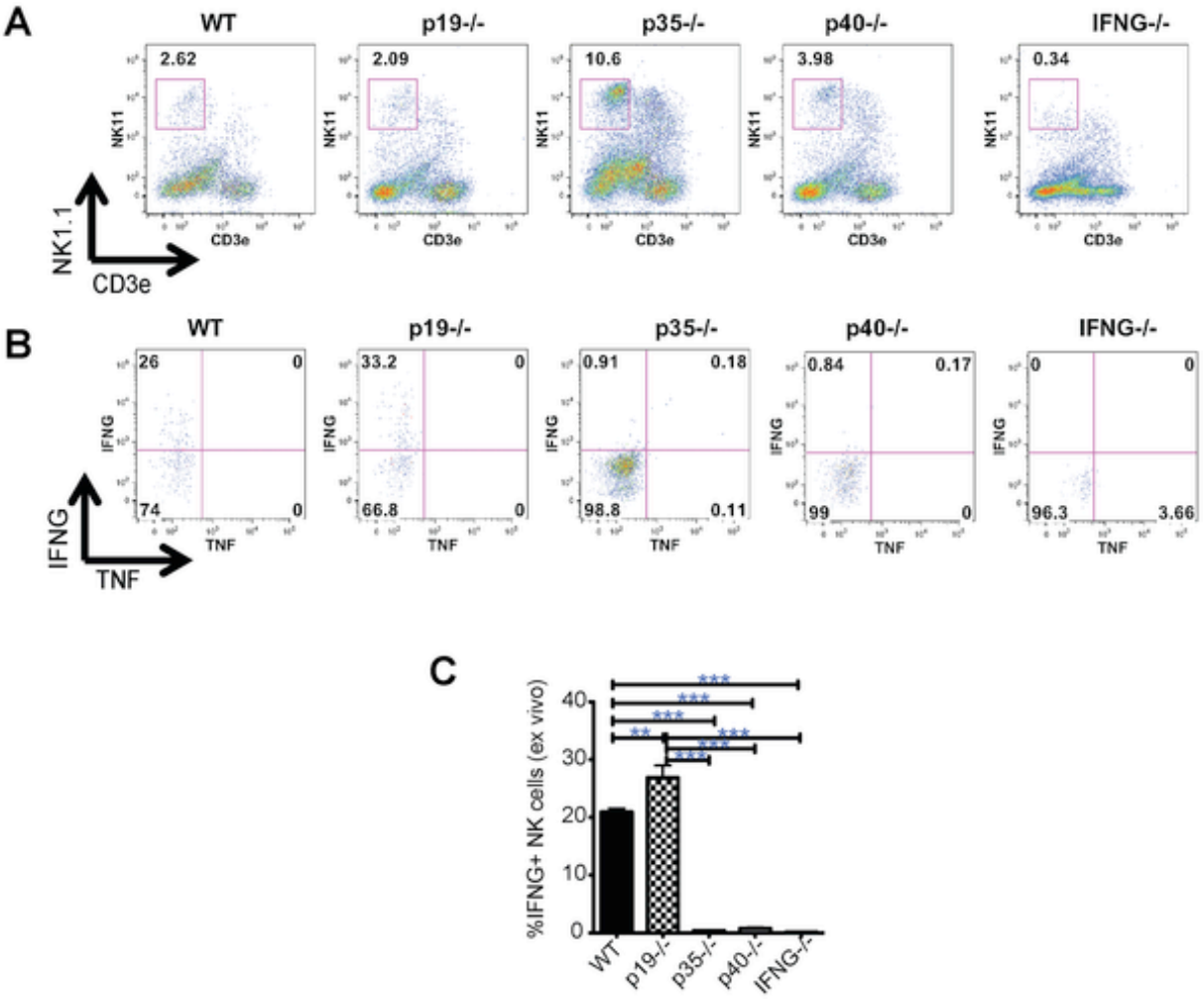


Figure 2-6: **IL-23 inhibits *Salmonella* induced IFN-γ production by lamina propria NK cells.**

(A) LP populations isolated from WT, p19$^{-/-}$, p35$^{-/-}$, p40$^{-/-}$ and IFN-g$^{-/-}$ mice were stained for surface expression of NK1.1 and CD3ε; numbers indicate the percentage of cells in the gated box. (B) Intracellular production of IFN-γ and TNF is shown for NK cells (the NK1.1^{+}CD3^{-} population gated in A). (C) The percentage of LP IFN-γ+ NK cells were quantified for the aforementioned mice at 3d *Salmonella* infection. Plotted are the mean ± SE for 3 mice per condition. Statistical significance was determined using the one-way ANOVA followed by Bonferroni post test. *:p<0.05, **:p<0.01, ***:p<0.001

Supplemental Methods:

Mice:

C57BL/6 mice have an inactivating G169D mutation in Nramp1, whereas A/J mice have the functional allele that transmits resistance to *S*. Typhimurium, *M. bovis* BCG and *Leishmania* infections [1]. The gene encoding Nramp1 (Slc11a1) is located on mouse chromosome 1. We generated C57BL/6 *SLc11a1*$^{G169/G169}$ congenic mice by crossing C57BL/6 mice with a line of C57BL/6 mice consomic for A/J chromosome 1 (C57BL/6J-Chr 1$^{A/J}$/NaJ, The Jackson Laboratory), and selecting offspring for optimal crossover events with Illumina murine SNP chips (Ilumina). After several generations of backcrossing, we derived heterozygous mice with A/J *SLc11a1*$^{G169/G169}$ allele flanked by 3 Mb from A/J chromosome 1, and all other chromosomes genotyped as C57BL/6. These mice were bred to homozygosity at *Slc11a1*$^{G169/G169}$, and are genetically 99.8% C57BL/6. The p19$^{-/-}$, p35$^{-/-}$, and IL-17RC$^{-/-}$ mice were backcrossed onto the *Slc11a1*$^{G169/G169}$ congenic C57BL/6 mice.

TUNEL/AB IHC:

Apoptotic cells were stained by the TUNEL method as previously described[138], on cecal paraffin sections using an Apoptag peroxidase *in situ* apoptosis detection kit (Millipore, Billerica, MA). Control slides were processed identically, but without the TdT. In order to stain for the acidic mucin positive goblet cells, paraffin embedded cecal sections were first rehydrated then stained with Alcian blue (Sigma). Lithium carbonate (Sigma), saffron (Sigma) and H&E were used for counterstains prior to dehydration for each section.

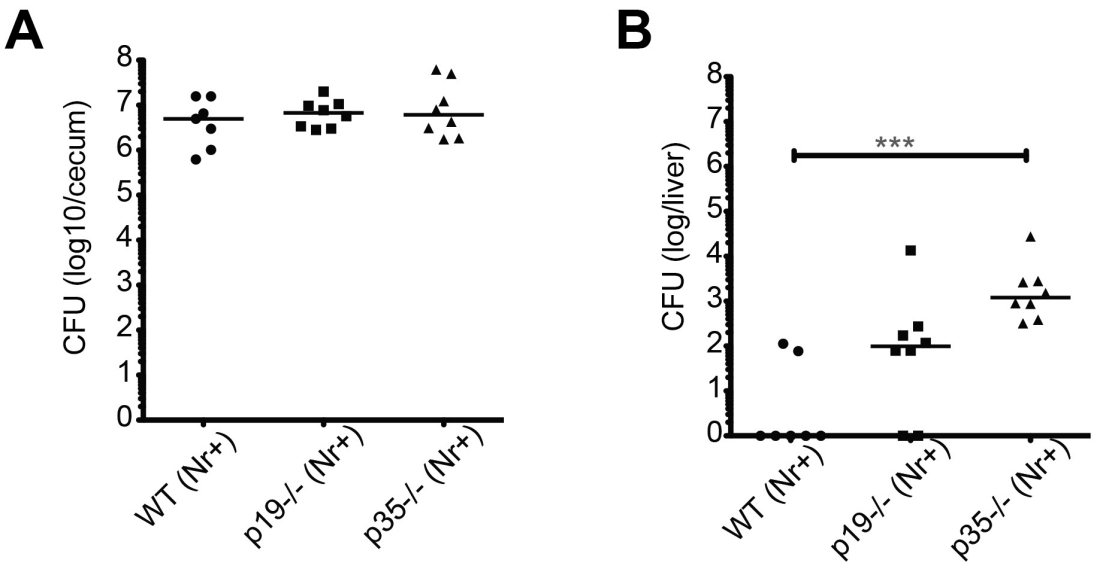
Lamina Propria Isolation:

Intestines were cut open lengthwise into 1cm pieces and washed 2X with CMF solution [10 mM HEPES, 25 mM sodium bicarbonate (Sigma), and 2% FBS (Atlas Biological, Fort Collins, CO) in Ca^{2+}, Mg^{2+}-free HBSS (Invitrogen), pH 7.2]. Tissue was transferred into EDTA/DTT/FBS/CMF solution [1.3 mM EDTA (Invitrogen), 1mM DTT (VWR,

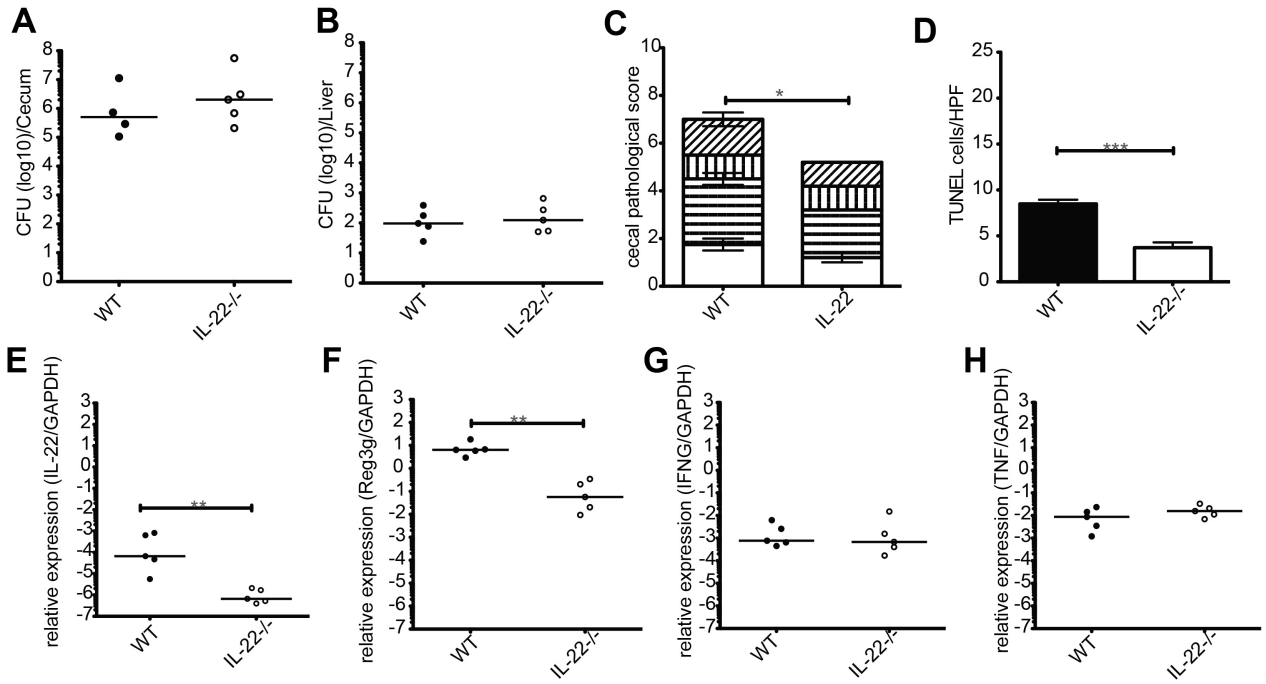
Radnor, PA), 10% FBS, in CMF supplemented with 1X HGPG (5 mM HEPES, 2 mM L-glutamine (Fisher Scientific), 20 U/ml penicillin/20 ug/ml streptomycin (Fisher Scientific) and20 ug/ml gentamicin (Invitrogen))]. After 20-min shaking at 250 rpm in 37°C for 20 min, samples were vortexed for 10s and epithelial cells from the supernatant were discarded. This step was repeated followed by two washes in CMF and the tissue was filtered through nylon mesh (70 mm nylon, Becton Dickinson (BD), San Jose, CA). LP suspensions were prepared from the EDTA treated de-epithelialized tissue by further incubation with collagenase solution [0.5 mg/ml of type II collagenase (Worthington, Lakewood, NJ), 1X HGPG and 5% FBS] at 37°C while shaking (250rpm) for 1 hr. The LP cells were isolated from the interface of a 40/80 Percoll gradient after centrifugation for 20 min at 2,400 rpm at RT. Cells were washed twice with supplemented RPMI 1640 medium [100 U/ml penicillin/100 ug/ml streptomycin, 100 ug/ml gentamicin (Invitrogen), 10% FBS, and 50uM β- mercaptoethanol (Sigma)].

