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Human Resistin Regulates Immunity to Helminth Infections and Sepsis

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ABSTRACT OF THE DISSERTATION

Human Resistin Immunoregulation of Helminth Infection and Sepsis

by

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Resistin-like molecules (RELM), a family of mammalian-conserved secreted proteins, have immunoregulatory effects in helminth infection, acting as a negative feedback loop to limit excess inflammation at the expense of increased parasite burden. This is through downregulation of the Th2 cytokines, IL-4 and IL-13, that are essential for alternative activation of macrophages and clearance of helminths. However, it is unclear how the human RELM proteins regulate immunity to helminth infections and inflammation. In this thesis, we have combine data from human studies and a transgenic mouse model, where human resistin and its regulatory region was inserted in mice (h*Retn*Tg^{+}). Human patients infected with filarial nematodes or soil-transmitted helminths had higher levels of circulating hResistin, and hResistin was associated with higher parasite burden. To assess whether hResistin directly increases helminth infection, we infected h*Retn*Tg^{+} mice with the murine hookworm, *Nippostrongylus brasiliensis* (*Nb*), which revealed that hResistin is upregulated by macrophages and we identified IL-4/STAT6 signaling

as a novel pathway for hResistin expression. Human resistin expression during helminth infection contributes to increased parasite burden and prolonged infection. RNA-seq of whole lung RNA demonstrated that h*Retn*Tg^{+} mice had increase proinflammatory cytokines and toll-like receptor (TLR) signaling. In human patients infected with helminths, hResistin is correlated with proinflammatory cytokines, suggesting that hResistin contributes to increased helminth infection through the upregulation of Th1 cytokines. Structural interaction predictions revealed that hResistin binds to TLR4 in the same binding pocket as MD-2, the adaptor protein that mediates lipopolysaccharide(LPS)-TLR4 interaction. Given that LPS-TLR4 interaction initiates septic shock, we hypothesized that hResistin may protect the host from endotoxic shock by outcompeting LPS binding to TLR4 and blocking subsequent proinflammatory cytokine production. Competitive cell binding assays confirmed that hResistin does indeed outcompete LPS for binding to TLR4, and thereby decreases Th1 cytokines in human PBMC. In a mouse model of LPS-induced sepsis, hResistin expression or exogenous administration resulted in protection against endotoxic shock, which was associated with a decrease in proinflammatory cytokines that promote sepsis progression. Finally, we show that *Nb*-induced hResistin reduces mortality from sepsis. In summary, we have identified hResistin as a key regulator of inflammatory responses in helminth infections and sepsis.

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CHAPTER ONE – Resistin-like Molecules Regulate Inflammation and Immunity to Helminth Infections

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Abstract

Helminths afflict two billion people worldwide, and have co-evolved with their hosts over millions of years to the benefit of both parasite and host. Since helminths are large extracellular parasites, the host is forced to limit tissue damage caused by the worms, rather than directly kill the worms. Thus, the type 2 immune response (Th2) evolved to expedite worm clearance from the host through mucus production, increase wound healing gene expression to repair tissue damage caused by the worms and regulate excess inflammation. This is characterized by the induction of Th2 cytokines, IL-4 and IL-13, which polarize macrophages to become alternatively activated. This contrasts with the Th1 immune response typically seen in bacterial or viral infection, where classically activated macrophages produce TNFα and IL-6 to combat intracellular pathogens. These immune responses often counter balance each other to prevent excessive inflammation, and more recently, Th2 induction has been proposed as a treatment for Th1-driven inflammatory diseases ranging from inflammatory bowel disease to sepsis.

In this chapter, we introduce the generation and regulation of type 2 immunity and its function in infectious diseases and wound healing. Next, we examine the ability of Th1 and Th2 immunity to balance each other, as understanding these counter-regulatory mechanisms could lead to better treatment of patients in both helminth infection and inflammatory diseases. Finally, we conclude with how resistin-like molecules, specifically hResistin, regulate of

Th2 immunity and inflammation, and we address the gaps in knowledge in the field.

Helminth infections

Helminths are a diverse family of large extracellular parasitic worms that can cause debilitating pathology, such as elephantiasis or blindness, and infect two billion people worldwide. Soil-transmitted helminths (STH) are the most prevalent group of helminths, infecting over 1 billion people, while filarial nematodes infect 150 million people, and trematodes infect over 200 million people [1]. The incidence of these parasites is predominantly confined to parts of the world where sanitation efforts are insufficient. Young children are most at risk for transmission, and their physical and cognitive development is often stunted by nutrient-stealing helminths. The soil-transmitted helminths that infect human patients include whipworms (*Trichuris*), roundworms (*Ascaris*, *Strongyloides*) or hookworms (*Necatur*). STH can be transmitted by fecal-oral transmission (whipworms), but some infective larvae can penetrate the skin of patients (roundworms or hookworms). These STH reside in the intestinal tract, where they reproduce and lay thousands of eggs per day, however some STH (hookworms) do have an intermediate life cycle in the lungs. On the other hand, filarial nematodes are dependent on a mosquito or black fly vector for transmission to the human host. A patient is infected when an infected mosquito takes a blood meal and deposits infective larvae into the host. The adult filarial nematodes reside in the lymphatics system, where they reproduce and release microfilariae into the bloodstream. Trematode (*Schistosoma)* cercariae can penetrate the skin of patients wading or swimming in contaminated water. These cercariae migrate

through the vasculature of the host and reproduce in the portal vein. Upon reproduction, eggs will then migrate to the intestine and get released in the feces.

Once the helminths are within the host, they are under enormous immune pressures to reproduce before they are expelled from the host. To that end, helminths have evolved mechanisms to maximize their fitness once they have reached the host. For example, *Schistosoma mansoni* development is stunted and reproductive activity is lost in immunodeficient mice, but can be restored by administration of the Th2 cytokine, IL-4 [2]. Thus, the host's own immune response triggers the accelerated reproductive activity of helminths. Helminths also secrete proteins that can delay the host's ability to expel the worms. *Nippostrongylus brasiliensis* secretes a cysteine protease inhibitor that decreases antigen processing and subsequent antigen-specific IgE production [3].

The host uses a number of pattern recognition receptors to identify helminth antigens that are on the cuticle (exoskeleton) or excreted/secreted by the helminths. The cuticle of helminths is coated with lipids, glycoproteins and collagen [4] that can be recognized through the pattern recognition receptors, C-type lectin receptors or toll-like receptors. For example, *Trichuris muris* antigens can be recognized by the mannose receptor, a C-type lectin receptor, and *Schistosoma* lysophosphatidylserine is recognized by TLR2 [5, 6]. Furthermore, helminth cuticle also contains chitin, a biopolymer that is known to induce the Th2 immune response necessary to clear helminths, although the receptor for chitin has not been identified. These antigens stimulate the production of Th2 cytokines that not

only expel the worm from the host but also heal tissue damage caused by the helminths.

Infected patients are treated with the anti-helminth drugs, ivermectin, praziquantel, diethylcarbamazine and albendazole. Many of these treatments inhibit metabolism of the helminths or cause paralysis by polarization of nerve or muscle cells. Development of vaccines has been unsuccessful for such diverse and complicated organisms and despite the availability of some anti-helminth drugs, resistance to these drugs is quickly rising and patients continue to get re-infected yearly. Thus, understanding how the host eliminates these pathogens and develops long-lasting immunity could offer new treatment strategies to eradicate parasitic disease.

Immune Response to Helminth Infections

Helminths elicit a Th2 immune response, dominated by IL-4 and IL-13, which lead to eventual expulsion of worms and healing of tissue that is damaged by the worms. This is initiated when tissue damage caused by helminths induce the production of danger-associated molecular patterns (DAMPs) by injured epithelial cells (Figure 1.1). IL-33, thymic stromal lymphopoietin (TSLP) and IL-25 have recently emerged as critical initiators of the Th2 immune response. IL-33 is released by necrotic epithelial cells and provides the initial signal that promotes Th2 cell recruitment and production of the canonical Th2 cytokines, IL-4 and IL-13 (Figure 1.2). Following helminth infection or exposure to allergens, TSLP promotes Th2 cell maturation in several ways, which include inducing dendritic cell surface expression of OX40L, inhibition of Th1/Th17 immune responses and mediating basophil activation to produce Th2 cytokines [7-10]. Likewise, IL-25 is produced by activated T cells and epithelial cells and mediates the production of IL-4 while inhibiting Th1/Th17 cytokine responses [11, 12]. IL-25 and IL-33 can also activate innate lymphoid cells to produce IL-4 and IL-13 early during helminth infection [12, 13]. Aside from Th2 cells and innate lymphoid cells, granulocytes, such as basophils, mast cells and eosinophils can be additional sources of Th2 cytokines [10, 14, 15].

IL-4 and IL-13 activate epithelial cells, which aid in expulsion of helminths from the host. Intestinal helminths can be expelled from the host by the “weep and sweep” method, characterized by the increase in production of intestinal mucus by

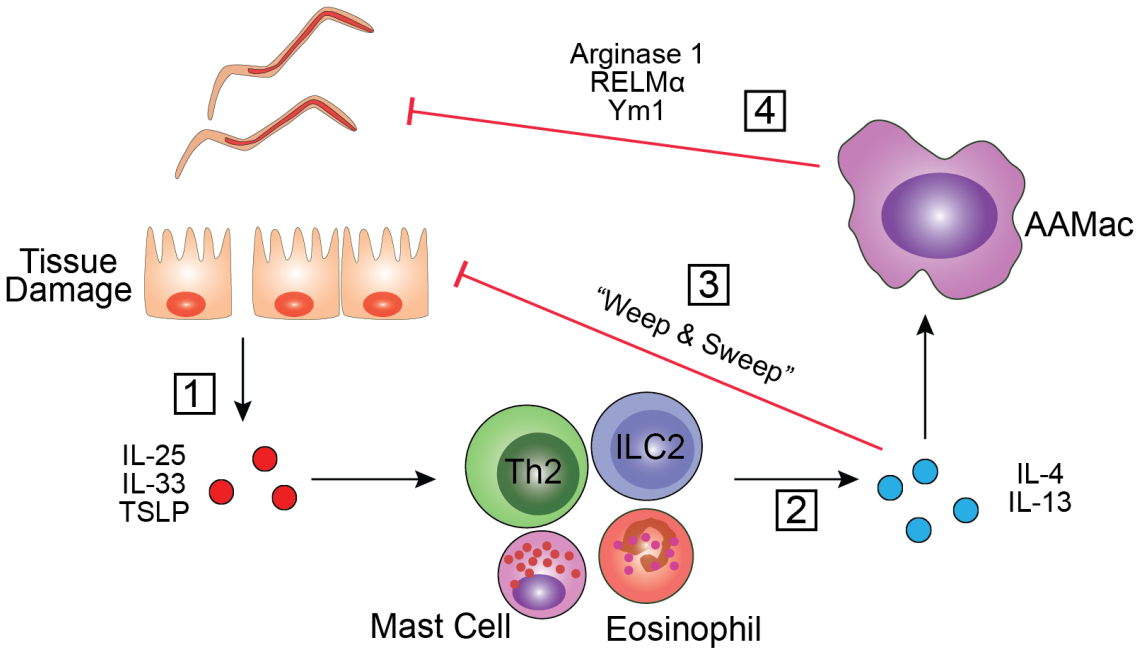

Figure 1: Th2 immunity during helminth infection. (1) Tissue damage caused by helminths induce epithelial cells to produce danger associated molecular patterns. (2) This leads to production of the Th2 cytokines, IL-4 and IL-13, by Th2 cells, innate lymphoid cells, mast cells and eosinophils. (3) IL-4 and IL-13 act on epithelial cells to clear helminth infections by "weep and sweep" mechanism. (4) IL-4 and IL-13 also polarize alternative activation of macrophages, which produce effector molecules to kill helminths and aid in wound healing.

goblet cells (“weep”) and epithelial cell contraction and hyperplasia (“sweep”) (Figure 1.3). This is dependent on IL-4/IL-13 signaling and phosphorylation of STAT6 [16, 17]. IL-4 also activates eosinophils to degranulate and release matrix metalloproteinases and growth factors that mediate wound healing, tissue remodeling and myofibroblast differentiation [18]. These Th2 cytokines also indirectly aid in helminth clearance and wound healing through alternative activation of macrophages (Figure 1.4).

Macrophages are a major component of the innate immune response to helminth infections. Originally discovered by Elie Metchnikoff, macrophages are a heterogeneous family of phagocytic immune cells capable of a battery of homeostatic, housekeeping, and infection-induced functions. These responsibilities include responding to and destroying pathogens, clearing debris caused by apoptotic cells, and regulating the host immune response. Following parasite infection, infection-induced signals, including pathogen associated molecular patterns (PAMPs) and cytokines, instruct macrophage activation.

Alternatively Activated Macrophages

Amongst many other Th2 cytokine-mediated effector mechanisms, including epithelial cell activation and turnover [19, 20], AAMacs contribute to immunity to certain helminths. In a secondary infection with *Heligmosomoides polygyrus*, or following infection with *Nippostrongylus brasiliensis*, AAMacs were critical in mediating worm expulsion [21, 22]. Both human and mouse AAMacs acted in concert with neutrophils to kill the human parasite *Strongyloides stercoralis* larvae [23]. Following *Trichuris muris* infection, macrophage-specific deletion in SH2-containing inositol 5'-phosphatase 1 (Ship1) resulted in impaired worm expulsion due to excessive CAMac activation [24], suggesting that the balance between classical and alternative activation is critically involved in immunity to helminth parasites.

AAMacs are characterized by the expression of several signature proteins including the mannose receptor, Arginase1, chitinases and RELMα, all of which are critically dependent on Th2 cytokines [25-27]. These AAMac signature proteins work in concert to combat helminth infection and limit helminth-associated inflammation. The macrophage mannose receptor (MMR) is a pattern recognition receptor of the innate immune system that binds to mannose, a sugar found on many pathogens including *Candida albicans* and HIV. Following helminth infection, MMR is involved in the immune recognition of *Schistosoma mansoni* larvae and *T. muris* [5, 28]. Arginase1 catalyzes the breakdown of arginine to prolines and polyamines. NOS2 and Arginase1 both compete for the substrate arginine to

create nitric oxide (NOS2) or ornithine/urea (Arginase1), respectively. In the context of helminth infection, Arginase1 dampens *S. mansoni* granulomatous pathology in the liver and Th2 immune responses [29]. Chitinase, an enzyme that cleaves and breaks down the chitin found on fungi, worms, and other organisms, consists of Ym1, a chitin-binding protein without chitinase activity, and acidic mammalian chitinase (AMCase). As an eosinophil chemotactic factor, Ym1 may mediate eosinophil accumulation in the infected tissue, although it is unclear whether Ym1 actually contributes to clearance of helminth infection. Similarly, AMCase, which is a functional chitinase, is highly upregulated in helminth infection and acts to breakdown chitin, thereby reducing the inflammatory effects of this allergen [30] and initiating Th2 immunity during *N. brasiliensis* and *H. polygyrus* infection [31]. The Resistin-like molecule (RELM) α is produced by AAMacs, eosinophils and epithelial cells, and is predominantly found in the lungs in response to allergens and helminth infections. Rather than help clearance of helminth infection, however, RELMα acts as a negative feedback loop to limit type 2 inflammation in multiple helminth infection models [32, 33].

Functional studies using mice are models to set a framework for helminth infections in humans. Similar to mouse models, $CD14^+$ blood monocytes from filaria-infected patients had increased AAMac gene expression compared to uninfected individuals. This included expression of Mannose receptor C type 1, macrophage Galactose type C lectin, Resistin and Arginase1 [34]. With well over 2 billion people infected with helminths, elucidating the immune response to these

parasites and the importance of AAMacs could have significant medical implications.

$CD4^+$ Th2 cells and innate cells such as eosinophils, basophils, mast cells and innate lymphoid cells secrete the cytokines IL-4 and IL-13, which induce alternative activation of macrophages (Figure 2.1). Additional cytokine/receptor pathways that contribute to AAMac activation include IL-21/IL-21R and IL-33/ST2 (Figure 2.2). During *N. brasiliensis* infection, IL-21 may promote AAMac activation indirectly by inducing macrophage expression of IL-4Rα and IL-13Rα1 expression, thereby promoting responsiveness to these Th2 cytokines [35]. IL-33 can directly induce AAMac gene expression and also activates innate lymphoid cells and $CD4^+$ Th2 cells to promote the type 2 cytokine environment allowing the control of helminths including gastrointestinal nematode *T. muris* [36, 37].

Both IL-4 and IL-13 bind to the common receptor IL-4Rα, activating the Signal Transducer and Activator of Transcription (STAT) 6 signaling pathway for AAMac specific gene expression and proliferation [38]. $STAT6^{-/-}$ mice exhibit defective Th2-mediated immune responses and AAMac expansion in numerous helminth infections including *N. brasiliensis* and *H. polygyrus* [39, 40]. While STAT6 directly promotes expression of AAMac signature genes, such as Arginase1 and RELMα, it also augments AAMac polarization indirectly, by binding to the promoter region of other transcription factors that induce AAMac gene expression (Fig. 2.3). These include Peroxisome Proliferator-Activated Receptor

(PPAR) γ, Krüppel-like Factor (KLF) 4, and Interferon regulatory factor (Irf) 4 [41-43].