Flow Cytometry:

For myeloid surface markers cells were stained with α-CD11c (FITC or PE-CY7), α-CDllb (PERCP-Cy5.5), α-I-A^b (FITC), and Ly6G (APC). For lymphoid surface markers cells were stained with α-CD3ε (APC), α-CD4 (PERCP), α-CD8 (PERCP), α-γδ TCR (FITC) and α-NK1.1(PE). Cells were washed with PBSA and resuspended in cytoperm/cytofix solution (BD) for 20 min on ice and then washed with Perm Wash (BD). For intracellular staining, myeloid sets were stained with α-TNF (Pacific Blue) and α-IL-6 (PE), while the lymphoid sets were stained with α-IFN-γ (PE-Cy7), α-TNF (Pacific Blue) and α-IL-17a (PE or FITC). Stained cells were washed several times with Perm Wash solution and resuspended in fresh 1% PFA solution.

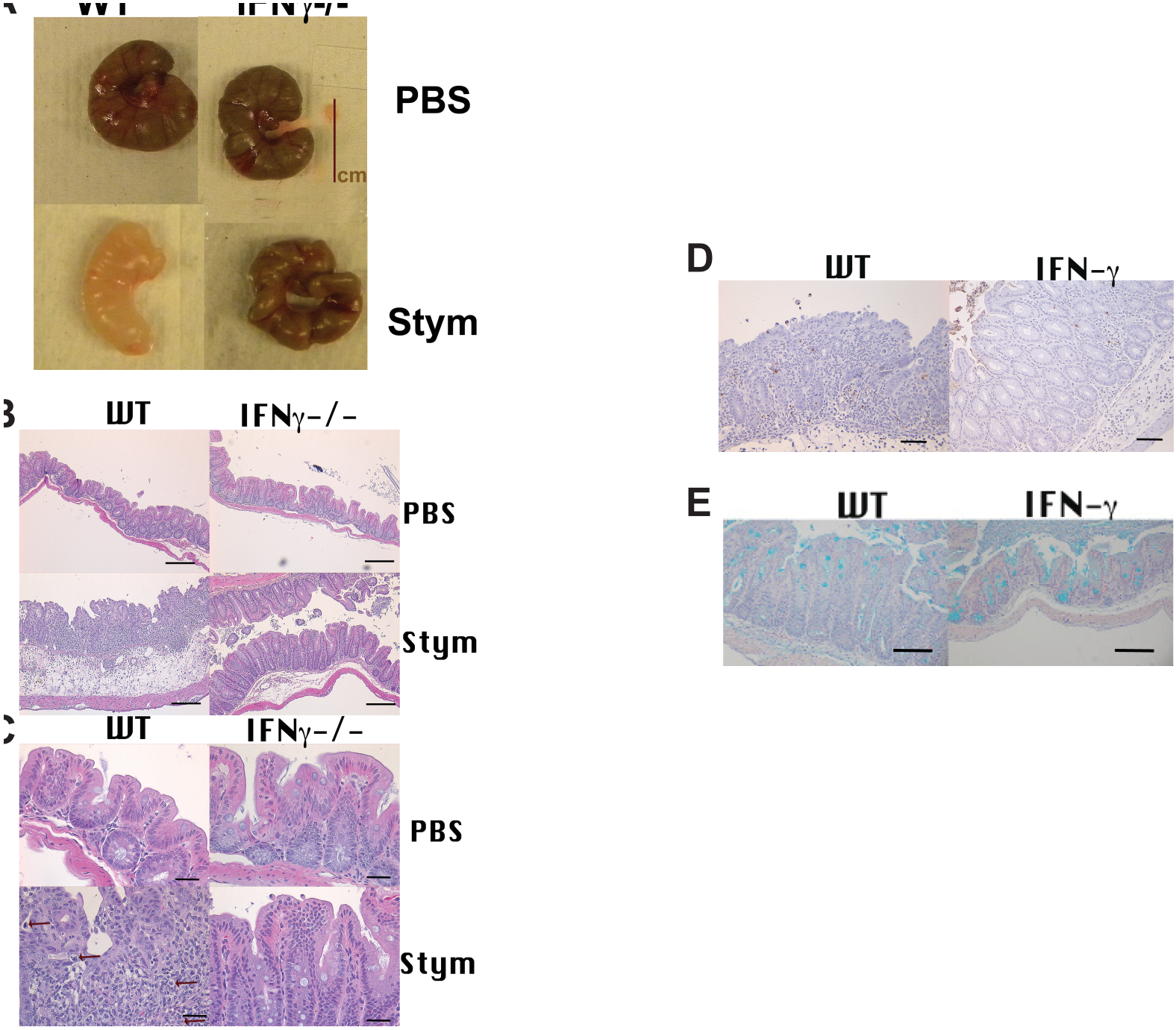


Supplementary Figure 2-1: **The role of IL-12 and IL-23 in protection against systemic CFU is independent of Slc11a1 genotype.**
Slc11a1$^{G169/G169}$ congenic WT, p19$^{-/-}$ and p35$^{-/-}$, mice were orally infected with 1x10^3 CFU of *S.* Typhimurium 1d after pretreatment with 20mg streptomycin. Bacterial burden was determined for (A) Cecum and (B) liver from mice at 3 days post infection. Bars represent the median bacterial load and the data are pooled from 2 separate infections. Statistical significance was determined using one way ANOVA with the Bonferroni post test ***:$p<0.001$.

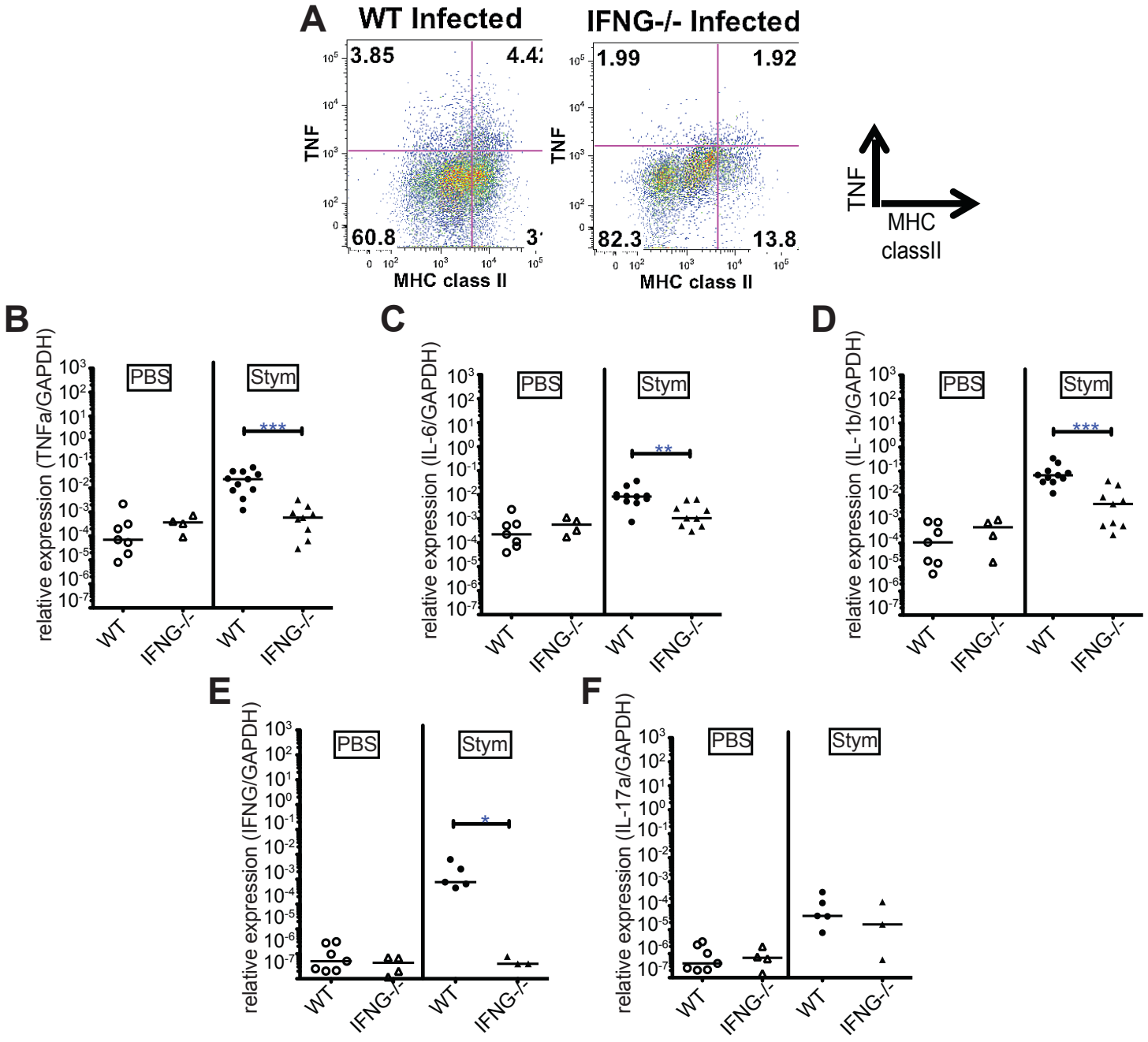


Supplementary Figure 2-2: **IL-22 promotes cecal inflammation and injury following *Salmonella* infection.**

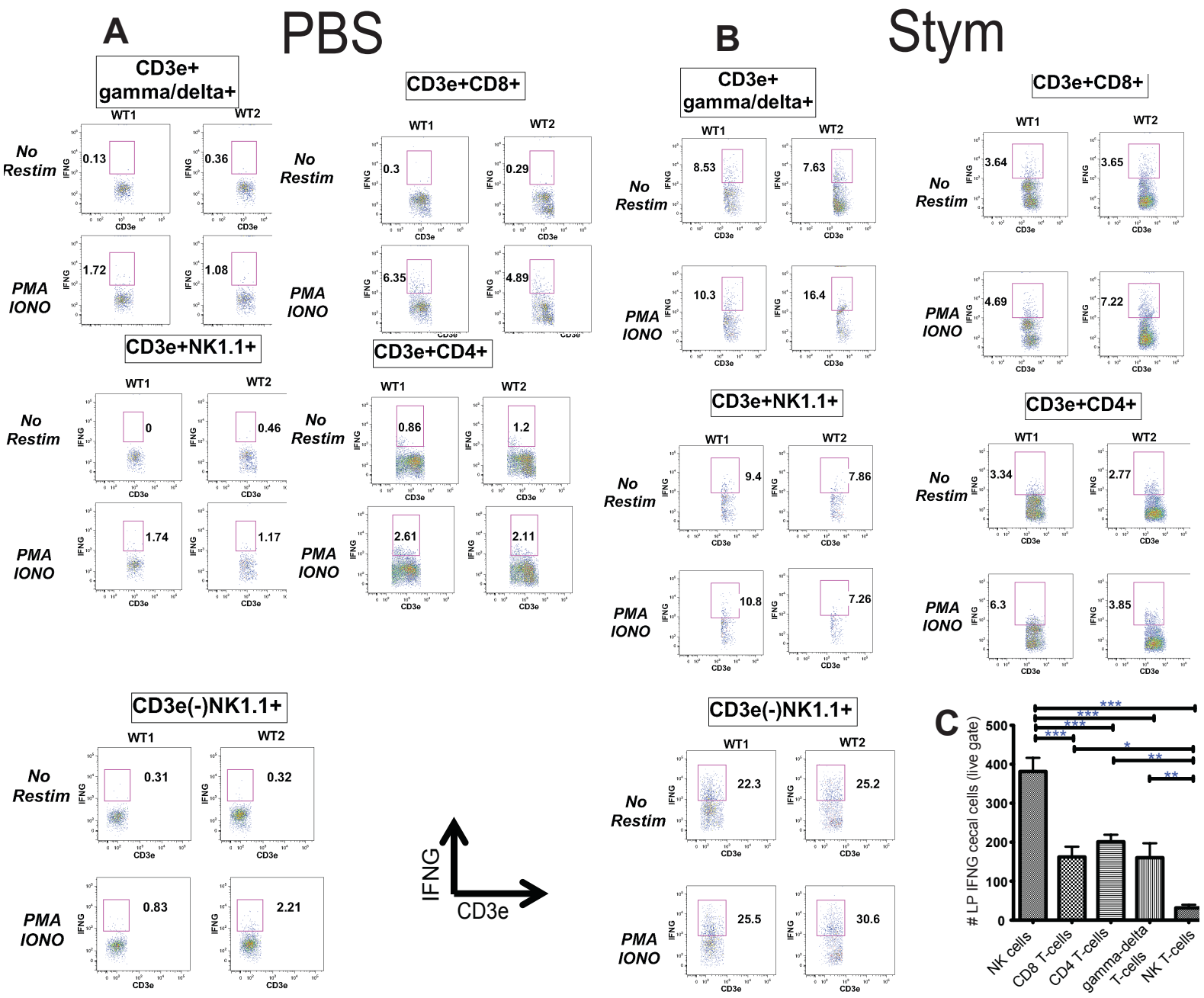
(A) Cecum and (B) liver *Salmonella* CFU from IL-22$^{-/-}$ and C57BL/6 mice. Bars represent the median bacterial load. (C) Scoring of inflammatory changes at 72hr p.i. from infected WT and IL-22$^{-/-}$ B6 mice. (D)TUNEL positive cells were quantified per high-power field in the ceca of at minimum 3 infected mice from each group. (E) IL-22, (F)Reg3g , (G)IFN-γ and (H) TNF cecal gene expression data at 3d after oral *Salmonella* infection from WT and IL-22$^{-/-}$ mice. Data are expressed as the ratio of mRNA levels of the gene of interest divided by GAPDH expression from the same RNA. Statistical significance was determined using the unpaired Student's t-test. *:$p<0.05$, **:$p<0.01$, ***:$p<0.001$.



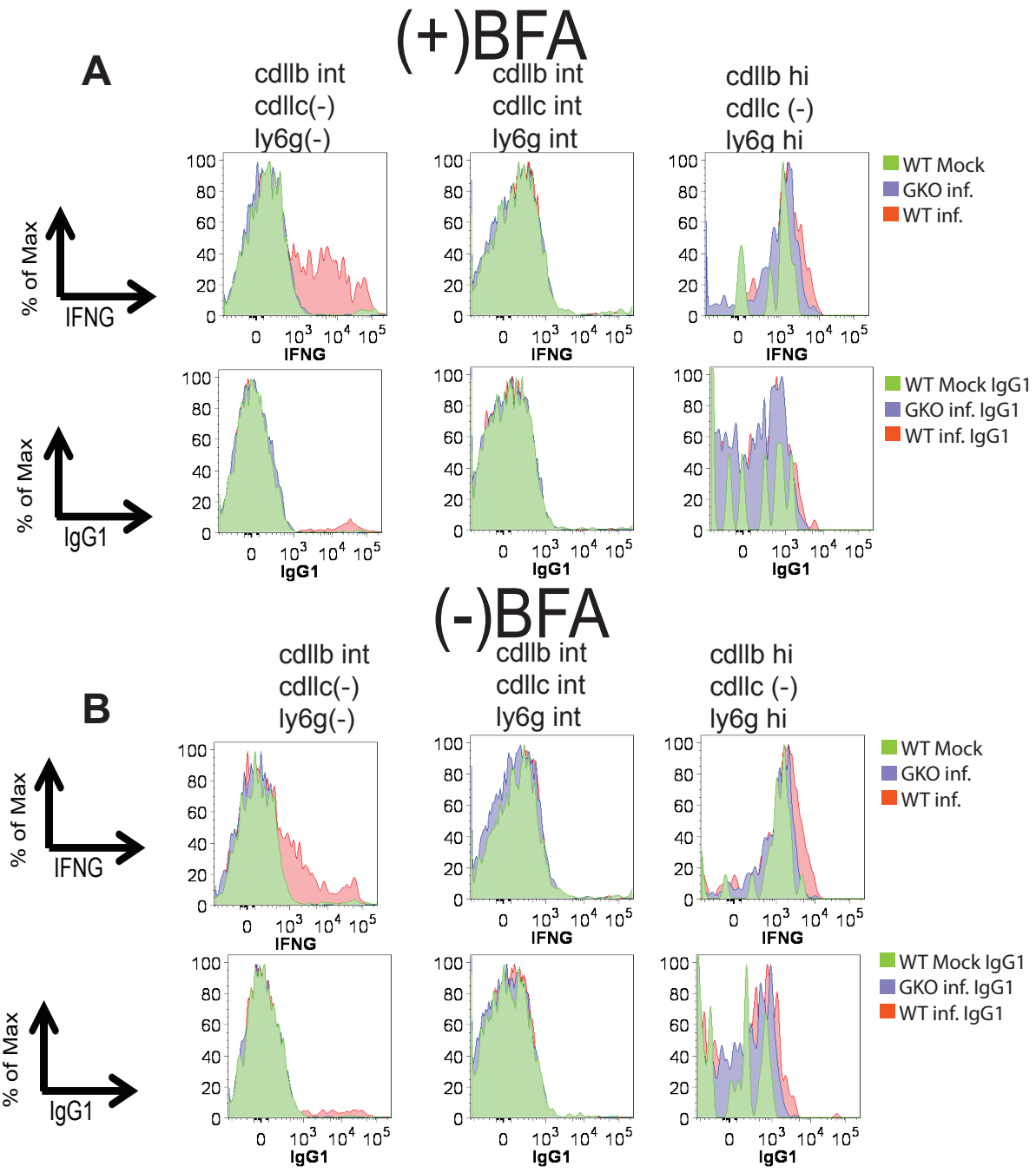
Supplementary Figure 2-3: **IFN-γ is required for *Salmonella* induced colitis**.
(A) Representative ceca from WT and IFN-$\gamma^{-/-}$ at 3d following *Salmonella* infection or mock treatment. The bar equals 1 cm. (B)Low and (C) high power H&E stained cecal sections of WT and IFN-$\gamma^{-/-}$ mice at 3d after infection. Arrows in (C) indicate dead cells. (D) TUNEL-positive (brown) and (E) Alcian Blue (AB)-PAS-stained (blue) cells of representative infected cecal sections. Bar in (B) represents 200 microns and (C-E) 50 microns



Supplementary Figure 2-4: **IFN-γ is essential for *Salmonella*-induced activation of inflammatory monocytes and pro-inflammatory cytokine expression.**
(A) LP cdllb^{hi}/cdllc^{mid} populations generated from C57BL/6, and IFN-γ$^{-/-}$ mice were assayed for their MHC class II expression and intracellular production of TNF. (B) TNF, (C) IL-6, (D) IL-1β, (E) IFN-γ, and (F) IL-17a gene expression in cecal tissue was measured by qPCR. Data are expressed as the ratio of mRNA levels of the gene of interest divided by GAPDH expression from the same RNA. Statistical significance was determined using the unpaired Student's t-test. *:p<0.05, **:p<0.01, ***:p<0.001.



Supplementary Figure 2-5: **NK cells have the largest proportion and number of LP IFN-γ producing cells among LP populations 3d following *Salmonella* infection.**
3d lamina propria cells from (A) mock or (B) *Salmonella* infected C57BL/6 mice were stimulated for 6hr with or without PMA and ionomycin in the presence of BFA. Cells were then stained for surface markers: CD3e, CD8, CD4, NK1.1, then fixed, permeabilized and stained for intracellular IFN-γ.



Supplementary Figure 2-6: LP monocytes and neutrophils are not major producers of IFN-γ.

After 3d mock or *Salmonella* infection LP cells were isolated from C57BL/6 and IFN-$\gamma^{-/-}$ mice, and treated BFA for 6hr (A) or left untreated (B). Cells were stained for CDllb, CDllc, and Ly6g, then permeabilized and stained for IFN-γ (top panels) or IgG1 isotype control (bottom panels). Green histogram: Mock C57BL/6; Blue histogram: Infected IFN-$\gamma^{-/-}$; Red histogram: Infected C57BL/6. Histograms are from pooled cells of 2 mice per group

Chapter 3: Protective Role of innate immune cytokines IL-12 and IL-23 in Adaptive Immunity to *Salmonella*

Introduction:

Individuals with defects in the IL12Rβ1 or IL-12p40 gene often suffer from unusual mycobacterial and NTS infections [171], [99]. The higher prevalence of *Salmonella* disease amongst patients with a deficiency in the IL-12/23 pathways compared to those deficient in IFN-γ suggests that the increased sensitivity to *Salmonella* infection in IL-12/23 defective patients is not simply due to defective IFN-γ production [171], [99], [172]. The human genetic studies are supported by murine studies regarding the role of IL-12 and IL-23 in resistance to *Salmonella* [64]. Mice deficient in p35 (no IL-12) had dramatically increased susceptibility to *Salmonella* and p40 deficiency (no IL-12 or IL-23) further augmented this susceptibility [100], [102]. These data, along with the human studies, suggest that IL-12/IFN-γ independent mechanisms also contribute to host defense against *Salmonella*.

In contrast to IL-12, IL-23 alone is dispensable for resistance to systemic *Salmonella* infection but its role is unmasked when IL-12 is absent [66]. The potential downstream targets of IL-23 include IL-22 [66] and IL-17A [101,145]. Because IL-23 is strongly associated with mucosal infections and immunity, it is possible that IL-23's influence may vary depending on the infection model. Previous studies to evaluate the protective roles of IL-12 and IL-23 have used either systemic or murine typhoid infection models, which lack prominent enteric infection and inflammation, which is characteristic of human NTS infections. In addition, the route of infection has been demonstrated to influence the derivation of T-helper subsets during infection with *Listeria monocytogenes*, another intracellular pathogen. Intravenous *L. monocytogenes* infection in mice generates TH1 cells, whereas intranasal infection produces mainly TH17 cells [173]. In contrast, oral infection of mice with *Salmonella* led to the transient production of flagellin specific TH1 and TH17 cells that were enriched at mucosal sites, and the late sustained production of TTSS-2 effector specific TH1 cells that were enriched at systemic sites [174]. These studies suggest that TH17 and TH1 cells may have distinct roles in mucosal versus systemic immunity, and that the generation of these cells is controlled by complex mechanisms that are influenced by the nature of the pathogen and the route of infection.