The PPAR family contains nuclear receptors found on many leukocytes, including macrophages and T cells, and binds to fatty acids and eicosanoids [44]. In macrophages, PPARγ can be activated by IL-4/IL-13 [45], or indirectly via prostaglandins (e.g. 15d-PGJ2) or eicosanoids (e.g. PGI2). As transcription factors, PPARs form heterodimers with the retinoid X receptor (RXR) [46] to promote AAMac gene expression (e.g. Arginase1) [45]. Phosphorylated STAT6 also induces expression of KLF4, a member of the Krüppel-like family of transcription factors that regulates cellular differentiation and growth. In $LysM^{Cre}/KLF4^{Flox}$ mice, there were significant increases in CAMacs and delayed wound healing, characteristic of a deficiency in AAMacs [42]. Similar to PPARγ, 15d-PGJ2 can activate KLF4, although this pathway is independent of PPAR but instead signals through MAPK and ERK [47]. IRF4, another target of STAT6, can also mediate alternative macrophage activation by directly binding to the promoter region of AAMac signature genes in response to the allergen, chitin [48]. *Irf4* gene expression is also epigenetically regulated by Jumonji domain containing-3 protein (Jmjd3), a H3K27 demethylation enzyme following *N. brasiliensis* [48]. Mechanistically, Jmjd3 demethylates the histone region in the *Irf4* promoter, allowing access for binding to phosphorylated STAT6 and subsequent *Irf4* expression [43] (Fig. 2.4).

AAMac activation can also occur independently of STAT6, and is mediated instead by phosphorylation of the transcription factor, CCAAT-enhancer-binding protein β (C/EBPβ). For example, adenosine, a purine nucleoside that is typically upregulated during hypoxia and tissue injury, can augment the polarization of AAMac via a C/EBPβ-dependent mechanism [49] (Fig. 2.5).

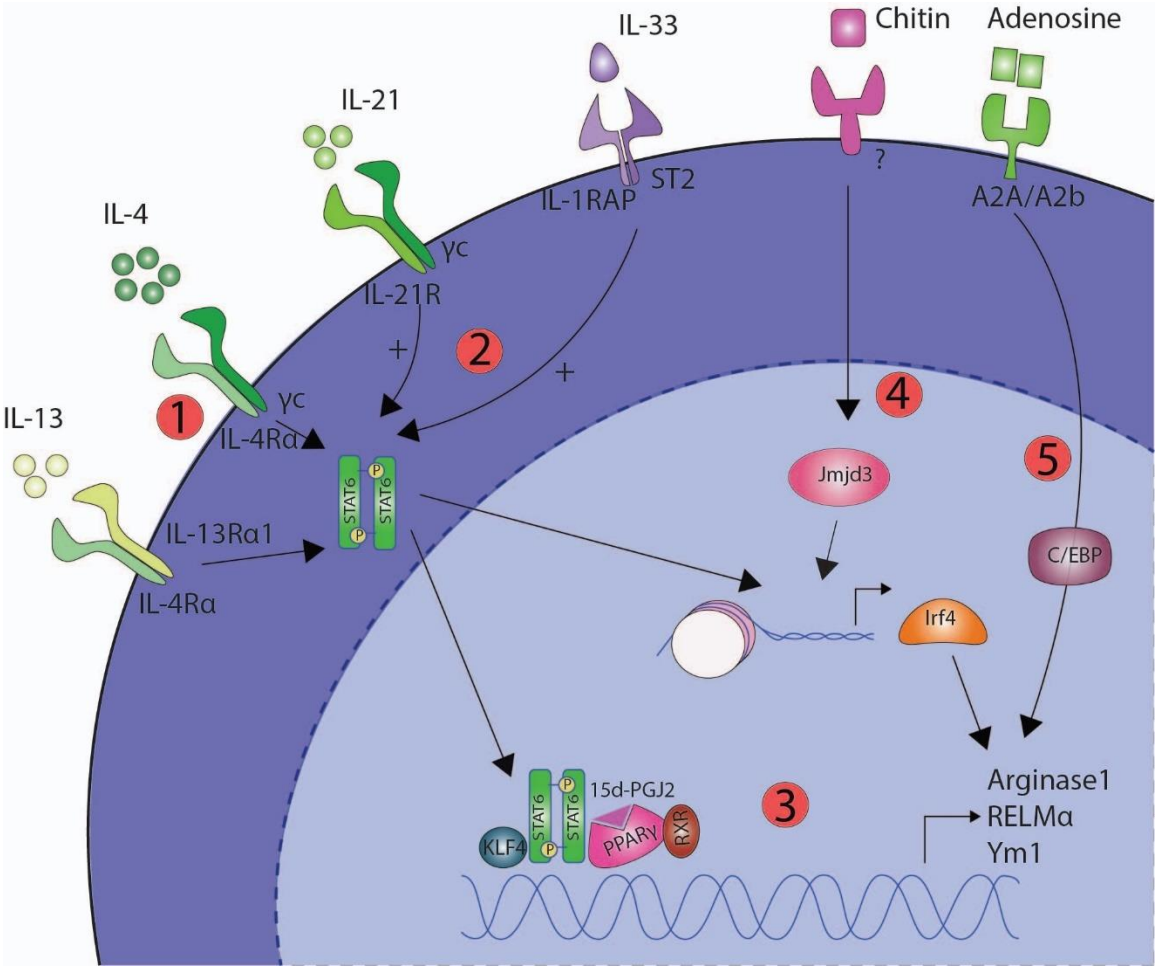

Figure 2: Polarization of alternatively activated macrophages. (1) IL-4 and IL-13, upon binding to their common receptor IL-4Rα, phosphorylates STAT6 to begin the induction of AAMac polarization. (2) IL-21 and IL-33 promote this signaling pathway. (3) STAT6 mediates expression of AAMac signature genes and activates other transcription factors such as PPARγ. (4) Helminths and chitin promote alternative activation of Jmjd3-mediated histone modification and expression of the transcription factor Irf4. (5) Adenosine promotes macrophage alternative activation through a C/EBPβ-dependent mechanism.

Th1/Th2 Balance

In contrast with Th2 immunity, the Th1 immune response occurs during infection with intracellular pathogens, including bacteria, viruses or protozoan parasites. Th1 immunity is initiated when pathogen-associated molecular patterns are recognized by pattern recognition receptors. For example, the toll-like receptor family can recognize antigens that are broadly shared between pathogens, such as single-stranded RNA (TLR7), flagellin (TLR5) or lipopolysaccharide (TLR2/4). Following activation of these receptors, proinflammatory cytokines, including reactive oxygen species (ROS), tumor necrosis factor (TNFα) or interferon (IFNγ), are produced to directly kill these pathogens or further recruit and activate other cells to combat these pathogens. Production of IFNγ by Th1 cells polarizes classical activation of macrophages (CAMacs), the counterpart to AAMacs, to produce ROS through nitric oxide synthase activity.

Th1 and Th2 immunity counterbalance each other to limit over production of any cytokines and collateral damage that may be caused to the host's cells and tissues (Figure 2). This is predominantly mediated by the cytokines IFNγ and IL-4/IL-13, which are produced by Th1 and Th2 cells, respectively. IFNγ and IL-12 production during *H. polygyrus* infection reprograms Th2 cells to produce more IFNγ and decrease their production of IL-4 [50]. Intratracheal instillation of LPS during OVA-induced allergy increased IL-12 production and decreased Th2 dependent eosinophilia [51]. One mechanism of macrophage-mediated

suppression may include expression of the inhibitory surface molecule Programmed Death Ligand 2 (PD-L2) [52]. PD-L1 and PD-L2 bind to the same receptor (PD-1) and are differentially expressed by CAMacs and AAMacs respectively [53]. These ligands bind to PD-1 found on T cells and inhibit their activity by inducing apoptosis. T cell transcription factors (Tbet in Th1, GATA-3 in Th2 cells) can also directly inhibit differentiation of the opposing phenotypes. Tbet expression leads to Th1 differentiation through regulation of GATA-3 function rather than directly inducing IFNγ expression [54]. Furthermore, CAMacs and AAMacs also regulate each other by direct competition for resources. They both catabolize arginine, CAMacs using nitric oxide synthase and AAMacs through Arginase 1, to produce ROS and polyamines, respectively.

Mechanistically, Th2 immunity dampens inflammation through the production of several immunoregulatory mediators including cytokines, receptors and enzymes. During Th2 immunity, regulatory T cells (T_{reg}) and AAMacs produce the anti-inflammatory cytokine, IL-10, which directly inhibits transcription of Th1 cytokines by activation of STAT3. In addition, AAMac-derived Arginase1 acts to inhibit inflammatory responses both by inhibiting proinflammatory cytokine expression and by blocking T cell proliferation. For instance, arginase catalyzes the production of polyamines, which can antagonize Th1 associated inflammatory genes [55]. In *S.mansoni* infection, arginase inhibited inflammation by blocking IL-12 and IL-23p40 production associated with neutrophil inflammation [56].

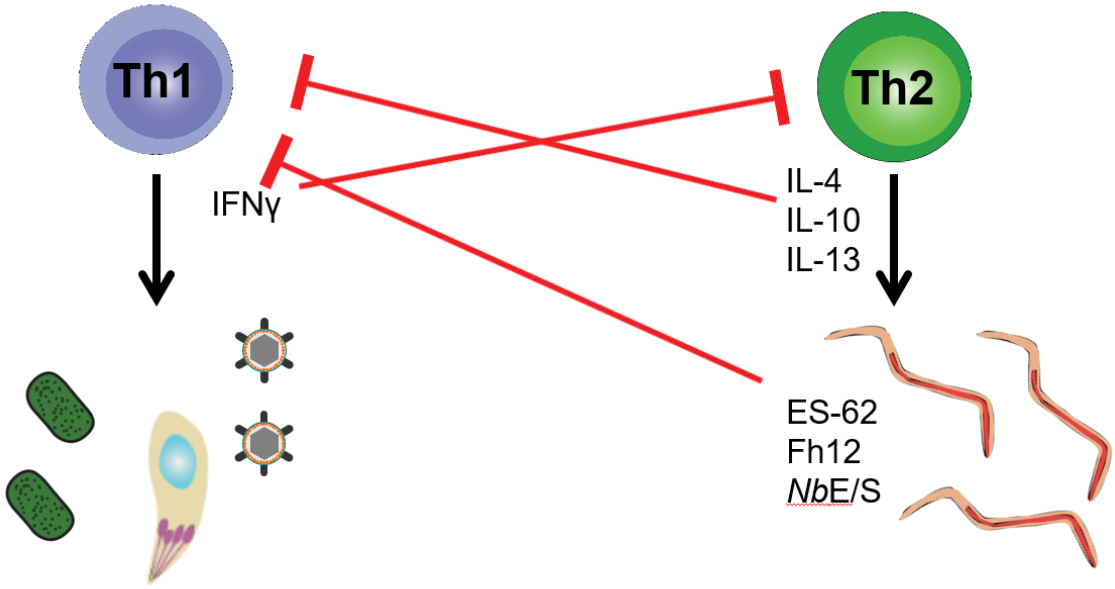

Figure 3: Mechanisms of Th1/Th2 balance.

Helminth antigens themselves can also downregulate the proinflammatory immune response. *N. brasiliensis* excretory/secretory (*Nb*ES) antigens directly inhibits neutrophil infiltration and proinflammatory cytokine production in a model of LPS-induced lung injury [57]. *F. hepatica* tegumental coat antigens bind to the mannose receptor on dendritic cells to limit T cell activity and inflammation [58]. Furthermore, several helminth antigens directly bind to TLR4 to inhibit the production of proinflammatory cytokines. These include the filarial nematode prosphorylcholine-containing excretory/secretory product, ES-62 [59], and *Fasciola hepatica* fatty acid binding protein [60]. Furthermore, *Trichinella spiralis* antigen can decrease myeloperoxidase activity, inducible nitric oxide synthase and IL-1β [61].

Although the Th1/Th2 balance prevents an excessive immune response, it is also strained when patients are co-infected with helminths and other pathogens. In endemic areas where helminth infections occur, infections with other parasites and pathogens is very high, and typically results in impaired immunity to both pathogens. Indeed, mice coinfected with *S. mansoni* or *N. brasiliensis* and *M. tuberculosis* have impaired resistance and have higher *M. tuberculosis* bacterial burdens [62, 63]. This is due to an increase in IL-4Rα mediated alternatively activated macrophages and IL-10 production, which suppresses the Th1 immune response necessary to control *M. tuberculosis* infection. In patients coinfected with helminths and *Plasmodium* spp. have increased risk of complications, anemia and increased *Plasmodium* parasite load [64, 65], although other studies have had

conflicting results [66, 67]. Patients coinfected with *Ascaris lumbricoides* had higher HIV viral load associated with an increase in *Ascaris* specific IgE [68]. Increased IL-12 and IFNγ production during *Plasmodium* and *H. polygyrus* co-infection contributes to chronicity of helminth infection and decreased Th2 cytokines [50, 69]. Furthermore, increased MAPK and NF-κB Th1 responses can lead to delayed parasite expulsion and increased immunopathology during filarial nematode infection [70, 71]. While co-infection with helminths and other pathogens is often detrimental to the host, helminths protect against inflammatory diseases by inducing anti-inflammatory cytokines.

Helminth Infection Protects Against Inflammatory Diseases

Harnessing the anti-inflammatory potential of helminths could provide new strategies to treat inflammatory diseases, such as sepsis. In areas where helminth infections are endemic, sepsis caused by secondary bacterial infections is common and can be fatal. This occurs in part because helminths introduce bacteria and cause damage to the intestinal wall, leading to “leakiness” of the intestinal barrier and dissemination of intestinal bacteria [72]. In patients infected with *S. stercoralis*, bacterial translocation leads to a systemic inflammatory response due to increased LPS and soluble CD14 in the serum of the host [73]. The gram-negative bacterial component, LPS, binds to toll-like receptor 4 (TLR4) and induces an NF-κB dependent inflammatory cascade resulting in excessive production of TNFα and IL-6. During viral and bacterial infections, T cells produce IFNγ to directly inhibit viral replication and induce reactive oxygen species production to kill bacteria. These proinflammatory cytokines are initially beneficial in clearance of pathogenic bacteria, but eventually damages the host’s cells and tissues. In cases where death occurs, excessive production of TNFα can lead to low blood pressure, vascular permeability and organ failure.

Helminth infections reduce mortality during sepsis in two ways, by stimulating production of anti-inflammatory cytokines or direct inhibition of TLR4 activity. Following *S. mansoni* infection, macrophage-specific IL-4R$\alpha^{-/-}$ mice (LysM^{Cre}/IL-4Rα^{Flox}) succumbed to acute infection associated with sepsis and

increased inflammation, including increased Th1 cytokine production, NOS2 activity [74]. For example, infection with the filarial nematode, *Litomosomoides sigmodontis*, protected mice from *E. coli*-induced sepsis by increasing phagocytosis of bacteria in a TLR2-dependent manner [75]. In addition, a filarial nematode-derived protein – chitohexaose – may have therapeutic properties in limiting septic shock and endotoxemia by blocking TLR4 signaling and instead promoting AAMac activation [76]. In human macrophages, *Trichuris suis* antigen can directly inhibit inflammatory signaling in a TLR4-dependent manner [77].

This anti-inflammatory cytokine production induced by helminths is also being exploited in inflammatory bowel diseases (IBD). Self-infection of an IBD patient with the whipworm, *Trichuris trichiura*, ameliorated symptoms of ulcerative colitis and sent the patient into remission. This was correlated with increased IL-22 and Th2 cytokines, which promoted goblet cell hyperplasia and mucus production [78]. Along with cytokine production, helminths also protect against IBD by altering the intestinal microbiota. In a mouse model of inflammatory bowel disease using *Nod2* deficient mice, infection with *Trichuris muris* resulted in a dominant Th2 cytokine and rescued mice from intestinal abnormalities and provided colonization resistance against pathogenic *Bacteroidetes* species [79]. Furthermore, infection with helminths increases microbiota diversity and promotes anti-inflammatory microbiota [80, 81]. These studies support an essential role for helminth-induced Th2 immunity in protection against lethal inflammation.

Resistin-like Molecules

One mechanism behind AAMac-mediated regulation of inflammation is the expression of RELM proteins. The resistin-like molecules (RELM) are a family of small (~10kD) secreted proteins that form multimers *in vivo*. RELMs are characterized by their conserved cysteine-rich motif that is localized to the C-terminal of the protein, which form disulfide bonds that are essential for proper formation of the protein [82]. RELM proteins have four murine homologs (resistin, RELMα, RELMβ and RELMγ) and two human homologs (resistin and RELMβ), each with distinct tissue distribution. Murine resistin is made by adipocytes, mRELMα by epithelial cells and AAMacs, mRELMβ by intestinal goblet cells and mRELMγ by epithelial cells and adipocytes [83]. Of the human RELM proteins, hResistin is produced by macrophages/monocytes and neutrophils, while hRELMβ is made by epithelial cells.

RELMs were originally identified as FIZZ (found in inflammatory zone) and HIMF (hypoxia-induced mitogenic factors) for their ability to induce cell proliferation in response to a hypoxic environment. However, they were renamed as resistin-like molecules after murine resistin was found to induce resistance to insulin and linked obesity to type 2 diabetes mellitus [84]. Despite the name, however, the other RELM proteins do not inhibit insulin activity and have pleiotropic effects in immunity.

RELMs in Helminth Infections

RELM proteins are heavily induced during helminth infection by both Th2 cytokines and helminth antigens. IL-4Rα and STAT6 signaling are essential in RELMα and RELMβ induction [19, 85]. Helminth antigen, such as *Fasciola hepatica* tegumental antigens can also induce AAMac polarization and RELMα production [86]. While Th2 cytokines induce RELMα production, RELMα acts as a negative feedback loop to limit excessive Th2 immunity [32, 33]. As RELMα can also bind to B7-H3 [87], an inhibitor of T cells [88], it is possible that the anti-inflammatory function of RELMα is dependent on the B7-H3 pathway. This results in delayed clearance of worms, but it also limits excessive pulmonary inflammation once the worms are expelled [89]. The function of RELMβ during helminth infection may be dependent on the helminth parasite. For example, RELMβ induced $CD4^+$ T cell production of IFNγ but had no effect on worm expulsion during *T. muris* infection [90]. On the other hand, RELMβ promotes expulsion of *N. brasiliensis* or *H. polygyrus* by increasing mucus production. Other studies have shown that RELMβ can bind to and inhibit the sensory function of *S. stercoralis* [91].

Human RELM proteins are also expressed in response to helminth antigens, although it is unclear what role they play in a Th2 environment. $CD14^+$ blood monocytes from filaria-infected patients had increased hResistin expression when stimulated with *B. malayi* antigen [34]. Given the expression of hResistin by human macrophages, we hypothesize that hResistin plays a key role in regulating the immune response to these parasites.

RELMs in Inflammatory Diseases

RELMs also modulate immunity in other inflammatory settings, such as allergen-induced airway inflammation or intestinal inflammation. In allergic lung inflammation, studies on the function of RELMα have been conflicting, with some reports demonstrating that RELMα does not contribute to allergic airway responses [92] and other reports showing that RELMα increase airway remodeling by inducing myofibroblast differentiation [93]. RELMα also enhances the expression of inflammatory cytokines, including IL-1β, IL-6 and TNFα, which promote lung injury in acute pancreatitis [94]. However, the proinflammatory effects of RELMα may depend on the tissue site and the infectious agent [95, 96]. In a dextran sodium sulfate (DSS) model of colitis, RELMα increased intestinal inflammation through IL-6 and TNFα, while inhibiting production of IL-10 [97]. RELMα also promotes a pathogenic Th17 proinflammatory response in a mouse model of intestinal inflammation induced by *Citrobacter rodentium*, which mimics enteropathogenic *E. coli* [96, 98].

In several models of intestinal inflammation (dextran sodium sulfate and 2,4,6-Trinitrobenzenesulfonic acid), goblet cell-derived RELMβ reduced inflammation through regulation of mucin and antimicrobial peptide production, but did not have any effect on cytokine production [99, 100]. Human RELMβ is also expressed in the lung during allergy [101] and acts on fibroblasts to increase airway remodeling [102]. This may be due to hRELMβ stimulating the proliferation

and activation of pulmonary endothelial and smooth muscle cells [103]. Although RELMβ is typically associated with the epithelial cells of the lung and gastrointestinal tract, hRELMβ is expressed by foam cells in atherosclerotic plaques and contributes to more severe atherosclerosis through induction of lipid accumulation and proinflammatory cytokines [104]. These studies suggest that the regulation of immunity by murine RELM proteins is extremely heterogeneous and may not necessarily reflect the function of human RELMs. Thus, mechanistic studies to examine the *in vivo* effects of human RELM proteins are necessary to provide alternative treatment strategies for infectious and inflammatory diseases.

Human Resistin

Despite ~60% sequence homology, murine and human resistin have functionally distinct roles in infectious and metabolic diseases. While murine resistin was initially identified for insulin resistance, the link between human resistin and type 2 diabetes is controversial [105-107]. This disparate function is likely due to their distinct expression patterns: murine resistin is expressed by adipocytes while human resistin is expressed by macrophages and neutrophils. Expression of hResistin by hematopoietic cells, which can traffic into and out of any tissue, suggests that hResistin expression likely occurs in any tissues where macrophages or neutrophils can be found.

Human resistin has been implicated in many inflammatory diseases. In patients diagnosed with atherosclerosis [108], rheumatoid arthritis [109] and sepsis [110], circulating hResistin expression is increased, where it's believed to contribute to foam cell formation in atherosclerosis [111, 112] and the inflammatory milieu during septic shock [113, 114]. Several clinical reports have even identified hResistin as a diagnostic marker of sepsis [110, 115, 116], periodontis [117] and systemic lupus erythematosus [118]. These correlative studies are supported by reports that LPS drives expression of hResistin and that hResistin can induce proinflammatory cytokine production *in vitro*.

Aside from inflammatory diseases, hResistin expression is also elevated in infectious diseases. *Streptococcus* spp., a gram-positive bacterium, can induce

hResistin release by neutrophil granules [119], and patients infected with Dengue virus also have elevated hResistin in the serum [120]. Furthermore, hResistin increases the production of proinflammatory cytokines and formation of neutrophil extracellular traps in the lungs during acute LPS-induced lung injury, leading to more severe lung injury [121]. Despite the association between hResistin and inflammation, it is unclear whether hResistin expression contributes directly to inflammation.

Resistin Transcription and Receptors

Although hResistin is elevated in many diseases, the factors that regulate hResistin expression are not well characterized. Indeed, *B. malayi* antigen increases hResistin expression in $CD14^+$ blood monocytes, although the mechanisms behind this activation are not understood. LPS, a component of the cell wall of gram-negative bacteria, can stimulate hResistin expression in human PBMC in an NF-κB dependent manner [122]. In humanized hResistin transgenic mice (h*Retn*Tg^+), LPS administration upregulated circulating hResistin levels as early as 2 hours [123, 124]. In addition to LPS, a high glucose environment can increase expression of hResistin in a human monocyte cell line through activation of MAPK and NF-κB [125]. Promoter sequence analysis and chromatin immunoprecipitation assays also revealed that the transcription factors, sterol regulatory element binding protein 1c (SREBP1c) and CCAAT enhancer binding protein α (C/EBPα), both bind to the hResistin promoter [126].

There are several single nucleotide polymorphisms (SNPs) that can alter hResistin expression, although rs1862513 is the best characterized SNP associated with hResistin [127]. SNP rs1862513 is located at -420 in the hResistin promoter, and a mutation from C to G increases hResistin expression in monocytes [128]. However, associations between rs1862513 and type 2 diabetes mellitus or heart disease have been conflicting [128-130]. Other SNPs have been found in the promoter region (-394) and within the coding region (+299) of hResistin [131], but it is unclear whether these SNPs are associated with

inflammatory diseases. Further studies will need to confirm these SNPs increase hResistin expression and inflammatory diseases.

Recent studies have identified two receptors for resistin, toll-like receptor 4 (TLR4) and adenylyl-cyclase associated protein 1 (CAP1) [132, 133]. TLR4 is a pattern recognition receptor that recognizes LPS and signals proinflammatory and antimicrobial peptide production during bacterial infection. Using transfected HEK293 cells, hResistin was only able to bind to TLR4 but not MD-2 or CD14, two adaptor proteins that mediate LPS-TLR4 interaction. In human PBMC, hResistin stimulates phosphorylation of NF-κB and MAPK, two transcription factors that promote inflammation, in a TLR4 dependent manner and competes with LPS for binding to TLR4 [134]. Furthermore, in gastric cancer cells, hResistin can induce stromal cell-derived factor-1 expression through TLR4 and NF-κB signaling [135]. In human monocytic cell lines (U937), hResistin induces expression of IL-12 and TNFα in an NF-κB dependent manner [136]. CAP1, expressed by monocytes, is an intracellular receptor that mediates cyclic AMP signaling and actin polymerization. CAP1 binds to the C-terminial of hResistin at its proline-rich SH3 binding domain to induce IL-6 and TNFα production. Signaling of hResistin through CAP1 involves upregulation of PKA activity, NF-κB signaling and cyclic AMP dependent activation [137]. However, these studies lack evidence of direct interaction between hResistin and receptor, and it is unclear which receptor is functionally relevant *in vivo.*

Conclusion

RELM proteins have immunomodulatory functions in a variety of infections and inflammatory diseases. These studies have revealed that RELMs have disparate functions in immunity, and how the human RELM proteins regulate inflammation is currently unknown. In the following chapters, we examine the function of human resistin in regulation of the immune response during helminth infections and endotoxic shock. Elucidating the importance of hResistin could have significant medical implications for billions of helminth-infected patients and millions of septic patients. To that end, we combine human studies with the use transgenic mouse model in which the human resistin gene, along with its entire regulatory region, was inserted into C57BL/6 mice using a bacterial artificial chromosome. In chapter two, we investigate hResistin in helminth infections, where other RELM proteins negatively impact the Th2 immune response necessary for worm clearance. In chapter three, we explore what factors regulate hResistin transcription in helminth infection. In chapter four, we identify hResistin as a critical mediator in regulating inflammatory diseases through balancing of Th1 and Th2 immunity.

CHAPTER TWO – Macrophage-Derived Human Resistin is Induced in Multiple Helminth Infections and Promotes Inflammatory Monocytes and Increased Parasite Burden

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Abstract

Parasitic helminth infections can be associated with lifelong morbidity such as immune-mediated organ failure. A better understanding of the host immune response to helminths could provide new avenues to promote parasite clearance and/or alleviate infection-associated morbidity. Murine resistin-like molecules (RELM) exhibit pleiotropic functions following helminth infection including modulating the host immune response; however, the relevance of human RELM proteins in helminth infection is unknown. To examine the function of human resistin (hResistin), we utilized transgenic mice expressing the human resistin gene (h*Retn*Tg^{+}). Following infection with the helminth *Nippostrongylus brasiliensis* (*Nb*), hResistin expression was significantly upregulated in infected tissue. Compared to control h*Retn*Tg^{-} mice, h*Retn*Tg^{+} mice suffered from exacerbated *Nb*-induced inflammation characterized by weight loss and increased infiltration of inflammatory monocytes in the lung, along with elevated *Nb* egg burdens and delayed parasite expulsion. Genome-wide transcriptional profiling of the infected tissue revealed that hResistin promoted expression of proinflammatory cytokines and genes downstream of toll-like receptor signaling. Moreover, hResistin preferentially bound lung monocytes, and exogenous treatment of mice with recombinant hResistin promoted monocyte recruitment and proinflammatory cytokine expression. In human studies, increased serum resistin was associated with higher parasite load in individuals infected with soil-transmitted helminths or filarial nematode *Wuchereria bancrofti*, and was positively

correlated with proinflammatory cytokines. Together, these studies identify human resistin as a detrimental factor induced by multiple helminth infections, where it promotes proinflammatory cytokines and impedes parasite clearance. Targeting the resistin/proinflammatory cytokine immune axis may provide new diagnostic or treatment strategies for helminth infection and associated immune-mediated pathology.

Introduction

Helminth infections are a major global health issue, causing severe morbidity and malnutrition in over a billion people worldwide [1]. Infection with lymphatic-dwelling filarial nematodes can result in lymphedema and elephantiasis, while soil-transmitted helminths (STH) can cause anemia, gastrointestinal hemorrhage or blockage, growth retardation and cognitive impairment. In human gastrointestinal helminth infections, although most infections have shown mild alterations to pathology, severe infections can result in colitis and dysentery [138]. Clearance of helminth infection has relied solely on anthelmintic drugs, including diethylcarbamazine, albendazole and ivermectin; however, there is currently no vaccine against human helminth pathogens and the rate of reinfection remains high. In mouse models, the T helper type 2 (Th2) immune response is essential in immunity to helminths, with $CD4^+$ T cells, innate lymphoid cells, eosinophils and basophils as the major producers of the Th2 cytokines, IL-4 and IL-13 [139-141]. In mice lacking IL-4, IL-13 or their receptors, there is impaired clearance of helminths, including the soil-transmitted helminth *Nippostrongylus brasiliensis* (*Nb*) and filarial nematodes [142, 143]. In addition to mediating immunity to helminths, Th2 cytokines are critically involved in suppressing infection-induced immunopathology. For instance, *Schistosoma mansoni* infection is lethal in IL-4R$\alpha^{-/-}$ mice and following *Nb* infection, IL4R$\alpha^{-/-}$ mice suffer from exacerbated inflammation in the lungs compared to wild-type mice [74, 144].

The balance between Th2 cytokines and type 1 proinflammatory cytokines, such as IFNγ and TNFα, is also an important consideration for the outcome of helminth infection as these cytokine pathways are counter-regulatory. For example, inhibition of IFNγ promotes expulsion of *Trichuris muris* by allowing the development of a protective Th2 immune response [145]. In filarial nematode infection, severe lymphatic pathology is associated with increased IFNγ and TNFα and conversely decreased IL-4 [146]. By promoting type 1 inflammatory cytokines, Toll-like receptor (TLR) signaling can negatively impact the host immune response to helminths by delaying both parasite expulsion and promoting immune-mediated pathology [147, 148]. Identifying factors that regulate the balance between type 1 and type 2 cytokines may offer new immune targeting strategies to promote protective immunity or ameliorate infection-associated pathology.

Resistin is a member of the resistin-like molecule (RELM) family of cysteine-rich secreted proteins that are conserved in humans and mice. In mice, there are four RELM proteins (resistin, RELMα, RELMβ, and RELMγ), however, there are only two RELM proteins in humans (resistin and RELMβ) [149]. Among the murine RELM proteins, RELMα and RELMβ proteins are potently induced following helminth infection. Studies from our lab and others have shown that RELMα suppresses Th2 cytokine responses induced by *S. mansoni* or *Nb* [32, 33]. On the other hand, the function of RELMβ during helminth infection may be dependent on the helminth parasite. For example, RELMβ induced $CD4^+$ T cell production of IFNγ

but had no effect on worm expulsion during *T. muris* infection [90]. In response to *N. brasiliensis* or *H. polygyrus* however, RELMβ was an effector molecule that could interact with the parasite and promote expulsion. Other studies have shown that RELMβ can bind to and inhibit the sensory function of *S. stercoralis* [91]. These studies, however, have only examined murine RELM proteins and to date, none have investigated the role of human RELM proteins such as resistin in helminth infection.

Murine resistin was initially identified as a hormone secreted by adipocytes that caused insulin resistance, a disorder that can result in type 2 diabetes [84]. Interestingly, although human resistin and mouse resistin have high sequence homology, resistin in humans is predominantly expressed by monocytes and macrophages and not adipocytes [150, 151]. In addition to diabetes, human resistin is also elevated in several inflammatory diseases such as atherosclerosis and rheumatoid arthritis [108, 152]. Indeed, in an inflammatory environment promoted by LPS stimulation, human resistin was upregulated and contributed to inflammation and insulin resistance [121, 124, 153]. Human resistin also mediated lung cancer cell migration, differentiation of osteoclasts [154], and VEGF-mediated angiogenesis [135]. Despite the many studies focusing on resistin in metabolic disorders and cancer, its function during infection is currently unknown. In human studies with filarial-infected individuals, *Brugia malayi* antigen increased resistin mRNA in human monocytes, particularly when these monocytes were isolated

from filarial-infected humans [34], prompting our investigation of the function of human resistin in helminth infection.

To examine the role of human resistin in helminth infection, we employed transgenic mice that express human resistin. These mice are deficient in murine resistin, and engineered to include the hResistin gene and its entire regulatory region (h*Retn*Tg^{+}) [124]. In the original study, h*Retn*Tg^{+} mice exhibited a similar hResistin expression pattern as that observed in humans. Additionally, h*Retn*Tg^{+} mice suffered from increased insulin resistance following LPS-induced endotoxemia. These results suggested that hResistin might be functional in mice, and that h*Retn*Tg^{+} mice provide a useful tool to study how hResistin expression is regulated and its function *in vivo*. Following *Nb* infection, we show that hResistin is significantly upregulated by macrophages in the infected lungs and intestine of h*Retn*Tg^{+} mice. Compared to littermate control mice that did not express hResistin (h*Retn*Tg^{-}), infected h*Retn*Tg^{+} mice suffered from exacerbated infection-induced inflammation, including increased weight loss and elevated monocyte infiltration in the lung. We performed RNAseq analysis to identify differentially expressed genes that may be responsible for hResistin-mediated effects. Gene set enrichment analysis revealed that hResistin induced global gene ontology pathways that were associated with the inflammatory response, chemokine signaling and TLR signaling. Additionally, exogenous treatment of naïve mice with recombinant human resistin could recapitulate the monocyte responses and gene expression profile observed in h*Retn*Tg^{+} mice, confirming that human resistin directly

promotes these inflammatory pathways *in vivo*. Moreover, hResistin-mediated stimulation of the inflammatory immune response led to increased parasite egg burdens and delayed worm expulsion. Finally, in human studies, we observed that patients infected with STH or the filarial nematode *Wuchereria bancrofti* had elevated serum resistin, and that resistin was positively correlated with parasite burden and serum proinflammatory cytokines. Taken together, our findings suggest that human resistin expression is an innate response to multiple helminths, where it promotes monocyte recruitment and a type 1 proinflammatory cytokine environment, leading to impaired helminth clearance and exacerbated infection-associated inflammation.