We hypothesized that IL-23 and IL-12 participate in the development of TH17 and TH1 effector/memory responses and are required for resistance to *Salmonella* infection. In this study,

we use the streptomycin pretreatment model of *Salmonella*-induced enterocolitis to define the roles of IL-12 and IL-23 in regulating late phase adaptive cellular and humoral immunity. Like systemic infection models, our data show that IL-12 and IL-23 mediate resistance against *Salmonella* largely through an IFN-γ dependent process with minor contribution of TH17 cells. IL-23 alone is dispensable in this model, but partially compensates for the loss of IL-12. The combined loss of IL-12/-23 leads to defective control of infection and diminished generation of antigen-specific CD4 T cells. Surprisingly, antigen-specific TH1 cells are readily detected upon rechallenge of immunized IL-12/23 deficient mice, indicating that these cytokines are not required for the generation of TH1 cells. Similarly, IL-12/23 deficient mice develop robust IgG2c antibody responses against *Salmonella*. Nevertheless, the IL-12/23-independent, antigen-specific TH1 and antibody responses are insufficient for optimal control of late phase *Salmonella* infection.

Results

IL-12 and IL-23 are required for resistance against attenuated S. Typhimurium infection via systemic and oral routes.

IL-12 has been shown to play a major role for protective T cell mediated immunity against many intracellular pathogens {Altare et al., 1998, #27942; Cooper et al., 2002, #61385; Freeman and Holland, 2007, #59054; Happel et al., 2005, #49400; Murphy et al., 2003, #61784}. p19-/- (IL-23 deficient) mice were resistant to intraperitoneal infection with 10^6 ΔaroA *S.* Typhimurium, while $p35^{-/-}$ (IL-12-deficient) mice were highly susceptible to infection (Fig. 3-1A). Deficiency in IL-12 and IL-23 ($p40^{-/-}$) further increased susceptibility, supporting the dominant role of IL-12 and auxiliary role of IL-23 for optimal bacterial clearance following systemic infection.

To investigate the roles of IL-12 and IL-23 in oral infection, we used the streptomycin pretreatment model to more closely mimic NTS infection in humans [16]. This model results in robust intestinal colonization and inflammation, and has revealed specific roles for IL-23 during acute infection [119,88]. WT and mutant mice were orally infected with 10^8 Δ*aroA S.* Typhimurium, and sacrificed at 24 d post-infection. Bacterial burden in IL-23 ($p19^{-/-}$) and IL-12 (p35-/-) deficient mice did not differ significantly from WT mice (Fig. 3-1 B and C). In contrast, IL-12/23 (p40-/-) deficient mice had increased bacterial burden in the cecum and liver compared to WT and p19-/- mice. Overall, the data indicated that IL-23 is not necessary to control gut or

systemic bacterial clearance (Fig 3-1 B&C). Similar to the systemic infection model, the combined action of IL-12 and IL-23 was essential to control infection.

Combination of IL-12 and IL-23 accounts for the partial IFN-γ mediated protection against oral *S. Typhimurium* infection

Human studies show a higher prevalence of *Salmonella* disease amongst patients with a deficiency in the IL-12/23 pathway to those deficient in IFN-γ, suggesting IFN-γ-independent factors also contribute to IL-12/23-mediated protection against *Salmonella* infection in humans. To better assess the role of IFN-γ in the mouse oral colitis model, we infected WT and mutant mice with $5X10^8$ CFU Δ*aroA S.* Typhimurium. WT mice and mice lacking IL-12 (p35-/-) or IL-23 (p19-/-) survived through the 35d study, whereas almost 40% of p40-/- died by 24 d (Fig. 3-1D). Strikingly, all IFN-γ-/- mice were moribund within 3 wk after oral colitis infection with Δ*aroA S.* Typhimurium (Fig. 3-1D). When compared to WT, both p40-/- and IFN-γ-/- mice were signifacntly more susceptible to oral attenuated *Salmonella* infection; interestingly, IFNγ-/- were significantly more susceptible than p40-/- suggesting IL2/23 independent pathways contribute to IFN-γ production. (Fig. 3-1D). The survival data parallels the bacterial clearance data (Fig. 3-1 B&C), and further supports the importance for IL-12 and IL-23 in protective immunity against oral *Salmonella* infection. In contrast to human studies, in mouse infections IFN-γ appeared to play a more significant role than the combination of IL-12 and IL-23.

IL-12 and IL-23 contribute to generation of *Salmonella* specific CD4 T cells

T-helper responses are critical for effective adaptive immunity to *Salmonella*, therefore we analyzed the contribution of IL-12 and IL-23 to CD4 T-cell responses. We orally infected mice with Δ*aroA S.* Typhimurium expressing the 2W1S peptide conjugated to OmpC (a gift from Mark Jenkins). The 2W1S peptide is highly immunogenic in C57BL/6 (B6) mice because of a relatively large population of naïve CD4 T cells with TCRs specific for the 2W1S:I-A^b complex [175]. At 24 d post-infection, we quantified the $CD44^+$/ $CD4^+$ effector/memory population in the peritoneum and spleen. Within the spleen, WT, IL-12 and IL-23 deficient mice had similar numbers of $CD4^+/CD44^+$ T cells (Fig. 3-2A), but IL-12/23 deficient mice (p40-/-) had significantly fewer $CD4^+/CD44^+$ T cells (Fig. 3-2A). Next we analyzed the peritoneal exudate cells (PECs). In contrast to splenocytes, IL-23 deficient mice had significantly more $CD4^+/CD44^+$ T cells in the peritoneum when compared to the other mice (WT, p35-/- and p40-/-) (Fig. 3-2B). This implies that in the presence of functional IL-12, IL-23 suppresses the accumulation of CD4+/CD44+ T cells in the peritoneum. Overall the combination of IL-12 and -

23, regardless of the immune compartment, contributed to the expansion of $CD4^+/CD44^+$ T cells during oral *Salmonella* infection.

CD4 T cell IFN-γ and IL-17 responses post-immunization

To evaluate the quality and quantity of the CD4 T-cell responses at 24 d post-infection, we stimulated splenocytes or PECs *ex vivo* with PMA and Ionomycin (P/I) or 2W1S peptide, and assessed for IFN-γ and IL-17 production by CD4+ T cells (Fig. 3-2 C-F). In general for both splenic and peritoneal compartments, all genotypes had greater numbers of IFN-γ+ than IL17+ CD4+ cells (Figure 3-2 C-F). In addition, for all genotypes a substantial subset of IFN-γ producing cells also produced TNF-α (data not shown). Splenocytes had a significant 2W1S-specific IFN-γ response; 2W1S-specific IL-17 responses were not detected in this compartment. Within the spleen, IL-23 deficiency did not alter the number of IFN-γ producing CD4 T cells. Surprisingly, increased numbers of IFN-γ producing CD4 T-cells were detected in splenocytes from p35-/- mice. The loss of both cytokines in the p40-/- spleens ablated 2W1S-specific IFN-γ responses (Fig. 3-2 C&D). Interestingly, p40-/- splenocytes had a small but detectable number of IFN-γ producing CD4 T cells when stimulated with P/I (Fig. 3-3C). Minimal but detectable levels of IL-17 producing cells were found only in p35-/- hosts after P/I stimulation, suggesting that IL-12 suppresses and IL-23 is required for IL-17 producing CD4 T cells. To evaluate IL-23 downstream targets, IL-17 and IL-22, we conducted a limited analysis IL-17RC and IL-22 deficient mice. At 24 d post-infection with Δ*aroA*, there were no significant differences in bacterial burden in the ceca and livers of IL-17RC or IL-22 deficient compared to WT mice. Similarly, no differences were observed in splenic 2W1S-specific T-helper response in either IL-17RC or IL-22 deficient mice compared to WT mice (supplementary Fig. 3-1), which supported the limited role for IL-23 in the context of functional IL-12 in splenocyte immune response.

As opposed to the spleen, p19-/- mice had increased IFN-γ^+ CD4 T cells in the peritoneum after P/I stimulation (Fig. 3-2E). The p19-/- mice also a significant increase in 2W1S-specific T-helper responses relative to either p35 or p40 deficient PECs, suggesting that IL-23 suppresses and IL-12 promotes IFN-γ responses by CD4 T cells in this compartment. Regarding IL-17 response, no significant difference was observed in the peritoneum. Interestingly, neither IL-22 nor IL-17RC deficient PECs showed a significant difference in 2W1S-specific IFN-γ or IL-17 compared to WT mice, implying that an IL-17/22 independent mechanism is responsible for IL-23 suppression of of CD4 T cells IFN-γ responses. Altogether, in the oral colitis model, IFN-γ is the dominant cytokine produced by CD4 T cells, and requires either IL-12

or IL-23. IL-12 suppresses TH17 responses detected in the spleen, and IL-23 suppresses TH1 responses detected in the peritoneum.

Anti-*Salmonella* antibody responses

Antigen-specific antibody responses are thought to play auxiliary roles to cellular immunity during primary *Salmonella* infection [54]. In C57BL/6 mice, IgG1 and IgG2c isotypes are thought to be driven by different T-helper subsets. TH1 cell derived IFN-γ promotes IgG2c production and TH2 cell derived IL-4 promotes IgG1 production after *Salmonella* infection [176]. 24 d after oral challenge with $5x10^8$ ΔaroA *S.* Typhimurium, we analyzed sera from mice for isotype specific anti-*Salmonella* antibody responses. All mice revealed high antibody titers compared to control mice (Fig 3-3A-D). Mice lacking both IL-12 and IL-23 had significant increases in all isotype-specific antibody responses relative to WT mice (Fig. 3-3A-D). No differences were seen in IgG2c titers between IL-12 deficient and WT mice, supporting previous studies [176]. Interestingly, IL-23 deficient mice had significantly decreased anti-*Salmonella* IgG1 relative to WT mice (Fig. 3-3C). IgA is thought to act primarily at mucosal surfaces, where it may limit pathogen access and protect from gut inflammation [177]. All mice had significant anti-*Salmonella* IgA responses, and surprisingly the p40-/- had significantly increased IgA responses relative to the WT mice (Fig. 3-3D). Thus p40-/- mice deficient in both IL-12 and IL-23 are highly susceptible to oral attenuated *Salmonella* infection despite robust antigen specific antibody responses.

Role of IL-12 and IL-23 in protection against rechallenge

Using the colitis model, we compared CD4 T cell responses in naïve and ΔaroA-2W1S immunized mice 3 d after challenge with 500 CFU WT-2W1S *Salmonella*. The 2W1S antigen specific responses were quantified with 2W1S pMHCII tetramers (a gift from Marc Jenkins) [175]. Day 3 after WT-2W1S *Salmonella* challenge, we looked at bacterial counts of both attenuated and WT strains in the ceca and liver of the respective animal groups. The bacterial burden of the ΔaroA-2W1S *Salmonella* was similar to that seen on day 24 (Fig. 3-4A&B; compare to Fig. 3-1 B&C). of the bacterial burden of WT *Salmonella* in the immunized mice differed between the two sites (Fig. 3-4 C&D). In the cecum, IL-12 or combined IL-12/23 deficiency led to decreased gut colonization, whereas IL-23 deficiency was indistinguishable from WT mice (Fig. 3-4C). In the liver there were no significant differences in bacterial counts from p19-/-, p35-/- and WT mice. In contrast, p40-/- mice were incapable of controlling dissemination of WT *Salmonella* and

had significantly higher liver CFU (Fig. 3-4D). These data indicate distinct roles for IL-12 and IL-23 in anti-*Salmonella* immunity, which are influenced by the site of infection.