Materials and Methods

Mice

Human resistin transgenic mice (h*Retn*Tg^{+}) were generated as previously described by Mitch Lazar [124]. Briefly, the human resistin gene, along with 21,300 bp upstream and 4,248 bp downstream of the human resistin start site, were engineered through a bacterial artificial chromosome. h*Retn*Tg^{-} mice were backcrossed onto WT C57BL/6 mice bred in house to generate mRetn$^{-/-}$ mice. In the resistin binding assay, CX3CR1^{GFP}PGRP^{dsred} mice were used as reporters for monocytes and neutrophils respectively [155]. Recombinant human resistin (Peprotech) was used for *in vivo* intraperitoneal injections (500ng/mouse at days -1 and/or day -3). In some experiments, mice were also treated intratracheally with 500ng hResistin. Cells from the peritoneal cavity were recovered at 24, 48, and 72 hours and analyzed by flow cytometry and real-time PCR. Recombinant hResistin was tested for endotoxin contamination using the Pierce *Limulus* Amebocyte Lysate assay (Thermo Scientific) by manufacturer's instructions under sterile conditions. For controls, mice were injected i.p. with PBS or the limit of detection concentration of LPS (1.5pg/mL). All animals in the experiment were age-matched (6-10 week old) and gender-matched, and housed five per cage under ambient temperature with 12 hour light/dark cycle.

Nippostrongylus brasiliensis Infection

Nippostrongylus brasiliensis (*Nb*) life cycle was maintained in Sprague-Dawley rats purchased from Harlan Laboratories. Mice were injected subcutaneously with 500 *Nb* third-stage larvae (L3) and sacrificed at days 2, 7 and 9 post-infection. Eggs in the feces of infected mice were counted using a McMaster counting chamber on days 6 – 9 following infection. Adult worms within the small intestine were enumerated after the entire small intestine of infected mice was cut longitudinally and incubated in PBS at 37˚C for >1 hr to allow worms to migrate out of tissue.

Cytokine Quantification

For sandwich ELISA, Greiner 96-well medium bind plates were coated with primary antibody to cytokines (Peprotech) overnight at room temperature. After blocking the plates with 5% NCS in PBS for 1 hr, sera or tissue homogenates were added at various dilutions and incubated at room temperature for 2 hr. Detection of cytokines was done with biotinylated antibodies for 2 hr, followed by incubate with streptavidin-peroxidase (Jackson Immunobiology) for 30 min. The peroxidase substrate TMB (BD) was added followed by addition of 2N H_2SO_4 as a substrate stop, and the optical density (OD) was captured at 450 nm. Samples were compared to a serial-fold dilution of recombinant cytokine.

Human studies

Serum and feces were collected from uninfected (n=51) or STH-infected school children (n=49) and helminth eggs in the feces were quantified by a modified Kato Katz or formol-ether concentration protocol [156]. For filarial-infected individuals, blood was collected and circulating microfilariae were previously quantified through filtration of 1 mL of whole blood into a Nucleopore 3µm polycarbonate filter as previously described [157]. The patient group consisted of infected patients (n=44) from the *Wuchereria bancrofti* island of Mauke in the Cook Islands and endemic normal individuals (i.e. those with no history of filarial infection at time of collection, n=17). Custom Luminex assay kits (analytes TNFα, Chi3l1, IL-6Rα, MCP2, Leptin, VCAM1, IL-6, VEGF, IFNγ, IL-1α, IFNγR1) from R&D Systems were run according to manufacturer's instructions and quantified on Luminex 200 (Luminex Corp.).

Real-time PCR

Tissue recovered for RNA extraction was first incubated overnight in RNAlater (Qiagen) at 4˚C. Tissue RNA was extracted with Trizol and RNA from cells was extracted on RNeasy columns (Qiagen). iScript Reverse Transcriptase (Biorad) was used for cDNA synthesis. Relative quantification of cDNA was measured by real-time PCR using the Biorad CFX Connect. Primer sequences for real-time PCR are: *Ccl2* F-TGGCTCAGCCAGATGCAGT,

R-TTGGGATCATCTTGCTGGTG; *Cxcl10* F-GCCGTCATTTTCTGCCTCA, R-CGTCCTTGCGAGAGGGATC; *Ccr2* F-TCAACTTGGCCATCTCTGACC, R-AGACCCACTCATTTGCAGCAT; *Irf7* F-CAGCGAGTGCTGTTTGGAGAC, R-AAGTTCGTACACCTTATGCGG. All other primers were purchased from Qiagen. Ensembl ID for each gene: *Irf7* (ENSMUSG00000025498), *Ccr2* (ENSMUSG00000049103), *Tnfa* (ENSMUSG00000024401), *Cxcl10* (ENSMUSG00000034855), *Tnfrsf1b* (ENSMUSG00000028599), *Retnla* (ENSMUSG00000061100), *Il-4* (ENSMUSG00000000869), *Il-5* (ENSMUSG00000036117), *Il-13* (ENSMUSG00000020383).

RNA Sequencing

RNA was originally extracted using Trizol reagent, and further purified on RNeasy columns (Qiagen). A total of 3 µg of RNA was used in synthesis of RNA library for sequencing, with periodic analysis of RNA and cDNA quality on the 2100 BioAnalyzer (Agilent Technologies). cDNA libraries from whole lung RNA were made using NEB ultra-directional RNA library kit with multiplexing primers. Multiplexed samples were sequenced on Illumina 2000 at the University of California, Riverside Genomics Core. Once sequenced, samples had 10-30 million total reads. Upon indexing and alignment through TopHat, read counts and normalization was done with R Bioconductor. Differentially expressed genes (DEGs) were defined in our study as being infection-induced, with a 1.5x fold change (h*Retn*Tg^{+} infected vs h*Retn*Tg^{-} infected), false discovery rate ≤ 0.05, and

a p-value ≤ 0.05. Raw data was normalized and analyzed using QuasR package on R Bioconductor. DEGs were then categorized by DAVID Functional Annotation Tools using Gene Ontology Enrichment Terms and KEGG Pathways. RNA-seq data have been submitted to GEO (accession number GSE60537).

Histology

Lungs were inflated with 1 mL 1 part 4% PFA/30% sucrose and 2 parts OCT and stored overnight in 4% PFA at 4˚C. After 24 hours, lungs were removed from 4% PFA and incubated another 24 hours in 30% sucrose. Lungs were then blocked in OCT and sectioned at 8 µm. H&E-stained lung sections were blindly scored on a 1-5 scale with 5 being the most severe score of pathology using criteria of leukocyte infiltration (1-5) and vascular inflammation/endothelial cell hyperplasia (1-5) for a total score out of 10. Scoring of the lung section was based on the following: 1 – absent, 2 – slight, 3 – moderate (covering up to 5% of total area), 4 – marked (>5% and <10% of total area), and 5 – severe (covering ≥10% of total area). For immunofluorescence staining, sections were incubated with rabbit anti-hResistin (1:400, generated by Mitchell Lazar), biotinylated *Griffonia simplicifolia* lectin (1:400, Vector Laboratories) overnight at 4˚C. Sections were incubated with appropriate fluorochrome-conjugated secondary antibodies for 2 hours at 4˚C and counterstained with DAPI. Sections were visualized under a Leica microscope (DM5500 B) and Volocity software (PerkinElmer) was used to quantify number of $hResistin^+$ and $DAPI^+$ cells.

Flow Cytometry

Lung tissue was cut up, incubated in 30µg/mL DNAse and 1mg/mL collagenase for 30 minutes in 37˚C shaking incubator and passed through a 70µm cell strainer to generate single cell suspension. Bronchio-alveolar lavage cells (BAL) were recovered through washing with 3mL of ice cold PBS. Peritoneal exudate cells (PEC) were recovered in a total of 5 mL of ice cold PBS. For flow cytometry, lung, BAL and PEC were blocked with 25µM Rat IgG and 25µM αCD16/32 (2.4G2, 5'), stained for 25' with antibodies for SiglecF (E50-2440), Ly6G (1A8), MHCII (M5/114.15.2) all from BD Biosciences; F4/80 (BM8), CD115 (AFS98), Ly6C (HK1.4), CD3 (17A2), CD11b (M1/70), CD11c (N418) all from eBioscience, Affymetrix; CD4 (RM4-5 from Invitrogen). Cells were then washed and analyzed on the LSRII (BD Bioscience), followed by data analysis using FlowJo v10 (Tree Star Inc.). Cells were sorted by flow cytrometry using FACSAria (BD Bioscience), and re-analyzed for purity on the LSRII (BD Bioscience).

hResistin Binding Assay

Dissociated lung cells were collected from *Nb* infected mice and washed with FACS buffer prior to 1 hour incubation with 0.5µg recombinant hResistin (Peprotech) or PBS for control. Cells were washed 2X in FACS buffer, incubated with Fc block (αCD16/32, 15'), stained with biotinylated αhRetn (Peprotech, 30'), followed by detection with BV421-conjugated streptavidin (BD Biosciences, 30').

Statistical Analysis

All statistics were analyzed by Graphpad Prism using where appropriate the student's t-test (for normal distribution data), Mann-Whitney nonparametric test (for asymmetric distribution data), two-way ANOVA (for analysis of more than one experiment), or nonparametric spearman correlation (for correlation analysis). *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.0001$.

Ethics Statement

All protocols for animal use and euthanasia were approved by the University of California Riverside Institutional Animal Care and Use Committee (https://or.ucr.edu/ori/committees/iacuc.aspx; protocol A-20120023B and A-20120024E), and were in accordance with National Institutes of Health guidelines. Animal studies are in accordance with the provisions established by the Animal Welfare Act and the Public Health Services (PHS) Policy on the Humane Care and Use of Laboratory Animals. For the human studies, protocols were approved by the Cook Islands government and the NIAID Institutional Review Board. Samples were collected following the informed written consent of the patient or guardian or parent of the school children. Multiplex cytokine assays and resistin ELISAs were performed at UCR with the approval of the University of California Riverside Institutional Review Board (HS-13-134 and HS-14-048).

Results

hResistin is upregulated in macrophages following *Nippostrongylus brasiliensis* infection

To study the function of human resistin in helminth infection, we utilized mice in which the human resistin gene along with its entire regulatory region was inserted using a bacterial artificial chromosome onto a murine resistin knockout background (h*Retn*Tg$^+$). Previous characterization of these mice showed that they provide a useful model for studying the effects of human resistin, as both humans and h*Retn*Tg$^+$ mice have comparable levels of circulating resistin [124, 153]. h*Retn*Tg$^+$ mice were infected with the STH *Nippostrongylus brasiliensis* (*Nb*), a parasite of mice and rats that infects the lungs and small intestine. *Nb* colonization of the lung and small intestine resulted in increased human resistin expression at the mRNA and protein level, as measured by real-time PCR and ELISA of the infected tissue respectively (Figure 4A, B). Using immunofluorescent (IF) staining of lung sections from naïve and infected h*Retn*Tg$^+$ mice, we quantified the frequency of hResistin$^+$ cells as a percentage of the total number of DAPI$^+$ cells. Compared to naïve mice, we observed significant increases in hResistin$^+$ cells in the lung as early as day 2 post-infection, a time point at which the parasites are present in the lung (Figure 4C). IF staining for hResistin (green) and *Griffonia simplicifolia* lectin (GSL), a macrophage-binding lectin (red) [158], revealed that hResistin was predominantly

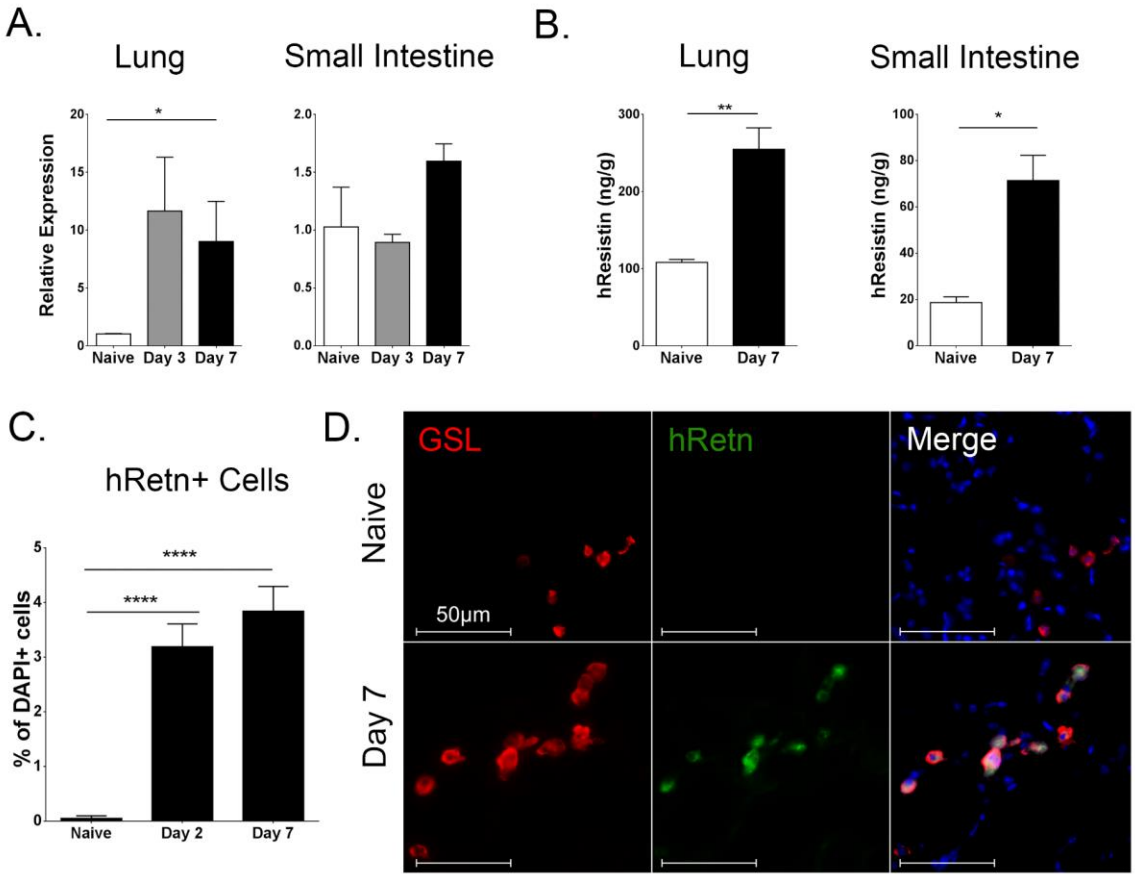

Figure 4: Detection of hResistin expression in *Nb*-infected transgenic mice. (A-B) hResistin expression in the lung and small intestine of naïve or *Nb*-infected mice was measured by real-time PCR analysis (A) or ELISA (B). (C) Quantification of hResistin expressing cells was performed on lung tissue sections. Data (mean ± SEM, n=4-6 per group) are representative of four separate experiments.

expressed by GSL^+ macrophages (Figure 4D). To systematically examine the cell-types that express hResistin, we sorted the dissociated cells from the lungs of *Nb*-infected Tg^+ mice followed by real-time PCR analysis for h*Retn* (Figure 5). Sorted cells were analyzed for purity by flow cytometry and hematoxylin and eosin (H&E)-stained cytospins, revealing >90% purity (Figure 5A). $CD11c^+F4/80^+$ macrophages were the cell-types of highest frequency in the lung that expressed h*Retn*. In addition to macrophages, monocytes and neutrophils but not eosinophils expressed detectable h*Retn* mRNA (Figure 5B). To our knowledge, this is the first demonstration that human resistin expression occurs in helminth-infected tissues and suggests that, in addition to metabolic disorders and cancer, human resistin may have a critical function in helminth infection.

Human resistin leads to exacerbated pulmonary inflammation during Nb Infection

To examine the functional significance of the increased hResistin during *Nb* infection, h*Retn*Tg^+ or littermate control h*Retn*Tg^- were infected with *Nb*. Compared to the control h*Retn*Tg^- mice, the h*Retn*Tg^+ mice exhibited more severe weight loss beginning at day 2 post *Nb* infection (Figure 6A). Histological examination of H&E-stained lung sections from h*Retn*Tg^- mice revealed that compared to naïve mice, *Nb* colonization of the lung resulted in hemorrhage and disruption of the lung alveolar architecture at day 2 post-infection, which was resolved by day 7 post infection, mirroring the weight recovery observed (Figure

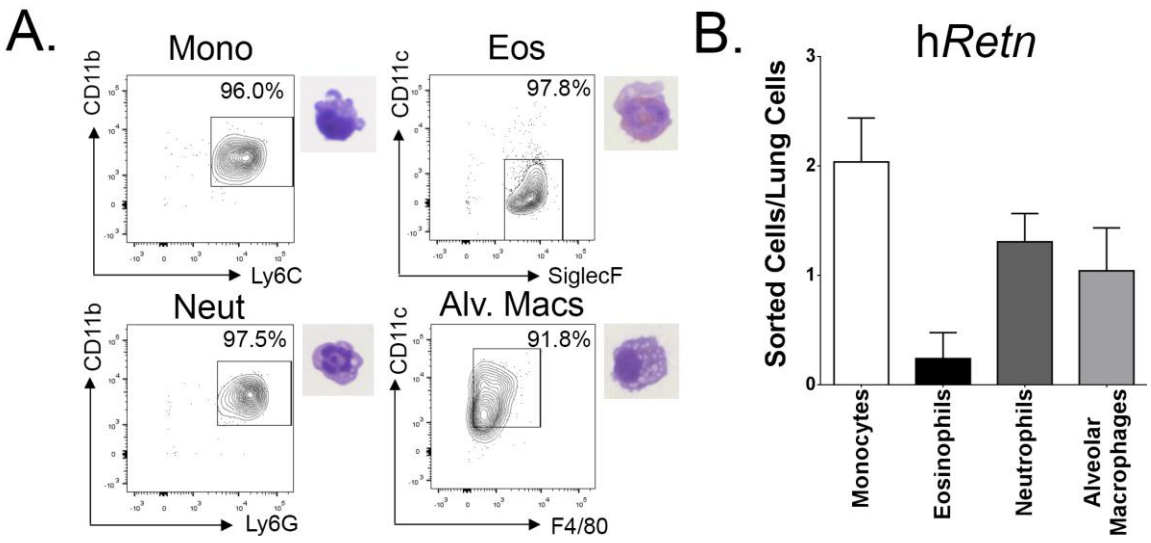

Figure 5: Monocytes, neutrophils and alveolar macrophages express hResistin in *Nb*-infected lungs. (A) Gating strategy for sorted lung cells from *Nb*-infected mice for CD11b+ Ly6C+ monocytes, SiglecF+CD11c- eosinophils, CD11b+Ly6G+ neutrophils and CD11c+F4/80+ alveolar macrophages and corresponding H&E stained cytospins. (B) Sorted cells were recovered for RNA and analyzed for h*Retn* by real-time PCR. Data (mean ± SEM, n=3-4 per group) are representative of three separate experiments.

6B, upper panels). In contrast, h*Retn*Tg$^+$ mice were unable to resolve inflammation by day 7 post-infection and instead exhibited increased leukocyte infiltration of the lung, characterized by the presence of large mononuclear cell-rich granulomas (Figure 4B, lower panels and inset). The exacerbated lung inflammation in the h*Retn*Tg$^+$ mice was confirmed by blind histology scoring (Figure 6C), and was associated with significantly increased numbers of cells from bronchio-alveolar lavage (BAL) (Figure 6D). To characterize the leukocyte infiltrates observed in the infected lungs, the lung tissue from h*Retn*Tg$^-$ or h*Retn*Tg$^+$ mice was dissociated and analyzed by flow cytometry. While there were no significant differences in eosinophils, neutrophils, CD4$^+$ T cells or alveolar macrophages (Figure 7A), we observed a significant increase in CD11b$^+$Ly6C$^+$ monocytes, suggesting that the granulomas observed in the lungs might consist of inflammatory monocytes (Figure 6E). In conclusion, these results demonstrate a unique and specific effect for human resistin in promoting *Nb* infection-induced inflammation and inflammatory monocytes in the lung.

Th2 cytokines are not altered by hResistin

Previous studies have shown that Th2 cytokines are critical in dampening the acute lung inflammation caused by *Nb* infection [144]. Additionally, studies from our group and others have shown that murine RELMα, which shares sequence identity and expression pattern with human resistin, suppresses Th2 cytokine responses in the lung [32, 33]. We therefore hypothesized that the

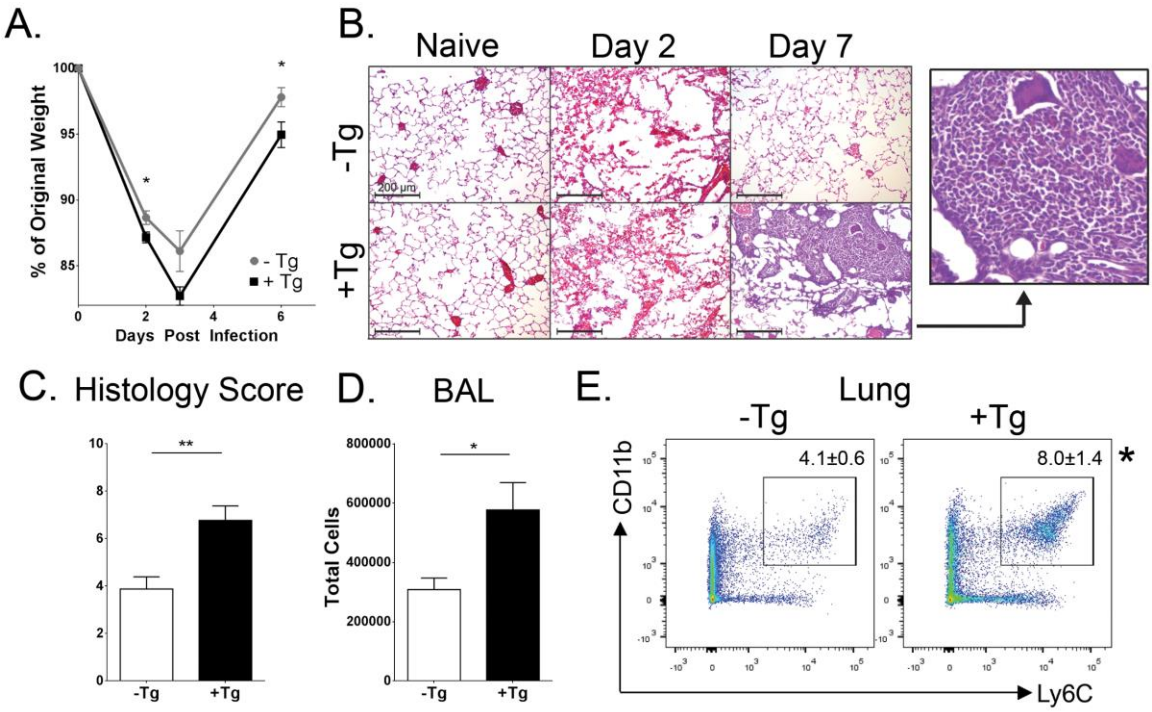

Figure 6: Expression of hResistin exacerbates lung inflammation. (A) h*Retn*Tg- mice or h*Retn*Tg+ mice were infected with *Nb* and weight loss was measured as a percentage of original weight. (B-C) Lung sections from naïve or *Nb*-infected mice were stained with H&E (B, scale bar=200µm) and pathology at day 7 post infection was assessed by blind scoring (C). (D-E) At day 7 post infection, total BAL cells were quantified (D) and dissociated lung cells were recovered for flow cytometry analysis of Ly6C+CD11b+ monocytes (E). Data (mean ± SEM, n=3-4 per group) are representative of three separate experiments.

exacerbated lung inflammation observed in the h*Retn*Tg^{+} mice was associated with reduced expression of Th2 cytokines or dysregulated RELMα expression. However, real-time PCR analysis of naïve or *Nb*-infected lung tissue from h*Retn*Tg^{+} or h*Retn*Tg^{-} mice revealed equivalent induction of RELMα and Th2 cytokines IL-4, IL-5 and IL-13 in both groups of mice (Figure 7B). These data suggest that human resistin mediates exacerbated lung inflammation through a mechanism that is independent of Th2 cytokines and RELMα.

Genome-wide transcriptional profiling reveals that global immune response pathways are triggered by hResistin

For an unbiased approach to identify gene expression pathways that are associated with hResistin-mediated inflammation in the lung, RNAseq analysis was performed on naïve or day 7 *Nb*-infected lung tissue from h*Retn*Tg^{+} or h*Retn*Tg^{-} mice. *Nb* infection resulted in global gene expression changes with 1662 genes upregulated by infection in the h*Retn*Tg^{-} mice, and a total of 2460 differentially expressed genes (DEG) (Figure 8A, left). Interestingly, infection of h*Retn*Tg^{+} mice resulted in a greater number of genes upregulated (2805) with almost double the number of DEGs (4552) as h*Retn*Tg^{-}mice (Figure 8A, middle). This suggests that hResistin has a significant impact on infection-induced gene expression changes in the lung. To assess infection-induced genes that were differentially expressed in response to hResistin, we compared *Nb*-infected

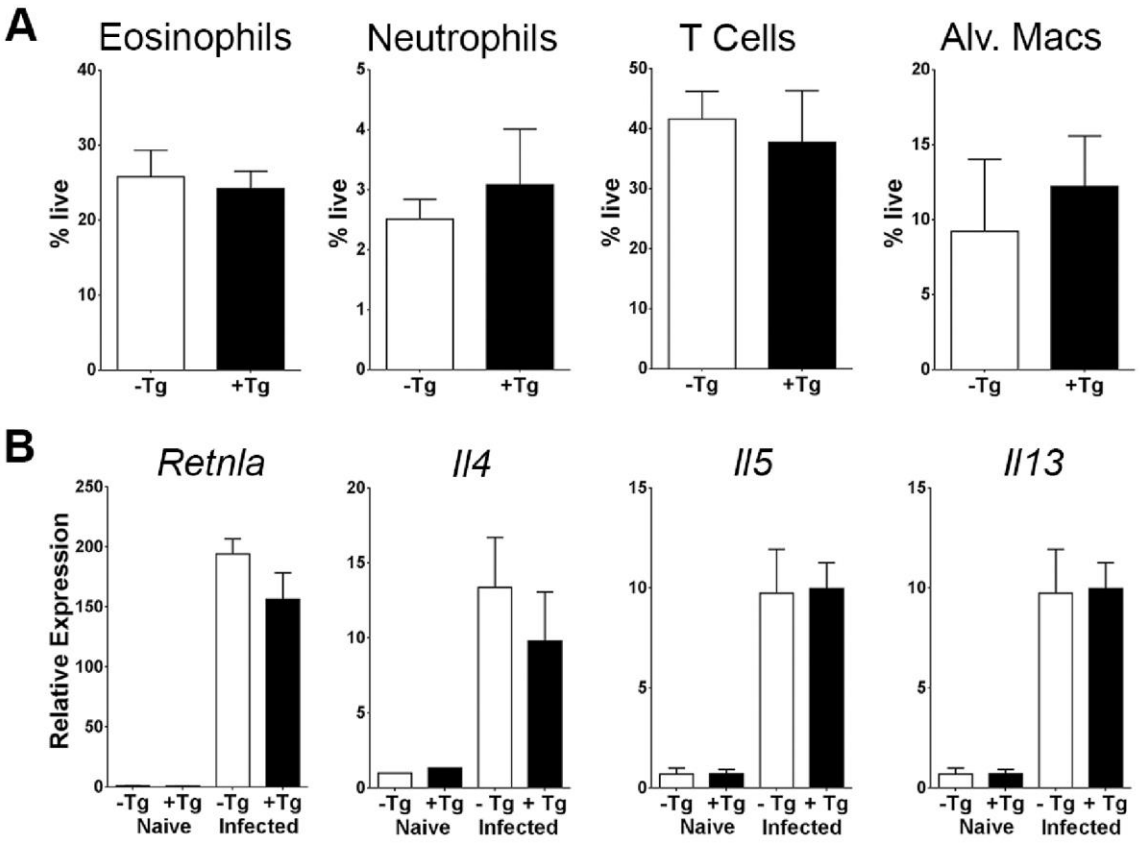

Figure 7: Analysis of brochio-alveolar lavage cells and Th2 immune responses in *Nb*-infected h*Retn*Tg- and h*Retn*Tg+ mice. (A-B) Flow cytometric analysis of BAL cell populations (A) and real-time PCR analysis of *Retnla* and Th2 cytokines normalized to *Gapdh* (B) were performed on lung tissue from naïve or day 7 *Nb*-infected mice. Alv. Mac, alveolar macrophages. Data (mean ± SEM, n=3-6 per group) are representative of three separate experiments.

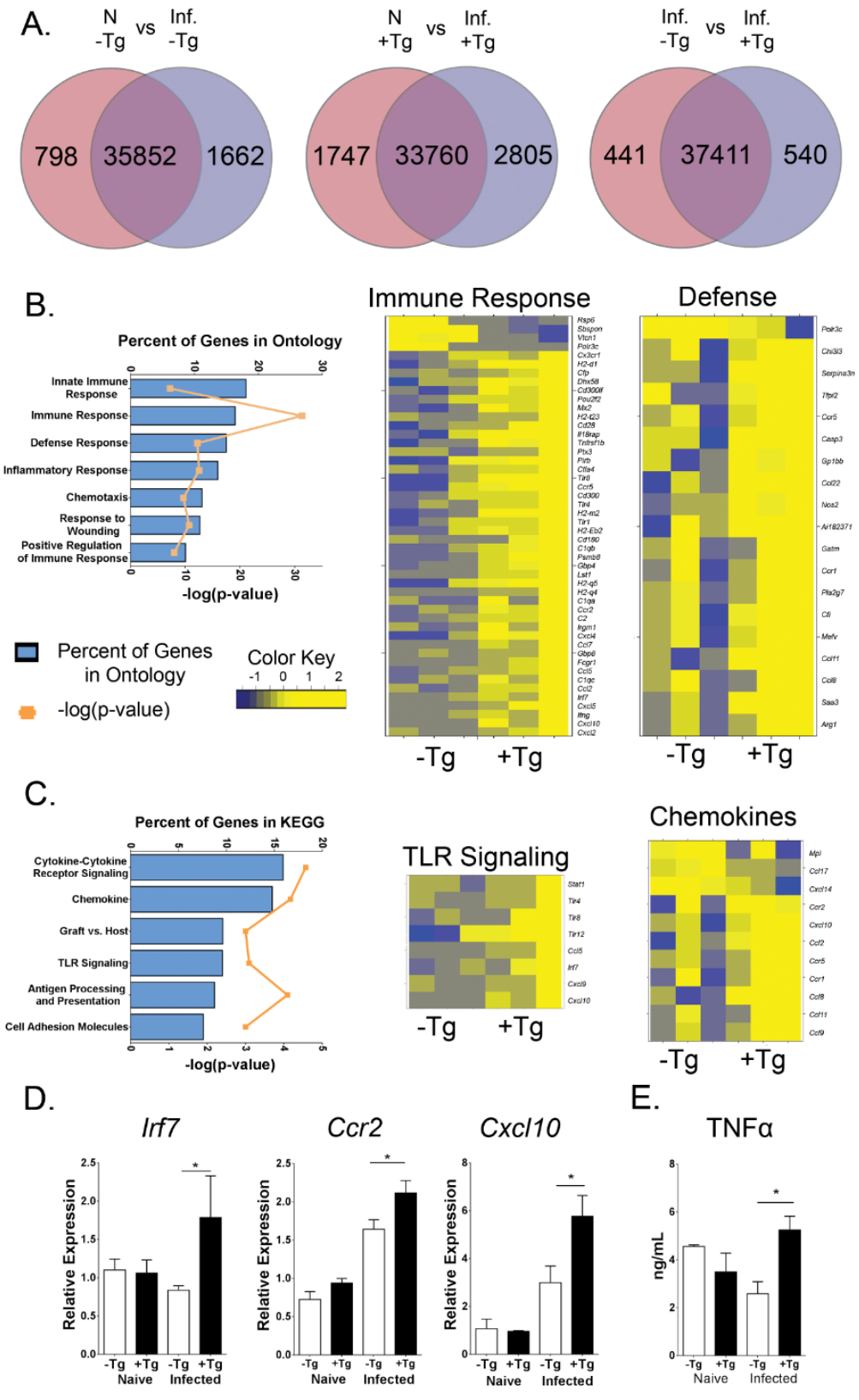
A.
N -Tg vs Inf. -Tg
798
35852
1662
N +Tg vs Inf. +Tg
1747
33760
2805
Inf. -Tg vs Inf. +Tg
441
37411
540
B.
Percent of Genes in Ontology
Innate Immune Response
Immune Response
Defense Response
Inflammatory Response
Chemotaxis
Response to Wounding
Positive Regulation of Immune Response
-log(p-value)
Percent of Genes in Ontology
-log(p-value)
Color Key
Immune Response
Defense
-Tg
+Tg
C.
Percent of Genes in KEGG
Cytokine-Cytokine Receptor Signaling
Chemokine
Graft vs. Host
TLR Signaling
Antigen Processing and Presentation
Cell Adhesion Molecules
-log(p-value)
TLR Signaling
Chemokines
D.
Irf7
Ccr2
Cxcl10
Relative Expression
-Tg +Tg -Tg +Tg
Naive Infected
E.
TNFα
ng/mL
Naive Infected

Figure 6: hResistin promotes type 1 inflammation, TLR signaling and chemokines. (A) Venn diagrams demonstrate infection-induced genes in h*Retn*Tg- and h*Retn*Tg+ mice. (B-C) Enriched biological pathways induced by h*Retn* were identified by Gene Ontology (B) and Kegg annotation (C) and represented as a graph and heatmap. (D) h*Retn*-induced TLR signaling and chemokine genes were validated by real-time PCR analysis of lung tissue. (E) Serum TNFα was quantified by ELISA. Data (mean ± SEM, n=3-4 per group) are representative of two separate experiments.

h*Retn*Tg^{-} with h*Retn*Tg^{+} mice (Figure 8A, right). We identified 540 genes upregulated in the h*Retn*Tg^{+} group and 441 genes downregulated for a total of 981 DEG. Gene set enrichment analysis revealed that the most significantly different gene ontology (GO) categories included the immune response, followed by the inflammatory response and defense response (Figure 8B). Heatmap analysis revealed that almost all the GO-defined immune response genes and defense genes were upregulated in the h*Retn*Tg^{+} group, suggesting a potent immune-stimulatory function for human resistin. For example, the gene *Tnfrsf1b*, a receptor for the proinflammatory cytokine TNFα was highly upregulated in the h*Retn*Tg^{+} group. KEGG pathways were used to further specify the differentially expressed categories based on molecular interactions and functions (Figure 8C). Specifically, two of the most upregulated pathways were chemokine signaling and TLR signaling. The chemokine signaling genes included *Ccr2* and *Cxcl10,* which are known to contribute to monocyte recruitment [159, 160]. Differentially expressed TLR signaling genes included *Irf7* and *Tlr4*, a previously identified receptor for hResistin [133]. We performed real-time PCR analysis of the naïve or infected lung tissue to validate the increased expression of some of the genes involved in TLR and chemokine signaling and observed significant upregulation of *Irf7*, *Ccr2* and *Cxcl10* in infected h*Retn*Tg^{+} mice compared to h*Retn*Tg^{-} mice (Figure 8D). Additionally, we observed elevated TNFα in the serum of infected h*Retn*Tg^{+} mice (Figure 8E), suggesting that hResistin promoted inflammatory cytokines systemically. In contrast, the pathways that were downregulated by hResistin were

not associated with the immune response (Table 1). Instead, we observed the downregulation of metabolic pathways such as arachidonic acid metabolism and oxidative phosphorylation. Both of these metabolic pathways have been associated with Th2 cytokine-activated alternatively activated macrophages [161, 162], which are protective in metabolic disorders such as insulin resistance or regulation of acute inflammation by Th2 cytokines [144, 163]. The downregulation of these protective pathways by hResistin are consistent with the exacerbated inflammation observed in h*Retn*Tg^{+} mice. Taken together, genome-wide transcriptional profiling and computational analysis suggest that hResistin initiates global gene expression changes in the *Nb*-infected lung tissue that contribute to increased TLR signaling and chemokines. These in turn promote a proinflammatory cytokine environment and exacerbated lung inflammation.