CD4 T cell IFN-γ and IL-17 responses during rechallenge

We determined the T-cell cytokine responses in the spleen to better understand the quality and quantity of the immune response to *Salmonella* in the rechallenged mice. Naïve infected C57BL/6 mice contained low numbers of 2W1S-specific CD4 T cells, whereas tetramer positive CD4 T cells were readily detected in immunized mice for all genotypes post-challenge (Fig 3-5A). CD8 tetramer-positive T cells were not detected in splenocytes using 2W1S class II tetramer (data not shown). As noted at day 24 following primary infection, there were significantly increased numbers of 2W1S-tetramer positive CD4 T cells in p35-/- compared to p19-/- and WT mice (Fig. 3-5A). Surprisingly, the immunized p40-/- mice had readily detectable tetramer(+) cells, which were comparable in number to WT mice (Fig. 3-5A).

We examined 2W1S-tetramer positive CD4 T cell responses by intracellular cytokine staining for IFN-γ and IL-17A. Once again IFN-γ-producing CD4 T cells were the predominant splenic population in both tetramer (+) and (-) groups (Figs 3-3 B&C, respectively), and loss of IL-12 resulted in a significant increase in tetramer (+) IFN-γ+ cells relative to WT, p19-/- and p40-/- mice. Surprisingly, tetramer (+) IFN-γ (+) CD4 T cells were readily detected in immunized p40-/- splenocytes at levels comparable to WT and p19-/- mice (Fig. 3-5B). The tetramer (-) cells showed similar results, with the additional finding that p19-/- and p40-/- immunized mice had about half the number of IFN-γ+ CD4 T cells compared to WT mice (Fig. 3-5C). IL-17+ and IFN-γ/IL-17+ cells were essentially undetectable in the tetramer (+) or (-) CD4+ cells from WT, p19-/- and p40-/- mice, and only detected in p35-/- mice (Fig. 3-5B and C).

Discussion

Patients with genetic deficiencies in IFN-γ and IL-12/23 receptor signaling, or receiving anti-IL-12 therapy, are more susceptible to *Salmonella* infections [123,143,178] indicating that TH1 responses are protective in human salmonellosis. Although lymphocytes from patients deficient in IL-12p40 or its receptor produce significantly less IFN-γ [179], these patients have a higher prevalence of non-typhoidal *Salmonella* (NTS) disease compared to patients deficient in IFN-γ

signaling. This suggests that susceptibility to salmonellosis in IL12 deficient individuals is not simply due to defective IFN-γ production in humans. In the present study, we use an oral colitis model, which simulates human NTS enteric disease, to explore the roles of the IL-12p40 dependent cytokines IL-12 and IL-23. Similar to the murine systemic infection models [66], we find the combined action of IL-12 and IL-23 are essential to control infection in systemic and mucosal sites during murine *Salmonella* enterocolitis (Fig. 3-1A-D). In contrast to human studies, our murine studies indicate that IFN-γ provides a more significant survival advantage than the combination of IL-12 and IL-23 (Fig. 3-1D). This observation may be due to intrinsic differences between murine and human immunity to *Salmonella* or the nature of the infection model.

TH1 cells are essential for protective immunity against *Salmonella* in both man and mouse [123]. Therefore we determined whether differences in the production of TH1 cells correlated with differences in susceptibility to attenuated *Salmonella* infection in the IL-12 and IL-23 deficient animals. We find that IL-12 and IL-23 were critical for the accumulation of CD44+ CD4 T-cells and IFN-γ-producing CD4 T-cells during primary immunization (Fig 3-2A+C). During either intraperitoneal or oral infection, either IL-12 or IL-23 were sufficient for the accumulation of IFN-γ producing CD4 T cells. During oral infection IL-23 deficiency did not affect IFN-γ producing CD4 T cells in the spleen, and even resulted in increased numbers of these cells in the peritoneum. The mechanism for IL-23 suppression of peritoneal IFN-γ producing CD4 T cells is undefined. In contrast to IL-23, IL-12 deficiency resulted in increased bacterial burden in the liver, although this was not significant in the oral infection model, despite preserved accumulation of IFN-γ producing CD4 T cells and increased numbers of IL-17 producing CD4 T cells. This is consistent with IL-12 independent T-helper mediated IFN-γ and IL-17 production that has been reported in other studies of attenuated *Salmonella* infection [100,176]; [66]. Nonetheless, the IL-23 dependent responses are not sufficient to compensate for IL-12 deficiency, and herein weshow that TH1 CD4 T cell response in IL-12 deficient mice is phenotypically distinct from WT mice. IL-12 deficient mice had increased numbers of CD4 T cells that produced IFN-γ, IL-17A and IFN-γ/TNF (data not shown) compared to WTmice, suggesting that TH1, TH17, and polyfunctional CD4 T cells (IFN-γ/TNF-producing) were generated in the absence of IL-12. The greater numbers of these cell types seen in the p35-/- mice probably reflects a combination of the increased bactrerila burden driving antigen specific T cell responses, and ability of IL-23 to support the accumulation of both TH1 and TH17 cells. The ontogeny of the CD4 T cell responses may also differ in these mice.

Humans with defects in the IL-12p40 pathway experience chronic and disseminated *Salmonella* infections. These individuals have decreased T cell proliferation and IFN-γ production, and administration of IFN-γ to these patients improves their disease [143]. In our study, the loss of both IL-12 and IL-23 severely attenuated the accumulation of CD4 T cell antigen specific IFN-γ responses in the spleen at 24 d post-immunization; however, p40-/- spleens contained a small but detectable population of IFN-γ producing CD4 T cells (Fig 3-2 C&D), as reported in the murine typhoid model [66]. Interestingly, p40 mice did not control systemic infection while p19-/- and p35-/- mice were able to do so, indicating that the small but detectable TH1 cell immunity in p40-/- mice is not sufficient to control infection with attenuated *Salmonella* or prevent systemic spread.

Humans with defects in IL-12p40 are chronically infected with *Salmonella* despite a robust antibody response [182]. In our study mice we show that similar to IFN-γ-/- mice[183], mice deficient in both IL-12 and IL-23 (Fig. 3-4) have augmented antibody production, suggesting that the lack of the protective TH1 cells results in increased antigen burden and higher antibody titers[182]. In addition to the global increase in antibody production in p40-/- mice, p40-/- mice also had substantial increases in IgA titers relative to the other mice. Utilization of antibody mediated immunity, albeit a less effective yet important means of protection, may contribute to the chronic nature of NTS infection in humans with defects in IL-12p40 or its receptor [99]. The increased antibody produced in p40-/- was not sufficient to provide protection against *Salmonella* infection, suggesting that IL-12 and IL-23 actions on additional cell types are required for protection.

Surprisingly, immunized p40-/- mice and p35-/- mice had significantly lower cecal CFU during rechallenge. This either implies that the loss of IL-12 alone or with IL-23 led to better control of gut bacterial infection. The p35-/- mice had augmented TH17 responses to *Salmonella*, suggesting that enhanced TH17-mediated immunity may help protect the gut from *Salmonella* infection. The p40-/- mice lacked TH17 responses, indicating that a separate mechanism may protect p40-/- mice from gut infection. Because the p40-/- had increased anti-*Salmonella* antibody titers, including IgA, relative to the other mice, the heightened antibody mediated immunity may limit cecal infection in immunized p40-/- mice. Additional studies looking at functional responses in lamina propria cells will help give a more detailed understanding of the cecal immune responses that control *Salmonella* infection and provide protective immunity.

Together these data indicate that either IL-12 or IL-23 are sufficient for generation of TH1 responses during oral *Salmonella* infection, and efficient protection. IL-12 is well known to promote TH1 immunity. Whether IL-12 acts solely on TH1 cells or other cell types to enhance protective host responses during *Salmonella* infection is undetermined. IL-23 was required to sustain TH17 responses, which were suppressed by IL-12. The TH17 responses may help limit *Salmonella* infection in the cecum. Combined IL-12 and IL-23 are essential for optimal control of *Salmonella* infection, and these cytokines also suppresses anti-*Salmonella* antibody responses, which may also limit cecal infection. IL-12p40-/- have been shown to produce Th2-type cytokine IL-4 [180] and this cytokine can induce mucosal IgA and serum IgG antibodies in response to infection [181]. Therefore, augmented antibody responses in p40-/- mice may reflect a combination of increased bacterial burden and TH2 cytokine stimulated B cell immunity.

Using tetramer staining for 2W1S specific CD4 T cells, we compared the responses of antigen-specific CD4 T cells between naïve and immunized mice upon challenge with WT *Salmonella*. These studies revealed similar numbers of tetramer (+) CD4 T cells in the spleens of p35-/- mice compared to WT mice and p19-/- (Fig. 3-3). Surprisingly, IFN-γ producing tetramer (+)CD4 T cells were increased in p35-/- mice, compared to WT and p19-/- mice. The increased number of TH1 CD4 T cells in the p35-/- mice may reflect their inability to effectively control the WT *Salmonella* infection relative to the WT and p19-/- mice. This interpretation is supported by the prominent numbers of tetramer (+) and (-) IFN-γ producing CD4 T cells that were also detected in the spleens of challenged p40-/- mice. The decreased ability of these mice to control WT infection may have resulted in greater antigen burden and T cell stimulation. The failure to detect substantial TH1 responses in immunized p40-/- mice at 24 d suggests that either IL-12 or IL-23 is needed to sustain TH1 responses during *Salmonella* infection. The markedly augmented TH1 response in immunized p40-/- mice post-challenge indicates that neither IL-12 nor IL-23 are required for the differentiation of TH1 cells during *Salmonella* infection. Thus *Salmonella*-specific TH1 CD4 T cell responses can develop in the absence of both IL-12 and IL-23. Our data suggest that IL-12 or IL-23 is required to sustain TH1 responses during *Salmonella* infection The proliferative capacity and functional properties, including memory development, of antigen specific TH1 cells that arise in the immunized p40-/- are unknown. In contrast to TH1 CD4 T cells, no significant TH17 CD4 T cell responses were detected in the p40-/- mice, suggesting that IL-23 is essential for the accumulation of *Salmonella*-specific TH17 CD4 T cells during primary immunization and challenge. Similar to IL-23, its downstream effector pathways, IL-17RC and IL-22 did not influence protection against oral *Salmonella* infection or the accumulation of IFN-γ and IL-17 producing CD4 T cells (suppl. Fig. 3-1). Our data also indicate that differences exist

between gut-associated (peritoneum) and systemic (spleen) immune sites, and suggest that IL-12 and IL-23 have differential effects on host immunity in different tissues.

In summary, our data uncover complimentary and unique roles for IL-12 and IL-23 during oral immunization and challenge with *Salmonella*. IL-12 suppresses and IL-23 is required for the development of TH17 CD4 T cells. Both IL-12 and IL-23 contribute to establishing sustained TH1 immunity to *Salmonella*, and lack of IL-12 may enhance TH1 immunity through failure to efficiently control infection and increased antigen burden. In contrast, neither IL-12 or IL-23 is required for the development of TH1 immunity to *Salmonella* infection, although both appear to be required to sustain this immune response. This indicates that factors other than IL-12 and IL-23 are sufficient to promote TH1 cell differentiation and activation during Salmonella infection, but that either IL-12 or IL-23 are required to sustain the TH1 immune response. The factors that influence TH1 cell differentiation during *Salmonella* infection are unknown, and similarly the ontogeny of the IFN-γ producing cells that are sustained by IL-12 or IL-23 have not been defined. Our data indicate that the IL-23 sustained immunity may be better at protecting the cecum against *Salmonella* infection, possibly via TH17 cell, and that IL-12 sustained immunity may be better at protecting the host against *Salmonella* infection in systemic sites. Whether these differences are due to functional differences in CD4 T cell immune responses, or the effects of these cytokines on other cell populations is an area for future investigation.

Material and Methods:

Mice

C57BL/6 (B6), IL-12p35-/-, IFN- γ-/-, and IL-12p40-/- mice were from The Jackson Laboratory and bred in-house. IL-23p19–/–, IL-17RC–/– and IL-22–/– mice bred onto the C57BL/6 background were kindly provided by Dr. Wenjun Ouyang (Genentech) {Ghilardi, 2004}, {Zheng, 2008}. Age-matched male and female mice between the ages of 8 and 12 wk were used in experiments. Mice were bred and housed in a specific pathogen-free facility at the University of Washington. All experiments were performed under Institutional Animal Care and Use Committee-approved protocols.

Bacterial strains

Salmonella enterica serovar Typhimurium strain SL1344 {Hoiseth, 1981} and SL1344 expressing 2W1S peptide (graciously provided by Marc Jenkins) were grown overnight at 37°C with shaking (200 rpm) in Luria-Bertani (LB) broth. The Δ*aroA* mutants were produced by P22 phage transduction into wild-type strain SL1344 or the SL1344-2W1S strain. Transductants were selected for tetracylcline resistance. *In vitro* studies show no difference in virulence and growth between SL1344 and SL1344-2W1S. These strains are all naturally resistant to streptomycin.

Animal infection experiments

Mice were fasted for 4–6 hr and pretreated with 20 mg of Streptomycin (ISC Bioexpress, Solon, Ohio) in 0.1 cc sterile phosphate buffered saline (PBS) by oral gavage. The following day mice were fasted for 4–6 hr and infected with 1-5×10^8 ΔaroA *S*. Typhimurium by oral gavage. For immunization studies (Fig. 3-3) Mice were orally immunized with 5×10^8 ΔaroA 2W1S or PBS 1 day after streptomycin pretreatment. After 28 d, mice ere pre-treated with streptomycin, and the following day mice were orally challenged with 500 CFU of WT SL1344 expressing 2W1S. For systemic infections, mice were injected intraperitoneally with 5×10^6 Δ*aroA* SL1344. At designated time points, mice were euthanized, PECs, whole cecum, spleen and liver were collected and weighed prior to further analysis. To assess bacterial burden sections of each organ were weighed and homogenized in PBS with 0.025% Triton X-100 (EMD chemicals, Darmstadt,

Germany), and serial dilutions were plated on streptomycin (50 μg/ml) containing MacConkey agar plates.

***Ex vivo* stimulation and endogenouse T-cell detection**

Single cell suspensions of the spleens were prepared by mincing and passing the tissue through a 70-μm mesh Cellstrainer (BD Falcon, New Jersey). The erythrocytes were lysed by resuspending and incubating the cell pellet for 2 min in ammonium chloride buffer (0.15 M NH4Cl; 0.1 mM EDTA disodium salt dihydrate; 10 mM NaHCO3; all reagents from Merck) at room temperature. The splenocytes were washed three times in cold PBS and resuspended in RPMI 1640 culture medium (Gibco, New York) supplemented with 10% heat-inactivated FCS, 2 mM l-glutamine (Biochrom, Berlin, Germany), 10 mM HEPES buffer, 100 U/ml penicillin, 100 μg/ml streptomycin, and 50 μM 2-ME (Sigma). After harvest from naïve or infected splenocytes or peritoneal exudate cells, these cells were *ex vivo* challenged for 6h with either PMA (50 ng/ml) and ionomycin (200 ng/ml), 10μg of 2W1S synthesized peptide (GenScript), or media (no restimulation) in the presence of 10 μg/ml brefeldin A (Sigma, Missouri) at 37°C. Following incubation, cells were treated with 2 mM EDTA in PBS for 10 min at 37°C to detach adherent cells. All future steps were incubated on ice. All antibodies used for flow cytometric analysis were purchased from BD Biosciences (San Jose, CA) or eBioscience (San Diego, CA). Cells were washed with PBSA (1% w/v BSA in PBS), and Fc receptors were blocked using α-CD16/32 for 15 min. Cells were stained with CD4 and CD44, fixed/permeabilized using the Cytofix/Cytoperm (BD) and stained for intracellular PERCP conjugated anti-IFN-γ or phycoerythrin-conjugated anti-IL-17A. We collected 1–3x10^6 gated events using a LSR II flow cytometer (BD). The data were analyzed using FlowJo software (Treestar, Inc., Ashland, OR).

Tetramer and cell surface staining.

Splenocytes from naïve or immunized mice were stimulated with PMA (50 ng/ml) and ionomycin (200 ng/ml) in the presence of brefeldin A (10 μg/ml). Cells were stained for 1 h at 25 °C with 2W1S:I-Ab–streptavidin-phycoerythrin, followed by surface staining with surface anti-CD4, then cells were treated with Cytofix/Cytoperm and stained with PERCP conjugated anti-IFN-γ and FITC conjugated anti-IL-17A. In all cases, cells were then analyzed on an LSR II (Becton Dickinson). Data were analyzed with FlowJo software (TreeStar).

Serum immunoglobulin analysis

Salmonella-specific IgG, and Ig isotypes IgA, IgG1 and IgG2c were measured by ELISA according to standard protocols {Engvall and Perlmann, 1972, #73999}.. Heat-killed *S*. Typhimurium were prepared by incubating bacteria (10^{10} CFU/ml in PBS) at 60 °C for 60 min and then stored at at −80 °C. Briefly, 192 well micro flat bottom ELISA plates (Costar, New Hampshire) were incubated overnight with 0.05 ml/well of h.k. SL1344 lysates (10ug/ml) in carbonate buffer (0.06M, pH 9.6) (Fisher-Scientific, NH) at 37°C overnight. Several wells in each plate were coated with only carbonate buffer as negative controls. Plates were washed three times with PBS/Tween, and then blocked with 100ul /well of PBS/T/FCS (PBS/Tween with 2% v/v fetal calf serum) for 1hr at room temp. Plates were washed again and then incubated with 0.05 ml/well of mouse sera serially diluted in wash buffer in duplicates, and the plates were incubated at room temp for 2 hrs. Plates were washed again, and then incubated with 0.05 ml/well peroxidase-conjugated goat anti-mouse IgG, IgA-, IgG1 and IgG2c (Source) diluted 1:1,000. Plates were incubated again at room temp for 2hrs. Plates were washed and then developed with 0.05 ml/well of TMB substrate sln (Invitrogen, New York) for 15min at room temperature, and stopped with 25ul of 2N sulfuric acid to each well. OD 450/490 were measured and mean OD values calculated for each serum dilution tested.

Statistics

We calculated statistical significance with Prism software (GraphPad, La Jolla, CA). Unless otherwise specified, all studies for which data are presented are representative of at least two independent experiments. Statistical analysis of more than two normally distributed groups was performed using ANOVA with Bonferroni post-test; comparison of two normally distributed groups was done using the unpaired Student's T-test with Welch's correction for unequal variance. For multiple comparisons of non-Gaussian distributed data, the groups were analyzed using the Kruskal–Wallis test followed by Dunn's post-test, and comparison of two groups of non-Gaussian distributed data was performed using the Mann–Whitney rank-sum test. Log-rank (Cox) test was used to compare the Kaplan Meier survival curves. Statistical significance was defined as a P value <0.05, and indicated in the figure legends.

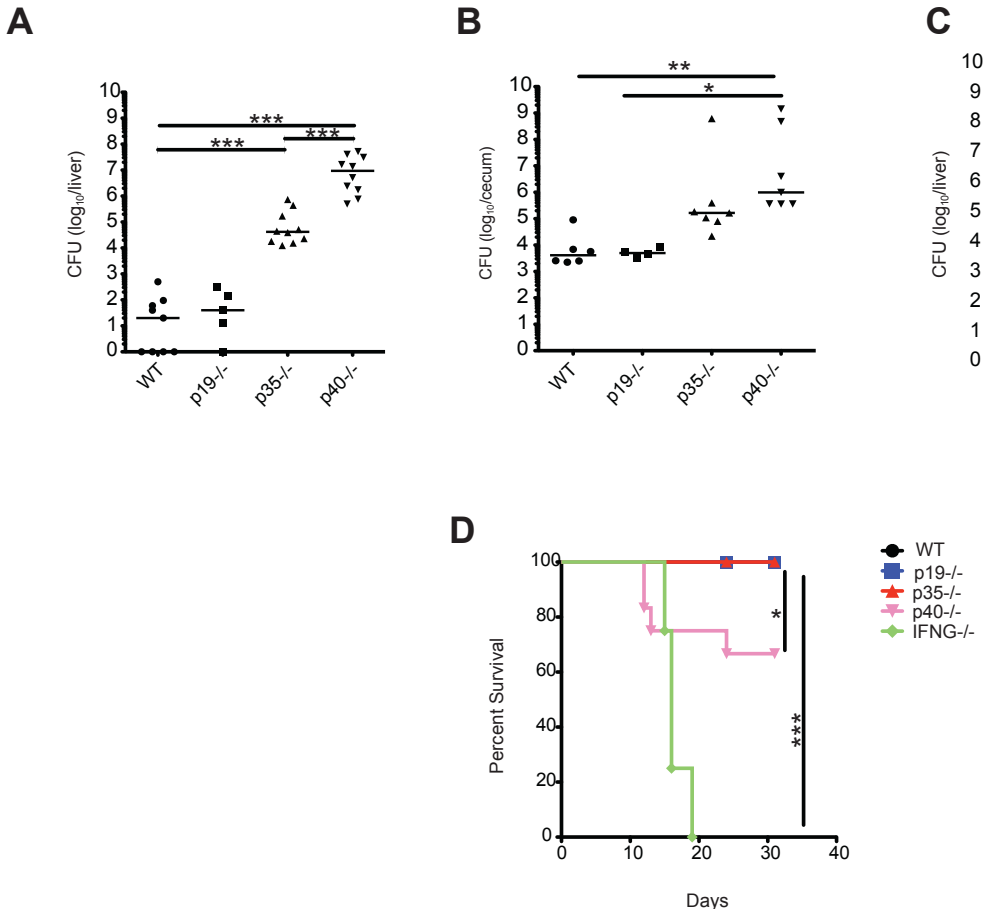


Figure 3-1: IL-12 and IL-23 are required for resistance against *S.* Typhimurium (Stym) by the systemic or oral routes of infection.

WT, p19$^{-/-}$(no IL-23), p35$^{-/-}$(no IL-12), and p40$^{-/-}$ (no IL-12/-23) mice were infected i.p. (A) or orally (B-D) with 5X10^{6} or 1-5×10^{8} CFU, respectively of ΔaroA *S.* Typhimurium. Orally infected mice were pretreated with 20 mg of streptomycin 1 day prior to infection. Liver (A&C) and (B) cecum were collected from infected mice 24 days post infection. Organs were weighed, homogenized and plated to determine CFU/organ. Bars represent the median bacterial load. Pooled data from 2 separate experiments are shown in A-C. Statistical significance was determined using the Kruskal Wallis test with Dunn multiple comparisons test (A-C). *: p<0.05, **: p<0.01, ***: p<0.001. Survival (D) was observed until day 35 after oral infection. Pooled data of two independent experiments are shown with the following: n = 12 (C57BL/6 mice), n = 10 (p19$^{-/-}$ mice), n = 7 (p35$^{-/-}$ mice), and n = 12 (p40–/– mice), and n=4 (IFN-γ$^{-/-}$). Log-rank (Cox) test was used to compare the five Kaplan Meirer survival curves.