hResistin recruits monocytes and promotes inflammatory gene expression *in vivo*

Given that *Nb* infection of h*Retn*Tg^{+} mice was associated with increased expression of monocyte attractant chemokines and monocyte infiltration of the lung, we hypothesized that human resistin directly stimulated the production of chemokines leading to recruitment of monocytes and the establishment of a proinflammatory cytokine environment. To test this *in vivo*, we treated naïve mice with PBS or recombinant human resistin intraperitoneally (i.p.) followed by examination of cell recruitment to the peritoneal cavity and gene expression

Term	P-value	Benjamini
Ribosome	3.2E-6	2.2E-4
Cardiac Muscle Contraction	1.2E-3	4.2E-2
Drug Metabolism	7.6E-3	1.5E-1
Arachidonic Acid Metabolism	1.1E-2	1.7E-1
Oxidative Phosphorylation	1.1E-2	1.4E-1
Metabolism of Xenobiotics by Cytochrome p450	3.1E-2	3.1E-1
RNA Polymerase	3.2E-2	2.7E-1
Retinol Metabolism	3.4E-2	2.6E-1
Huntington's Disease	4.2E-2	2.8E-1
Parkinson's Disease	5.0E-2	3.1E-1

Table 1: KEGG pathway analysis reveals gene sets downregulated by hResistin.

analysis. Recombinant human resistin treatment led to the rapid and brief recruitment of $Ly6C^{+}CD115^{+}$ inflammatory monocytes at day 1 post-injection, followed by a return to the monocyte frequency observed in PBS-treated mice at day 2 and 3 post-injection (Figure 9A). This recruitment of leukocytes is specific for inflammatory monocytes, as no other cell population was increased at day 1 post infection (Table 2). In contrast, real-time PCR analysis revealed a gradual significant increase in genes associated with TLR-signaling and chemokines that occurred after the peak in monocyte recruitment, culminating at day 3 post injection (Figure 9B). To test that the effects of hResistin were specific and not caused by potential LPS contamination of the recombinant protein, we performed a limulus test to measure endotoxin levels in recombinant protein. The endotoxin levels in the concentration of hResistin used were below the limit of detection of the assay. However, as a more appropriate control, we injected mice with LPS at the limit of detection of 1.5pg/mL. In comparison to LPS treatment, hResistin induced monocyte recruitment and proinflammatory gene expression (Figure 10), suggesting that hResistin effects were independent of LPS. Overall, this data corroborates the timing of *Nb* infection, as we see an increase in hResistin expression at day 3 post-infection (see Figure 4A), with a subsequent increase in proinflammatory cytokines by day 7 post-infection (see Figure 8).

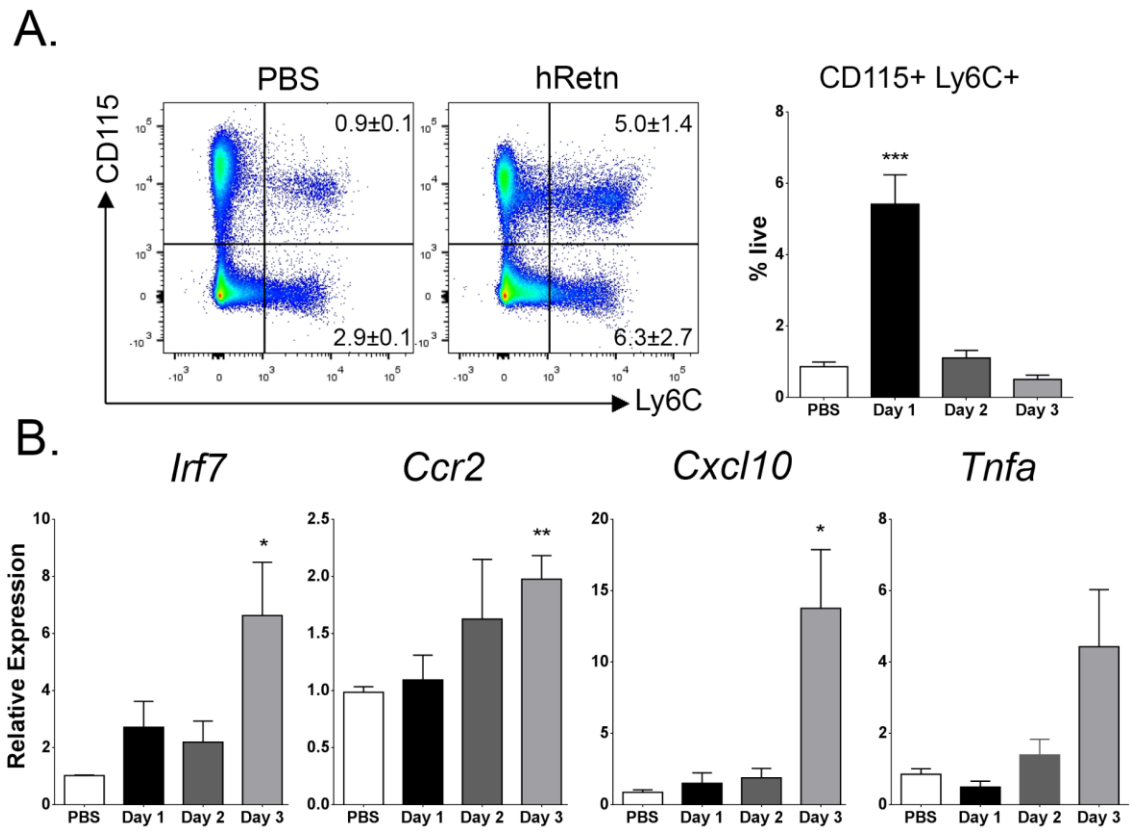

Figure 9: hResistin recruits inflammatory monocytes. (A-B) Naïve C57BL/6 mice were treated with recombinant human resistin (500ng) followed by flow cytometry analysis of CD115+Ly6C+ inflammatory monocytes (A) and real-time PCR analysis of PEC RNA (B). Data (mean ± SEM, n=3 per group) are representative of two separate experiments.

	PBS (%live ±SEM)	hResistin (% live±SEM)	p-value
Monocytes	0.92±0.13	5.0±1.4	0.0006
T Cells	6.14±0.51	4.01±0.81	0.0916
Eosinophils	24.97±2.73	22.26±7.77	0.7586
Neutrophils	0.85±0.47	1.61±1.18	0.5797
Macrophages	28.70±9.22	40.65±9.85	0.4555

Table 2: Cell composition in the peritoneal cavity following recombinant hResistin injection

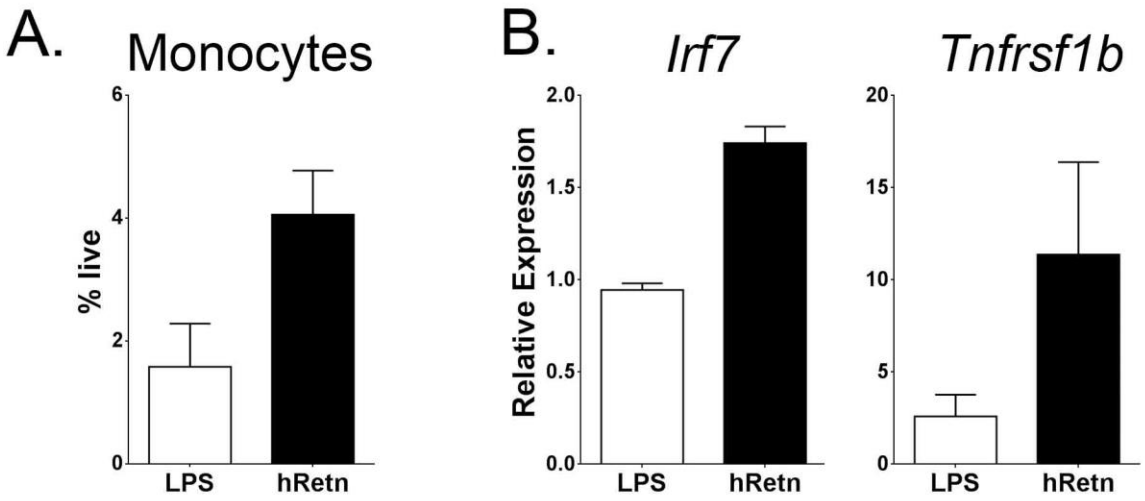

Figure 10: Effects of recombinant hResistin are independent of potential LPS contamination. Naïve mice were treated i.p. with 500ng recombinant hResistin or 0.15pg LPS. (A-B) PECs were recovered at day 3 and measured for monocytes by flow cytometry (A) or proinflammatory cytokines by real-time PCR (B).

Monocytes and neutrophils are the main cellular targets of hResistin during Nb infection

In both *Nb* infection and treatment with recombinant hResistin, we observed increased monocyte responses, however, whether monocytes are the direct cellular target of hResistin in *Nb* infection is unclear. To examine this, we performed a resistin binding assay on dissociated lung cells from *Nb*-infected mice according to previously established methodologies from the lab [33]. Cells were incubated with recombinant hResistin or PBS, followed by detection with biotinylated anti-hResistin antibody. Compared to control PBS, hResistin preferentially bound lung monocytes with a distinct population of hResistin-bound monocytes (Figure 11A, 13.2%). Further characterization of the hResistin-bound cells confirmed that monocytes constituted the highest proportion of resistin-bound cells (57.8%) followed by neutrophils (21.5%) and alveolar macrophages (15%) (Figure 11B). The other cell-types examined, including eosinophils (SiglecF^{+} CD11c^{-}), T Cells (CD3^{+}), dendritic cells (MHCII^{hi} CD11c^{+}) and interstitial macrophages (MHCII^{mid} CD11b^{+}) did not exhibit significant binding to hResistin, confirming the that hResistin binding assay was specific.

To assess the functional consequence of hResistin binding for expression of proinflammatory cytokines, we performed cell sorting of the lungs of *Nb*-infected h*Retn*Tg^{+} and h*Retn*Tg^{-} mice for monocytes (CD11b^{+} Ly6C^{+}), neutrophils (CD11b^{+} Ly6G^{+}) and alveolar macrophages (CD11c^{+} F4/80^{+}) (Figure 11C). Real-time PCR

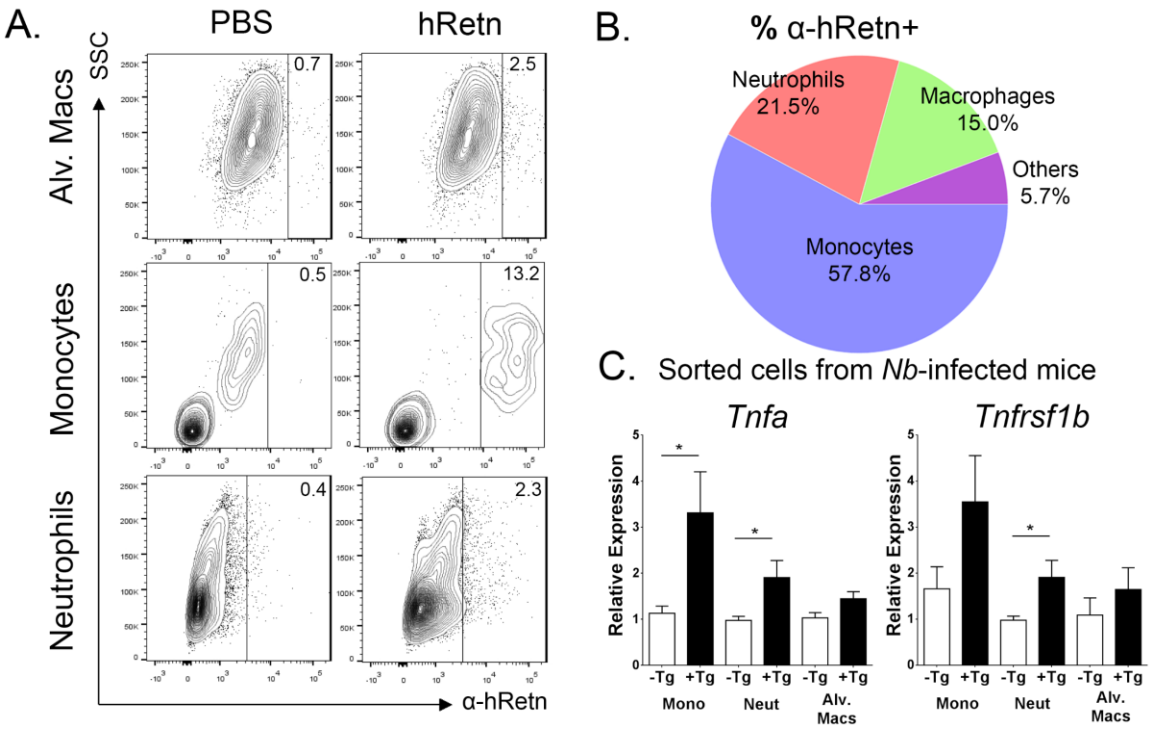

Figure 11: Monocytes and neutrophils are the main cellular targets of hResistin during *Nb* infection. (A-B) Lung cells from *Nb*-infected mice were incubated with recombinant hResistin followed by detection with α-hRetn to determine which cells can bind hResistin compared with control PBS (A). Surface expression of the hResistin-bound cells identified monocytes as the main cell-type that bound hResistin (B). (C) Sorted lung cells from *Nb*-infected h*Retn*Tg- and h*Retn*Tg+ mice were analyzed for proinflammatory gene expression by real-time PCR. Data (mean ± SEM, n=3-4 per group) are representative of two separate experiments.

analysis for inflammatory genes *Tnfa* and *Tnfrsf1b* revealed that monocytes from the h*Retn*Tg^{+} mice exhibited the highest expression of proinflammatory genes, followed by neutrophils. In contrast, alveolar macrophages did not exhibit hResistin-mediated increases in proinflammatory gene expression. Together, these data implicate inflammatory monocytes and neutrophils as the cellular targets of hResistin-mediated expression of proinflammatory genes.

h*Retn*Tg^{+} mice exhibit increased Nb egg and worm burdens

In human studies, a type 1 skewed immune response during helminth infection has been correlated with increased susceptibility to gastrointestinal nematode infection [164]. Additionally, mice deficient in TLR-4 are highly resistant to the gastrointestinal nematode, *Trichuris muris* [148]. We hypothesized that the type 1 proinflammatory environment and the increased TLR-4 signaling mediated by human resistin might therefore impair intestinal immunity to *Nb*. To this end, we monitored the time course of *Nb* colonization in the small intestine of h*Retn*Tg^{+} and h*Retn*Tg^{-} mice by assessing the parasite egg burdens in the feces followed by sacrifice of the mice at day 9 post-infection. Throughout the course of infection, h*Retn*Tg^{+} mice exhibited more than two-fold increases in parasite egg burdens (Figure 12A). At day 9 post-infection, the majority of the h*Retn*Tg^{-} mice had expelled the *Nb* worms whereas the h*Retn*Tg^{+} mice had an average of 100 worms in the small intestine (Figure 12B). In addition to infection-induced resistin expression in the small intestine (see Figure 5), we examined if systemic hResistin

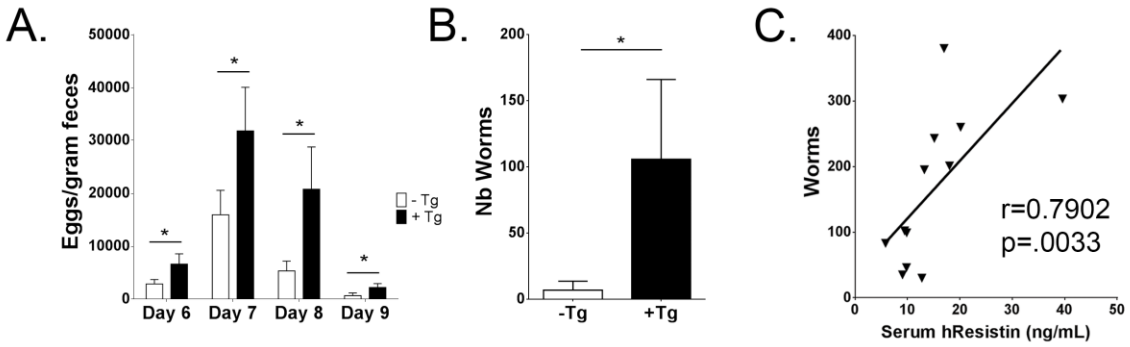

Figure 12: h*Retn*Tg^{+} mice are more susceptible to *Nb* infection. (A-B) h*Retn*Tg^{+} mice (black bars) and control h*Retn*Tg^{-} mice (white bars) were infected with 500 L3 *Nb* worms followed by parasitology analysis by fecal egg counts (A) or adult parasite numbers in the intestine at day 9 (B). Data (mean ± SEM, n=3-6 per group) are representative of three separate experiments. (C) Serum hResistin concentration in h*Retn*Tg^{+} mice is positively correlated with *Nb* worms at day 7 post infection (n=12).

expression could be predictive of higher worm burden. Analysis of serum human resistin levels in infected h*Retn*Tg^{+} mice from 3 combined experiments and a total of 12 mice revealed a significant and positive correlation between systemic human resistin expression and intestinal parasite burden (Figure 12C). Overall, these data suggest that *Nb* parasites induce hResistin infection locally and systemically, adversely affecting parasite expulsion.