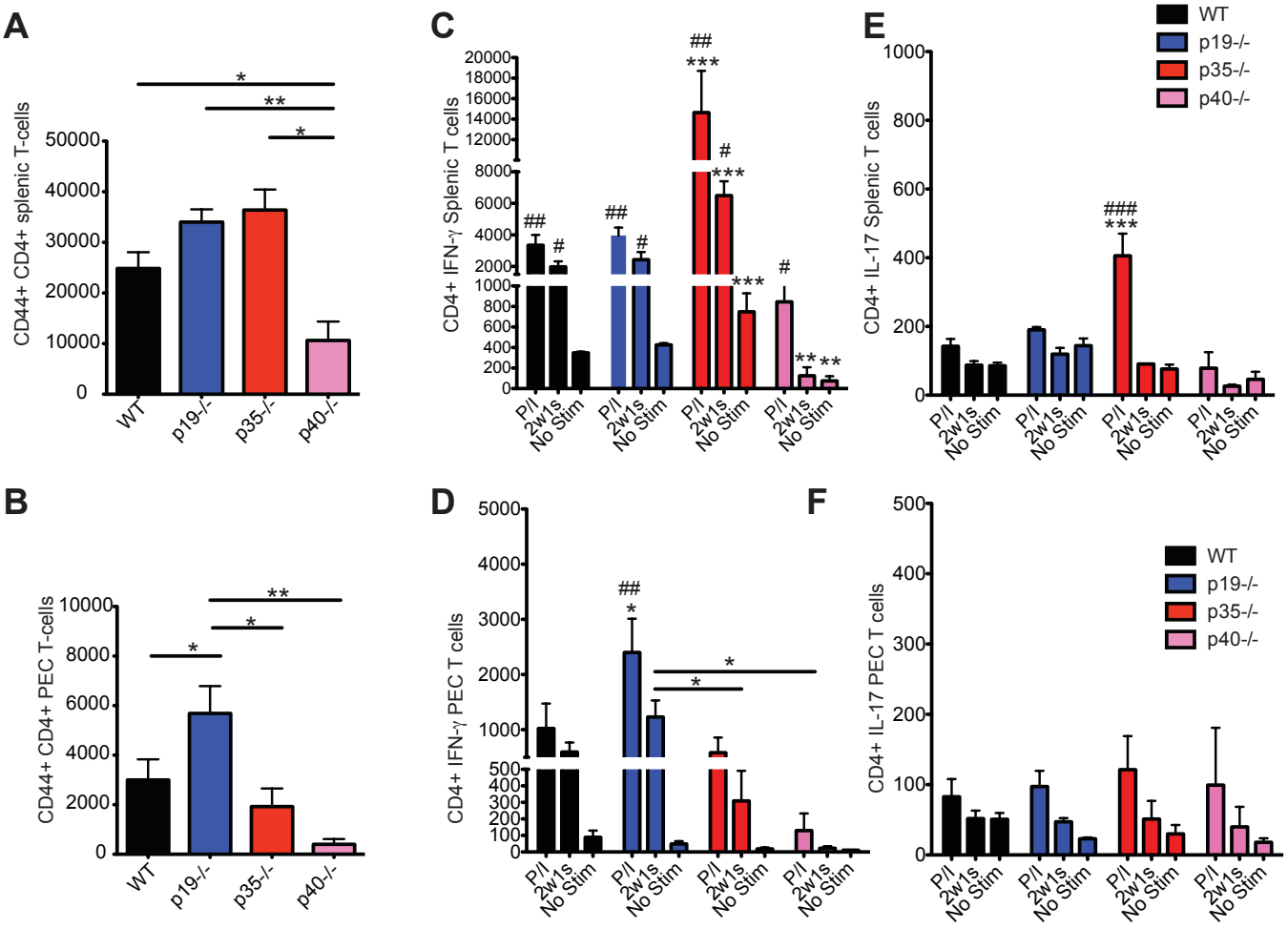


Figure 3-2: Differential regulation of CD4 T-cells lineages by IL-12 and IL-23.

24d after oral infection with 5×10^8 ΔaroA SL1344 expressing 2w1s (ΔaroA Stym2w1s), we stimulated splenocytes (A, C, E) or peritoneum exudate cells (PECs)(B, D, F) with PMA and Ionomycin (P/I), 10μg of 2W1S peptide, or media (no restimulation). Cells from both compartments were treated for 6 hrs ex vivo with BFA, followed by surface staining with CD4 and CD44 and intracellular IFN-γ and IL-17a . Quantification of splenic and PEC expression of CD44+ (A&B), IFN-γ (C&D) and IL-17a (E&F) mean positive CD4 T cells/mouse. Bars represent the mean counts. In A&B, * statistical significant differences amongst CD44/CD4 positive populations amongst the WT and mutant groups. In C-F, # represents a signifcant difference between stiumulated and unstimulated cells within each genotype and * represents a significant difference between cells from WT mice and cells from the other genotypes for each stimulation. * and bar draws differences between groups not relative to WT mice Statistical significance was determined using one way ANOVA followed by Newman-Keul's post test. */#: $p<0.05$, **/##: $p<0.01$, ***/###: $p<0.001$. Combined data from X experiments with Y mice per group.

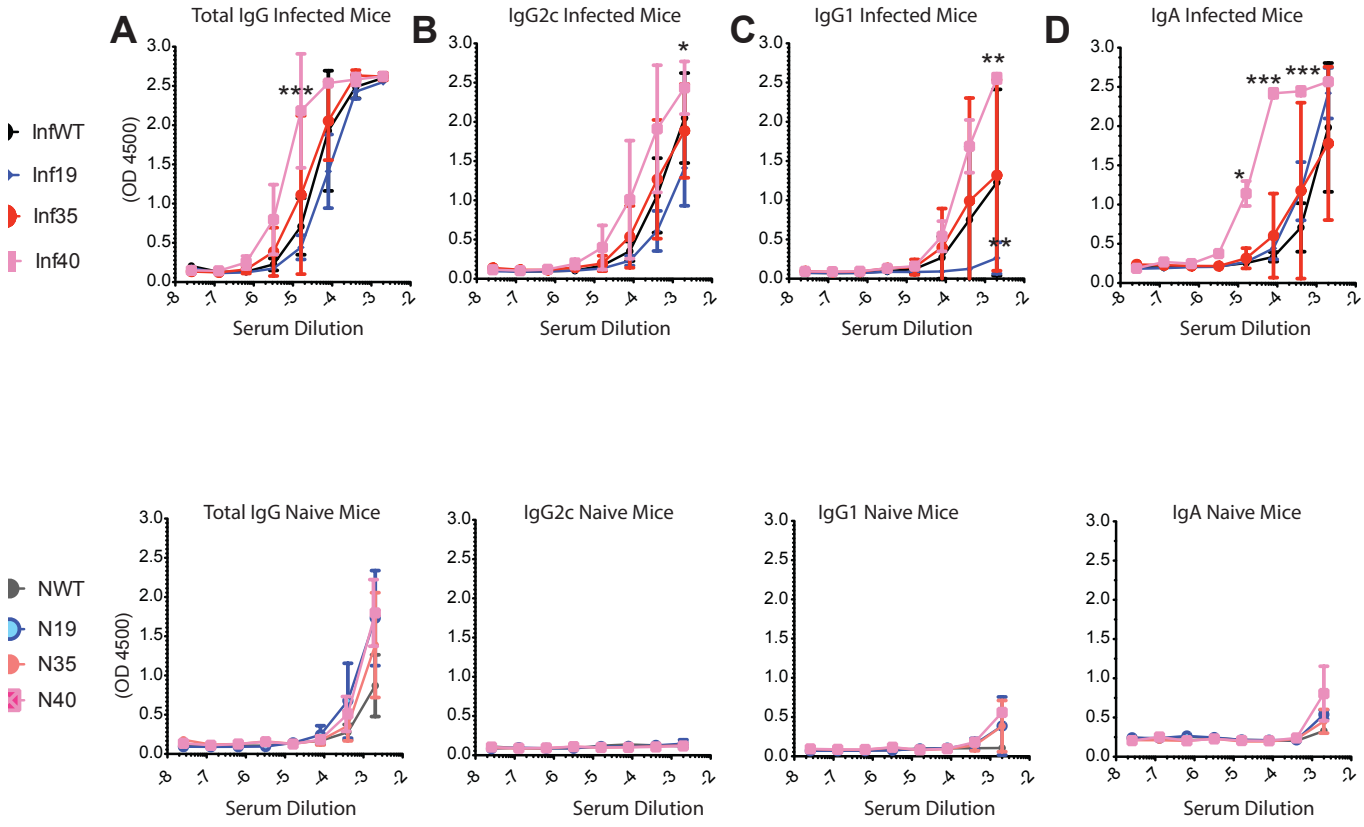


Figure 3-3: *Salmonella* specific antibody production is augmented in the IL-12/-23 deficient mice after oral infection.

Antibody titers of Salmonella-specific total IgG, subclass 2c and G1 and IgA in the serum of naïve mice (N) mice (lower row) 24d after treatment with PBS or immunized mice (upper row) 24 days after 5×10^8 oral ΔaroA SL1344 infection. These results show one out of two similar independent experiments containing 4-8 mice per group. * represents a significant difference between mouse groups from WT mice at each dilution. Statistical significance was determined using two-way ANOVA followed by Bonferroni post test. *: $p<0.05$, **: $p<0.01$, ***:$p< 0.0001$

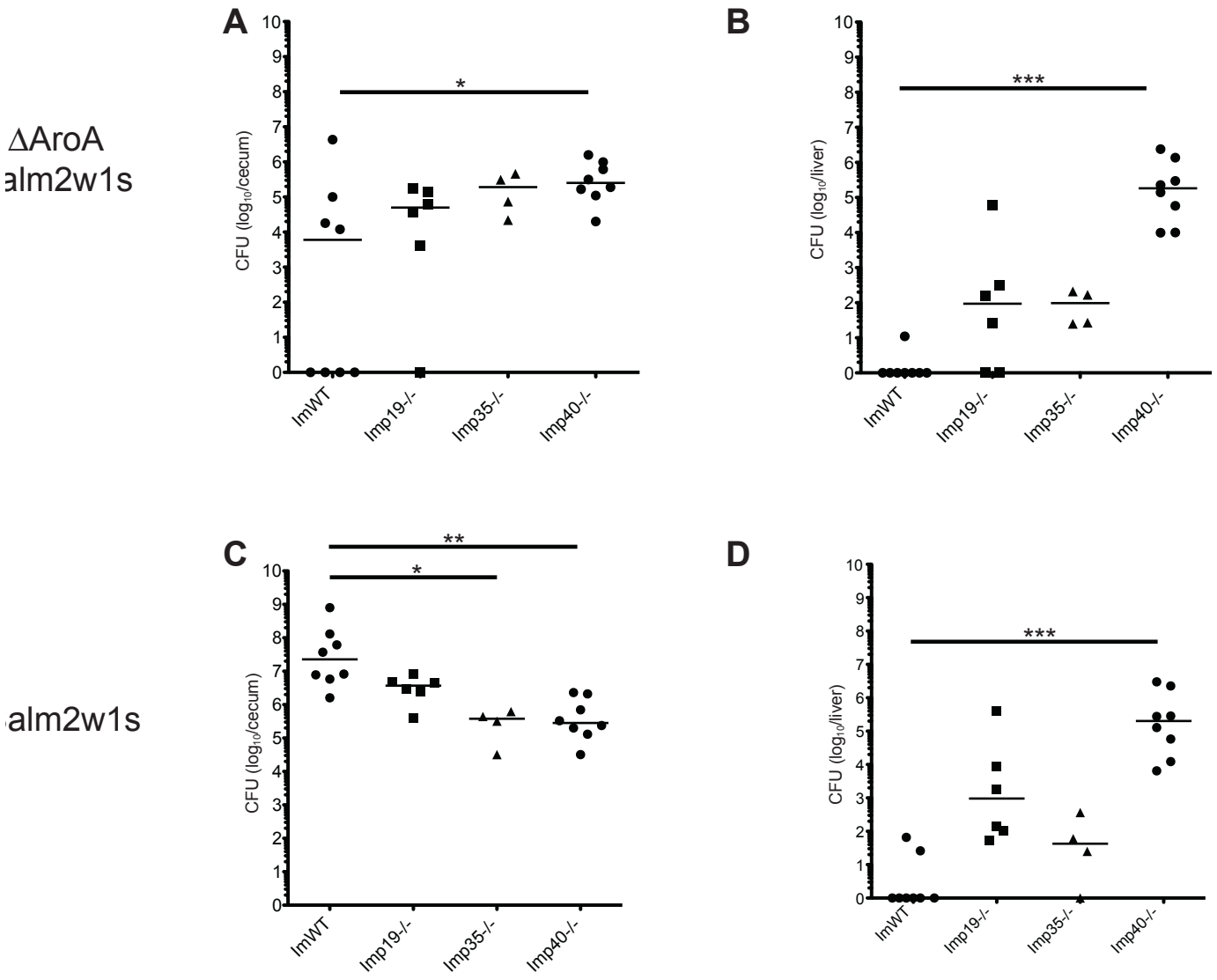


Figure 3-4: IL-12 and IL-23 are required for resistance against *S.* Typhimurium (Stym) systemic or oral infection.