Murine resistin does not affect Nb-induced inflammation or parasite burden

Given that human resistin caused dramatic effects in the immune response to *Nb*, we investigated if murine resistin mediated similar inflammatory effects. However, *Nb* infection of wild-type (WT) C57BL/6 mice did not induce murine resistin in the infected tissue, and we observed no difference in weight loss or parasite burdens between WT and m*Retn*$^{-/-}$ mice (Figure 13). It is likely that the difference in expression pattern between murine resistin, which is expressed in adipose tissue, and human resistin, which is expressed by monocytes/macrophages, accounts for the functional differences between murine and human resistin during helminth infection. Taken together, we have identified a unique function for human resistin in promoting type 1 inflammation and impeding parasite clearance during *Nb* infection.

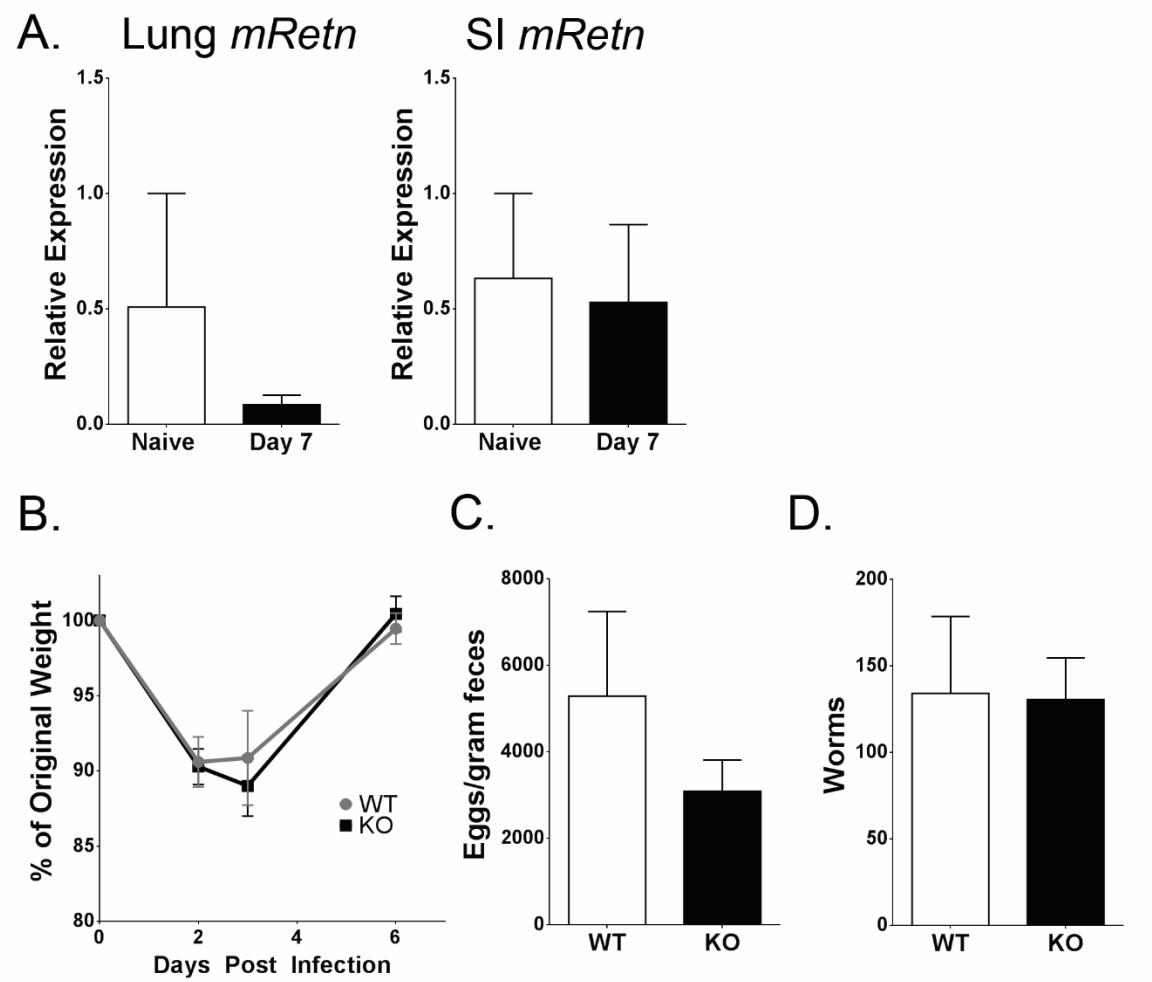

Figure 13: Expression and role of murine resistin in *Nb*-infected mice. Control C57BL/6 and murine resistin knockout (KO) mice were infected with 500 L3 *Nb* worms and sacrificed on day 7 post infection. (A) Real-time PCR analysis of mResistin expression in C57BL/6 mice following *Nb* infection was performed. (B) Weight loss following infection was monitored. (C-D) Fecal egg burden (C) and adult *Nb* worms (D) were quantified on day 7 post infection. Data (mean ± SEM, n=4 per group) are representative of three separate experiments.

Resistin is upregulated in human soil-transmitted helminth infection and is positively correlated with proinflammatory cytokines

STH are the most widespread helminth infection in humans, affecting an estimated 1 billion people worldwide. In many of these cases, environmental and economic factors promote high rates of co-infection with various parasites and reinfections by STH [165]. As *Nb* is an STH, we sought to investigate the clinical significance of our findings in the h*Retn*Tg^{+} mouse model. We compared serum collected from STH-infected school children resident in Ecuador (age 5-15, average 9 years) to uninfected, endemic normal school children from the same cohort [156]. The cohort included 49 infected and 51 uninfected school children. Within the infected cohort, all were infected with *A. lumbricoides,* many were infected with *T. trichiuria,* and only a few were infected with *A. duodenale,* showing that *A. lumbricoides* was the most prevalent STH in that region [156]. Consistent with the h*Retn*Tg^{+} mouse model, infected individuals exhibited significantly increased expression of resistin compared to uninfected children (Figure 14A). Given our previous observation that human resistin initiated a proinflammatory cytokine environment and impaired parasite expulsion following *Nb* infection, we tested if serum resistin expression was predictive of increased parasite burden or proinflammatory cytokine expression in the infected school children. We observed a significant and positive correlation between serum resistin expression and the *A. lumbricoides* egg burden (alepg) in the feces (Figure 14B, left). Next, we performed luminex assays to measure the

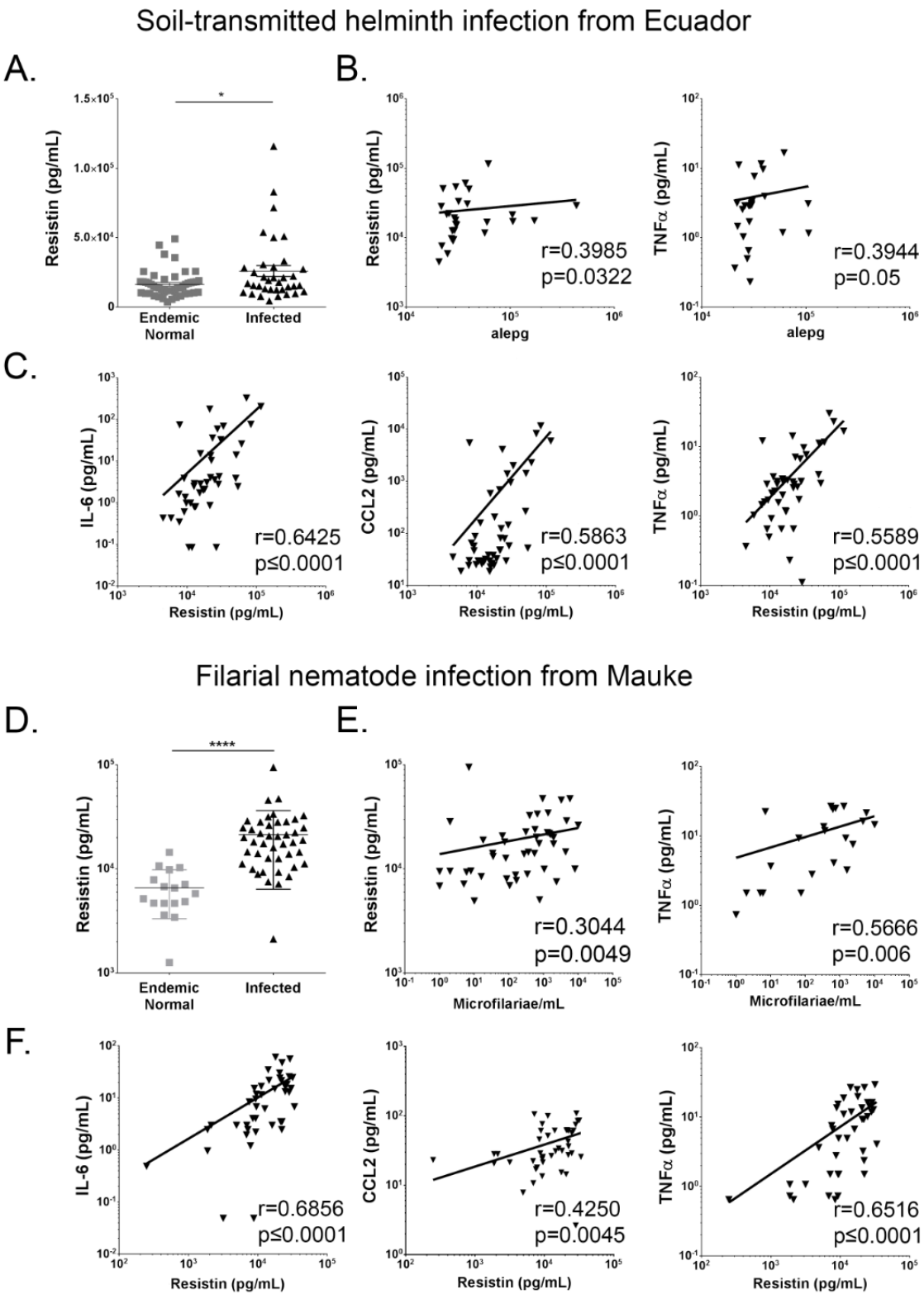
Soil-transmitted helminth infection from Ecuador
A.
B.
C.
Resistin (pg/mL)
Endemic Normal
Infected
*
alepg
r=0.3985
p=0.0322
TNFα (pg/mL)
r=0.3944
p=0.05
IL-6 (pg/mL)
r=0.6425
p≤0.0001
CCL2 (pg/mL)
r=0.5863
p≤0.0001
r=0.5589
p≤0.0001
Filarial nematode infection from Mauke
D.
E.
F.
****
Microfilariae/mL
r=0.3044
p=0.0049
r=0.5666
p=0.006
r=0.6856
p≤0.0001
r=0.4250
p=0.0045
r=0.6516
p≤0.0001

Figure 14: Expression of resistin in human patients infected with STH or filarial nematodes. (A) Serum resistin from uninfected (n=51) or STH-infected children (n=49) was measured. (B) The fecal egg burdens in infected children was quantified as *A. lumbricoides* eggs per gram feces (alepg) and plotted against serum resistin and TNFα. (C) Proinflammatory cytokines were positively correlated with resistin in the serum of STH-infected children. (D) Serum from uninfected endemic normal individuals (n=17) or patients infected with *Wuchereria bancrofti* (n=44) was analyzed by ELISA for resistin. (E) In infected individuals, a positive correlation was observed between the circulating microfilariae and resistin or TNFα. (F) Proinflammatory cytokines were positively correlated with resistin levels in the serum of infected patients.

concentration of multiple cytokines in the serum. We chose proinflammatory cytokines (e.g. TNFα, CCL2, IL-6), which were initially identified in the h*Retn*Tg^{+} mouse model, and other factors that have been associated with helminth-associated morbidity for a total of 11 analytes. We observed that elevated TNFα serum levels were associated with increased parasite egg burdens in the feces (Figure 14B, right). Given our transgenic mouse studies showing that resistin directly promotes TNFα expression, we hypothesized that helminth-induced resistin expression in the infected children promoted a proinflammatory cytokine environment that was responsible for impaired parasite clearance. We tested associations between serum resistin and proinflammatory cytokines, and observed a significant positive correlation between resistin and IL-6, CCL2 and TNFα (Figure 14C), and other proinflammatory cytokines (Table 3). Interestingly, higher resistin levels also correlated with a higher concentration of wound healing factors, such as Chi3l1 and VEGF (Table 3), reflecting the RNAseq data (see Figure 8). Overall, these results support our findings from the h*Retn*Tg^{+} mouse model, and confirm that our mouse model can be appropriately applied to human helminth infections.

Resistin expression is elevated in human filarial nematode infection

Given that we have previously observed the induction of RELM proteins in various helminth infections of mice [90, 166, 167], we hypothesized that human resistin expression may be an indicator of multiple helminth infections. After STH

	STH Infection		Filarial Nematode Infection	
	Spearman rho	P-value	Spearman rho	P-value
Chi3l1	***0.4825***	***0.0005***	0.0302	0.8475
VEGF	***0.4184***	***0.0031***	***0.3751***	***0.0132***
IL-1α	***0.4034***	***0.0120***	0.2439	0.1196
IFNγR1	***0.2699***	***0.0636***	***0.5809***	***≤0.0001***
IFNγ	0.3913	0.1491	0.1383	0.3763
IL-6Rα	0.0953	0.5193	***0.4369***	***0.0034***
VCAM1	0.0670	0.6510	0.2497	0.1108
Leptin	-0.0048	0.9740	0.1640	0.2933

Table 3: Many inflammatory factors are positively correlated with resistin in humans infected with soil-transmitted helminths or filarial nematodes.

infections, filariasis is the second most prevalent nematode infection, affecting an estimated 120 million people worldwide [1]. We examined serum samples collected from 44 filarial-infected individuals and 17 non-infected endemic normal individuals from the *Wuchereria bancrofti*-endemic island of Mauke in the south Pacific, where levels of circulating microfilariae were previously quantified in the blood [157, 168]. We observed that circulating resistin levels were increased in infected microfilaremic individuals by almost three-fold compared to the non-infected endemic normal population (average 6749 pg/mL in endemic normal versus 21361 pg/mL in infected individuals) (Figure 14D). Microfilariae counts were also positively associated with resistin and TNFα (Figure 14E). Finally, elevated resistin was associated with higher concentration of IL-6, CCL2 and TNFα (Figure 14F) and inflammatory factors (Table 3), suggesting that the resistin/proinflammatory cytokine immune axis we identified in mice is also present in human nematode infections. Protective immunity to helminths is typically associated with type 2 cytokine responses [169], therefore we investigated if resistin expression was inversely correlated with parasite-specific type 2 immune responses in the microfilariae-positive patients. There was no significant correlation between resistin expression and parasite-specific antibody titers for the Th2-associated isotype IgG4, nor did we observe significant correlations with IL-5 or IL-10 cytokine production (Table 4). This suggests that the downstream effects of resistin are independent of type 2 cytokines, and instead involve proinflammatory cytokine induction. Taken together, these human studies support

a model where resistin is an innate response to multiple helminths that is correlated with elevated inflammatory cytokines and impaired parasite clearance.

	Spearman rho	p-value
Serum		
IgG4 Mf	0.1026	0.4782
PBMC stimulated with Mf antigen		
IL-10	0.038	0.802
IL-5	-0.099	0.511
IFNγ	0.279	0.061

Table 4: Type 2 immunity is not altered by resistin in filarial-infected individuals

Discussion

Some of the earliest studies of RELM proteins identified these as potently induced genes in mouse helminth infection models [166]. Optimal expression of RELMα and RELMβ in helminth infection is dependent on Th2 cytokines [19, 166]. Recent studies, however, have reported that other stimuli, such as bacterial infections and presumably LPS, can induce RELMα and RELMβ gene expression [96, 170]. Similarly, human resistin expression is induced in a variety of inflammatory settings including endotoxemia, metabolic disorders and cancer [153, 154, 171]. However, the function of human resistin in infection has not been defined. Here, we combined studies utilizing a transgenic mouse model and human data to demonstrate that human resistin is induced following both STH and filarial nematode infection, where it promotes a proinflammatory cytokine environment. In the mouse model, the effects of resistin were detrimental to the host, and data from both the mouse and human studies demonstrated a positive correlation between resistin, proinflammatory cytokines and parasite burdens.