WT, $p19^{-/-}$(no IL-23), $p35^{-/-}$(no IL-12), and $p40^{-/-}$ (no IL-12/-23) mice were orally immunized with 5×10^8 ΔaroA Stym2w1s or PBS 1 day after streptomycin pretreatment. 28 d later, after another streptomycin pretreatment, mice from either immunized or naïve groups were orally infected with 500 CFU of WT SL1344 expressing 2W1S. Cecum (A+C) and Liver (B+D) were collected from mice 3d after WT challenge and either ΔaroA Stym2w1s (A&B) or WT Stym2w1s (C&D) were counted from each organ. Organs were weighed, homogenized and plated to determine CFU/organ. Bars represent the median bacterial load. 1 representative of 2 experiments is shown. Statistical significance was determined using the Kruskal Wallis test with Dunn multiple comparisons test (A-C). *: $p<0.05$, **: $p<0.01$, ***:$p< 0.0001$

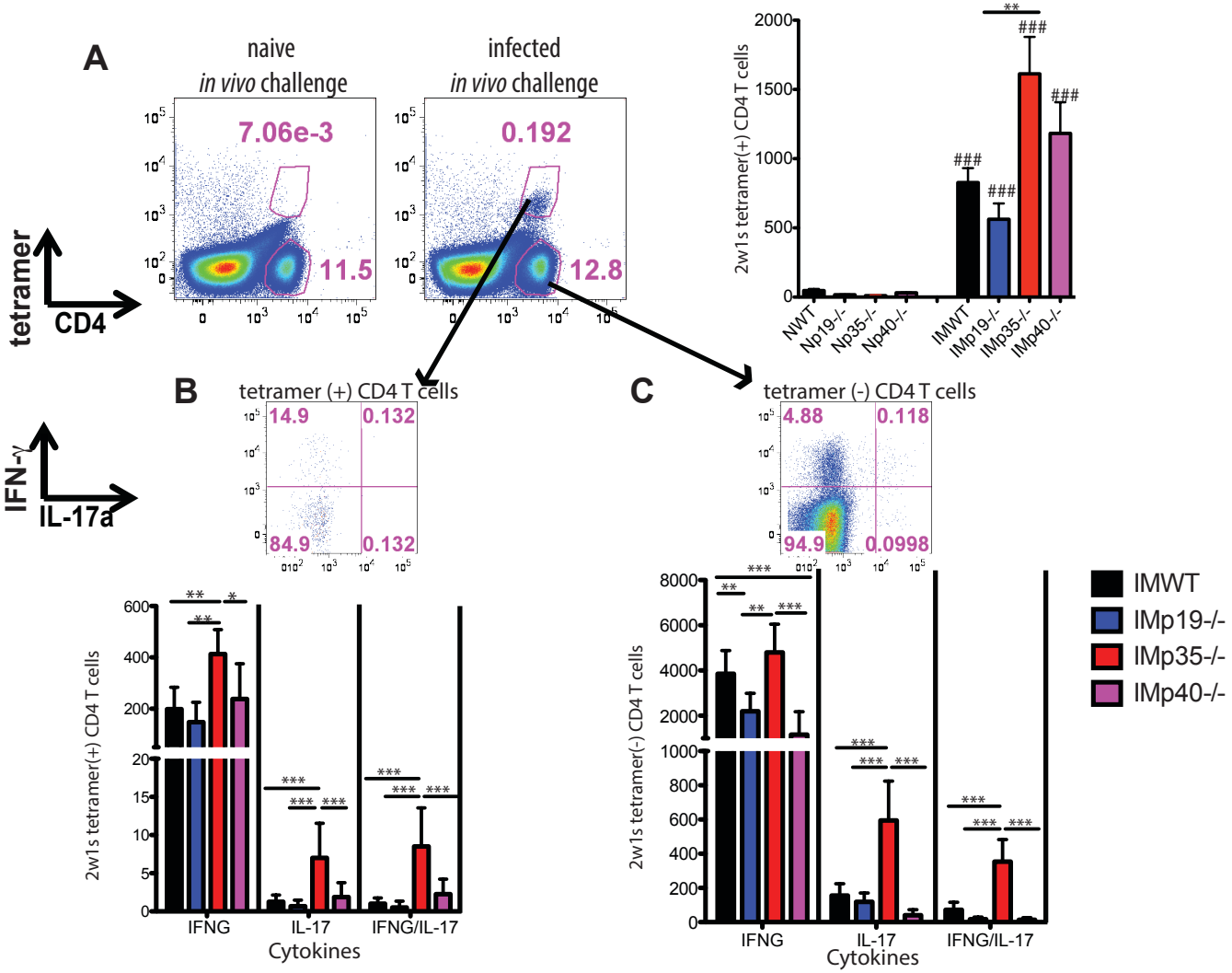
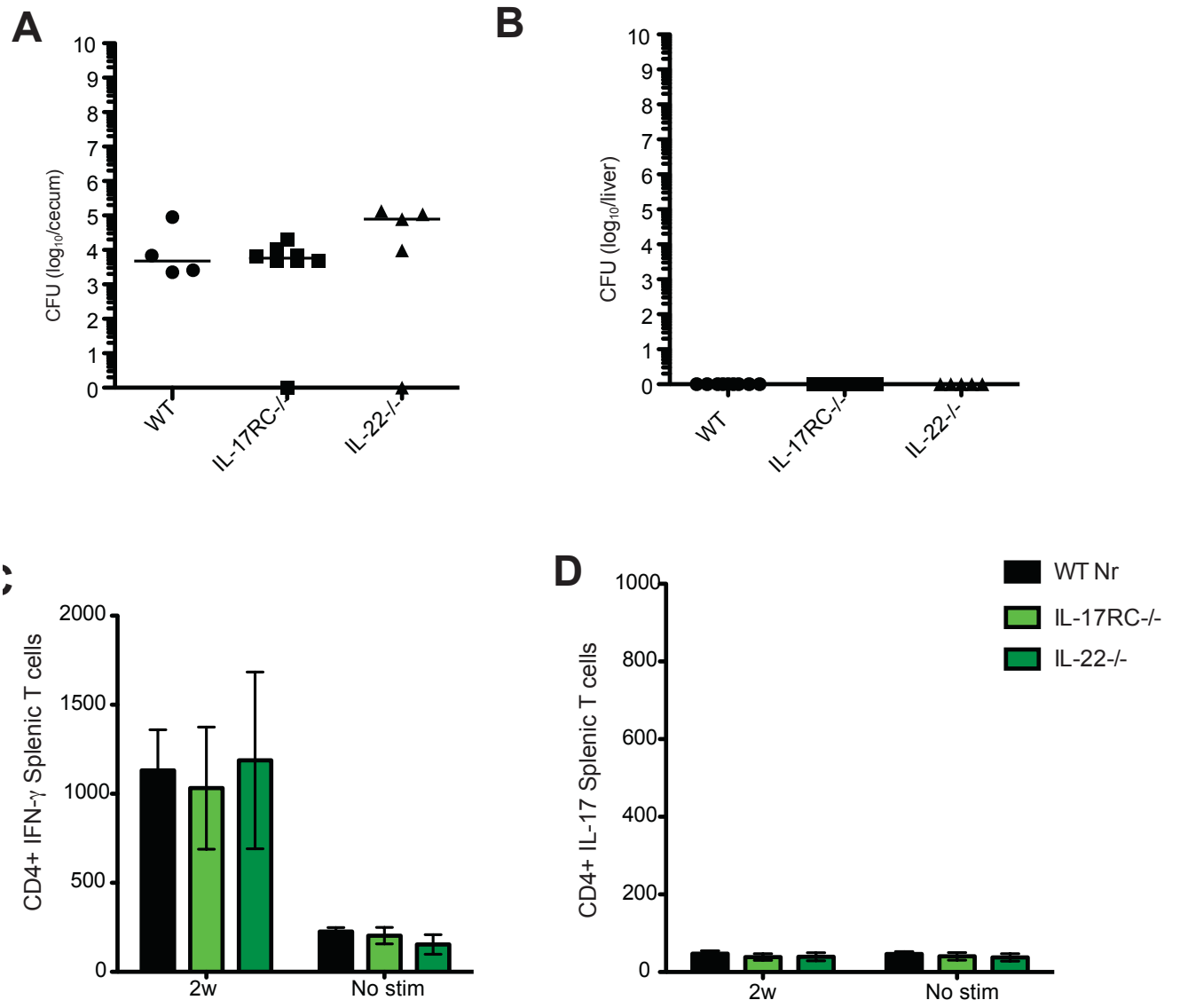


Figure 3-5: IL-12/-23 dependent and independent antigen-specific IFN-γ and IL-17 production differentially regulated

C56BL/6 mice deficient in IL-12, IL-23 or both were orally immunized with 5×10^8 ΔaroA Stym2w1s or PBS 1 day after streptomycin pretreatment for 28d. Then 1 day after another streptomycin pretreatment, mice from either immunized or naïve groups were orally infected with 500 CFU of WT SL1344 expressing 2W1S. We harvested splenocytes at d3 from naïve or immunized animals and stimulated for 6h with phorbol 12-myristate 13-acetate and ionomycin in the presence of brefeldin A and analyzed the 2W1S antigen specific response, with a WT naïve or immunized representative mouse shown in A. Quantification of splenic mean 2W1S:I-Ab–specific CD4 T cells/spleen in mice based on the gates above. From this gate 2w1s tetramer (+) (B) or (-) (C) IFN-γ, IL-17, or dual producing CD4 T cells were quantified. In A-C the * represents differences in 2w1s tetramer expression (total or individual cytokines) amongst the various immunized mouse groups. Numbers in A, represents the differences between naïve and immunized mice for each genotype. Statistical significance was determined using one way ANOVA followed by Newman-Keul's post test. */#: $p<0.05$, **/##: $p<0.01$, ***/###: $p<0.001$. Data are representative of two independent experiments.



Supplementary Figure 3-1: Minimal role for IL-17R or IL-22 in Protection against Oral *Salmonella*

WT, IL-17 receptor RC-/- and IL-22$^{-/-}$ were orally infected with 5×10^8 CFU ΔaroA *S.* Typhimurium. Cecum (A) and liver (B) were collected and prepared for CFU counts similar to Figure 3-1. Bars represent the median bacterial load. Pooled data from 2 separate experiments are shown in A&B. Statistical significance was determined using the Kruskal Wallis test with Dunn multiple comparisons test. E*x vivo* challenge of harvested splenocytes (C&D) with10μg of 2W1S peptide, or media (no restimulation) Cells were treated for 6 hrs ex vivo with BFA, followed by surface staining with CD4 and intracellular IFN-γ and IL-17a. Quantification of splenic IFN-γ (C) and IL-17a (D) positive CD4 T cells. Bars represent the mean counts. Statistical significance was determined using one way ANOVA followed by Newman-Keul's post test. */#: $p<0.05$, **/##: $p<0.01$, ***/###: $p<0.001$.

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