Previous studies showed that resistin mRNA was upregulated in macrophages from filarial-infected patients following exposure to filarial antigen [34, 172]. In h*Retn*Tg^{+} mice and the serum of STH and filarial nematode-infected individuals, we demonstrate that resistin protein is significantly elevated in the infected tissue and systemically in the circulation. Although many studies have shown that LPS can induce resistin expression both *in vitro* and *in vivo*, our data

implicates resistin expression as a common innate response to multiple helminth infections. This suggests that hResistin is not only functionally relevant during type 1 inflammatory settings induced by bacterial antigens or metabolic disorders, but may have functional consequences during a Th2-biased helminth immune response.

Human resistin did not significantly alter the Th2 cytokine gene expression, or associated Th2 cell activation. Instead, we demonstrate through genome-wide transcriptional profiling and functional studies that resistin promoted the recruitment of inflammatory monocytes and the establishment of an innate proinflammatory cytokine environment, including increased expression of TLR-signaling associated genes and monocyte chemoattractants. This is consistent with previous *in vitro* studies utilizing human monocyte/macrophages [134, 136, 173]. In a co-culture system with endothelial and smooth muscle cells, resistin induced the expression of monocyte chemoattractants including CCL2, which led to increased monocyte migration [174]. Given that *Nb* infection of the lung causes dramatic injury and hemorrhage (see Figure 4), it is likely that there is activation of endothelial and smooth muscle cells to produce monocyte chemoattractants. Indeed, lung monocyte infiltration in the h*Retn*Tg^{+} mice was associated with significantly elevated expression of CCL2, and serum resistin concentration in both STH and filarial nematode-infected individuals was positively correlated with CCL2. Interestingly, *in vivo* treatment of naïve mice with recombinant resistin led to rapid monocyte recruitment that preceded increased expression of monocyte

chemoattractants. This suggests that monocytes are directly recruited by resistin and in turn begin to express chemokines as part of a positive feedback loop to increase inflammation.

Previous studies have identified several putative receptors for human resistin *in vitro*, including TLR-4 and CAP1, which can also induce NF-κB dependent induction of proinflammatory cytokines TNFα, IL-6 and IL-1β [133, 134, 137]. Our RNA sequencing studies revealed that human resistin promoted expression of TLR4 (and other TLRs), but not CAP1. Additionally, direct injection of recombinant human resistin in naïve mice resulted in a marked increase in inflammatory genes downstream of TLR signaling, including IRF7. This suggests that resistin may act through TLR-4 and not CAP1 during *Nb* infection, and the importance of CAP1 in resistin-mediated effects may be more significant in metabolic disorders.

Altogether, our data suggest that resistin expression could have significant implications for the disease outcome of helminth-infected individuals. In humans, increased resistin expression could be predictive of impaired immunity to helminths or exacerbated inflammation following infection. Recent studies have shown that filarial nematode and STH-infected individuals demonstrate increased circulating microbial products [72, 175]. It is possible that this endotoxemic phenotype promotes resistin expression, which in turn acts to increase the proinflammatory cytokine environment. In filarial nematode infection, the establishment of chronic

infections typically involves the downmodulation of innate and adaptive immune responses [176]. Therefore, it is somewhat surprising that there was a significant and positive correlation between microfilarial burdens, resistin and proinflammatory cytokines in the chronically-infected patients. It is possible that the resistin-mediated effects on proinflammatory cytokines operates independently of regulatory mechanisms such as regulatory T cells and IL-10 that also contribute to chronicity of infection. One debilitating consequence of filarial nematode infections is the development of lymphatic dysfunction, lymphedema and ultimately elephantiasis. This pathology has been associated with elevated TLR signaling [147], and resistin may initiate this by overcoming the suppressive mechanisms that typically occur in filarial infections. Although there were not sufficient filarial nematode-infected individuals with lymphatic pathology in our cohort to test this hypothesis, our data suggest that future studies examining resistin correlation with lymphatic pathology may be warranted. Additionally, our studies of the transgenic mouse model and STH-infected children have provided a useful framework for longitudinal studies to examine whether high resistin expression leads to an increased likelihood of infection with STH. In summary, our studies contribute to the growing evidence of the pleiotropic effects of human resistin in several human diseases, and demonstrate a critical function for this protein in helminth infection. We show that expression of human resistin in mice recruits inflammatory monocytes to the site of infection, promotes type 1 inflammation, and is correlated with increased STH and filarial nematode burden in humans. To our knowledge,

this is the first report on the ability of the human resistin to impair immunity to helminths and promote inflammatory responses that may mediate pathology. Targeting resistin may therefore provide new approaches to host-directed treatment strategies for helminth infection and amelioration of the associated immune-mediated pathology.

CHAPTER THREE – Human Resistin Expression is Regulated by IL-4 and STAT6

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Abstract

Toll-like receptor 4 (TLR4) signaling plays a critical role in hResistin function, both as a receptor for hResistin and as an inducer of hResistin expression following LPS stimulation. We previously showed that hResistin expression is also increased during helminth infection. However, in a Th2 environment such as helminth infection, it is unclear whether LPS initiates hResistin transcription or if there are alternative mechanisms. To investigate this, we generated TLR4$^{-/-}$/h*Retn*Tg^{+} mice and infected them with the murine hookworm, *Nippostrongylus brasiliensis*. hResistin is still upregulated following helminth infection in the absence of TLR4, suggesting a previously unidentified pathway for hResistin expression. As an unbiased approach to identification of the signaling pathways that mediate hResistin expression, we used the hResistin promoter sequence to predict transcription factor binding sites. This *in silico* approach revealed two STAT6 binding sites in the hResistin promoter. Treatment of h*Retn*Tg^{+} mice and human PBMC reveal that hResistin can be stimulated by the Th2 cytokine and STAT6 activator, IL-4. This upregulation was dependent on STAT6 phosphorylation, as cells treated with IL-4 and a STAT6 inhibitor were unable to produce hResistin. This data suggests that the IL-4/STAT6 axis is essential for hResistin expression in Th2 immunity and helminth infection.

Introduction

Human resistin expression is elevated in many inflammatory diseases, including atherosclerosis, arthritis and sepsis [108, 113, 152], and infectious diseases, including bacterial infection (*Streptococcus* spp.) and viral infection (Dengue virus) [119, 120]. *In vitro* studies reveal that NF-κB and C/EBPβ, two transcription factors downstream of LPS stimulation, are the only known transcription factors that promote hResistin expression. However, our lab demonstrated that hResistin expression is also increased in a multitude of helminth infections, where it is produced by alveolar macrophages, neutrophils and monocytes. Given that LPS stimulation is minimal during helminth infection, we sought to identify the mechanisms that regulate hResistin expression.

Due to their large size, helminths cause damage to pulmonary or intestinal tissue when they burrow through tissue. When epithelial cells are damaged, they produce danger-associated molecular patterns and cytokines to induce wound healing genes, including IL-25, IL-33 and TSLP. This initial burst of cytokines stimulates the production of the Th2 cytokines, IL-4 and IL-13, by innate lymphoid cells, Th2 cells, eosinophils, basophils and mast cells. These Th2 cytokines then signal through their common receptor, IL-4Rα, to polarize alternative activation of macrophages. This is predominantly mediated by STAT6 phosphorylation and translocation into the nucleus to induce AAMac gene expression, including murine RELMα. Given that hResistin expression profile is most similar to RELMα and they

are both produced by macrophages, it is possible that they are regulated by the same mechanisms.

In addition to Th2 cytokines, helminth antigens can also directly stimulate RELM protein expression. *Fasciola hepatica* tegumental antigens can stimulate RELMα production by AAMacs [86]. This may be mediated through chitin, a common allergen found on helminth cuticles, that can induce AAMAc polarization and RELMα production. In human $CD14^+$ macrophages, *B. malayi* antigen can stimulate AAMac genes, including hResistin [34], although these mechanisms have not been characterized. On the other hand, *S. mansoni*-derived hemozoin downregulated RELMα expression in macrophages, which could have inhibitory effects on the subsequent Th2 immune response [177]. Furthermore, *H. polygyrus* excretory/secretory products can decrease Th2 immunity, including RELMα [178]. Thus, helminths have evolved mechanisms to directly modulate the host's immune response, and could lead to hResistin expression.

Incidence of helminth infection is often concurrent with bacterial infections, and occurs because helminths often cause damage to the lungs and intestinal wall, leading to the dissemination of opportunistic bacteria [72]. Indeed, in patients infected with *S. stercoralis*, bacterial translocation results in an increase in circulating LPS and proinflammatory cytokines [73]. Furthermore, filarial nematodes can introduce the symbiotic bacteria, *Wolbachia*, which induce TLR2 and TLR4 signaling pathways [179]. This increase in LPS could stimulate hResistin

expression during helminth infection. Indeed, the bacterial wall component, LPS can upregulate hResistin expression both *in vitro* and *in vivo* in an NF-κB dependent manner.

To examine the signaling pathways that regulate hResistin expression during helminth infection, we generated TLR4$^{-/-}$/h*Retn*Tg^{+} mice and infected them with the hookworm, *Nippostrongylus brasiliensis* (*Nb*). Human resistin expression was similar even in TLR4 deficient mice, suggesting an alternative pathway must activate hResistin transcription. As an unbiased approach to identify this alternative pathway, we performed an *in silico* search for potential transcription factor binding sites in the regulatory region upstream of the hResistin start site. We confirmed binding sites for NF-κB, the downstream transcription factor of TLR4, and additionally identified two potential STAT6 binding sites. Cells were treated with IL-4±AS1517499, a STAT6 inhibitor, which revealed a STAT6-dependent expression of hResistin. Furthermore, injection of IL-4 complex promoted hResistin expression, confirming that IL-4 also regulates hResistin expression *in vivo*. This data suggests that key components of Th2 immunity, specifically IL-4 and STAT6, promote hResistin expression during helminth infection.

Materials and Methods

Mice

Human resistin transgenic mice (h*Retn*Tg^{+}) were generated as previously described by Mitch Lazar [124]. Briefly, the human resistin gene, along with 21,300 bp upstream and 4,248 bp downstream of the human resistin start site, were engineered through a bacterial artificial chromosome. h*Retn*Tg^{+} mice were crossed with TLR4$^{-/-}$ mice to generate TLR4$^{-/-}$/h*Retn*Tg^{-} and TLR4$^{-/-}$/h*Retn*Tg^{+} mice. Mice were then infected with 500 L3 *Nb* by subcutaneous injection. At day 7 post infection, lung and small intestine tissue was homogenized and hResistin expression was quantified by ELISA.

Cytokine Quantification

For sandwich ELISA, Greiner 96-well medium bind plates were coated with primary antibody to cytokines (Peprotech) overnight at room temperature. After blocking the plates with 5% NCS in PBS for 1 hr, tissue homogenates were added at various dilutions and incubated at room temperature for 2 hr. Detection of cytokines was done with biotinylated antibodies for 2 hr, followed by incubate with streptavidin-peroxidase (Jackson Immunobiology) for 30 min. The peroxidase substrate TMB (BD) was added followed by addition of 2N H_2SO_4 as a substrate stop, and the optical density (OD) was captured at 450 nm. Samples were compared to a serial-fold dilution of recombinant cytokine.

IL-4 complex

IL-4 complex (IL-4c) extends the activity of IL-4 *in vivo*, and results in a slow continuous release of cytokine. IL-4c consists of a 2:1 molar ratio of recombinant IL-4 (Peprotech) and anti-IL-4 antibody (clone 11B11; BioXCell). Mice were injected in the peritoneum with 5µg of IL-4c at day 0 and day 2. At day 4, peritoneal cells were recovered in 5mL PBS and stored in RLT buffer (Qiagen) for RNA extraction.

Immunofluorescent Staining

Lungs were inflated with 1 mL 1 part 4% PFA/30% sucrose and 2 parts OCT and stored overnight in 4% PFA at 4˚C. After 24 hours, lungs were removed from 4% PFA and incubated another 24 hours in 30% sucrose. Lungs were then blocked in OCT and sectioned at 8 µm. For immunofluorescence staining, sections were incubated with rabbit anti-hResistin (1:400, generated by Mitchell Lazar), anti-IL-4Rα (1:100, BD) or anti-pSTAT6 (1:100, Abcam) overnight at 4˚C. Sections were incubated with appropriate fluorochrome-conjugated secondary antibodies for 2 hours at 4˚C and counterstained with DAPI. Sections were visualized under a Leica microscope (DM5500 B).

Human PBMC

Human buffy coat was commercial purchased from Zen-bio and peripheral blood mononuclear cells were isolated by density gradient. Buffy coat was overlaid

on top of Histopaque-1077 and spun at 700g at 25˚C with no brake and PBMCs were recovered from the interphase. PBMCs were then plated on a 96-well plate and stimulated with recombinant IL-4 (40ng/mL, Peprotech) ± AS1517499 (10ug/mL, Axon Chemical) or dexamethasone (1:50). Cells were incubated for 16hrs at 37ºC, 5%CO_2. After incubation, supernatant was removed and cells were lysed for RNA extraction (Qiagen) and real-time PCR.

Results

Human resistin expression is TLR4-independent during helminth infection

We previously showed that hResistin expression is increased during helminth infection, but the helminth-induced factors that promote hResistin are unknown. Previous reports have demonstrated that h*Retn* gene expression is induced in response to LPS, and is NF-κB dependent [180]. To examine whether hResistin expression is dependent on TLR4 during helminth infection, we generated TLR4$^{-/-}$/h*Retn*Tg^{+} mice and TLR4$^{-/-}$/h*Retn*Tg^{-} control mice. BMMacs from these mice were generated and stimulated with LPS (100ng/mL). Since LPS can stimulate both TLR2 and TLR4, ultrapure LPS (Invitrogen) was used, which only stimulates TLR4. Following LPS stimulation, cells were lysed and RNA was recovered to analyze hResistin expression by real-time PCR. In TLR4$^{+/+}$ macrophages, hResistin expression is induced by LPS, but this upregulation is abrogated in TLR4$^{-/-}$ cells (Figure 15A). This confirms that hResistin expression following LPS stimulation is also regulated through TLR4 in our mouse model. To test whether hResistin expression is TLR4 dependent during helminth infection, TLR4$^{-/-}$/h*Retn*Tg^{+} mice were infected with 500 L3 *Nb* larvae, and hResistin expression was quantified by ELISA. In naïve tissue, hResistin expression is the same in TLR4$^{-/-}$ and TLR4$^{+/+}$, although it was under the limit of detection in the small intestine. This suggests that under homeostatic condition h*Retn* is not regulated by TLR4. Following *Nb* infection, hResistin expression is increased in

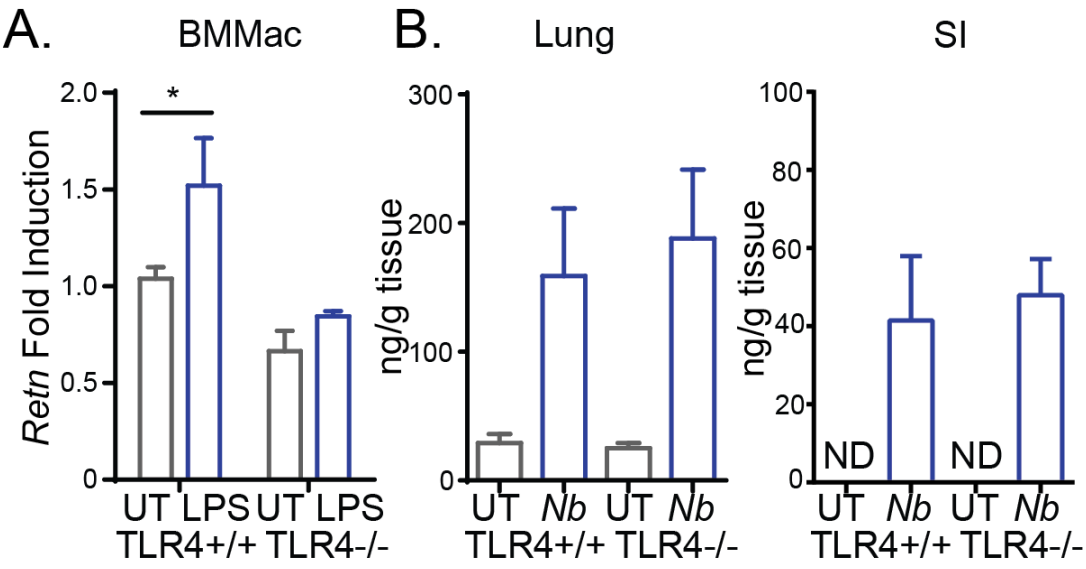

Figure 15: hResistin expression is not regulated by TLR4 during helminth infection. (A) h*Retn* mRNA in TLR4$^{+/+}$ and TLR4$^{-/-}$ mice was measured in LPS-treated BMMacs. (B) h*Retn* expression in naïve and Nb-infected TLR4$^{+/+}$ and TLR4$^{-/-}$ mice was measured by ELISA. Data (mean±SEM) are representative of 2 separate experiments (n=4-5).

the lungs and small intestine in both the TLR4$^{+/+}$ and TLR4$^{-/-}$ (Figure 15B). This suggests a previously unidentified TLR4-independent pathway for hResistin expression.

Identification of alternative transcription factors that regulate hResistin transcription

Since TLR4 signaling is not important in hResistin expression during helminth infection, we took an unbiased approach to identify other possible transcription factors that might regulate hResistin expression. Using MatInspector software, we predicted potential transcription factor binding sites in the hResistin promoter region based on the sequence. We confirmed two NF-κB binding sites in the hResistin promoter. However, we also discovered two STAT6 binding sites (Figure 16A) which is phosphorylated by IL-4Rα signaling [38]. This suggested to us that hResistin expression may be regulated by the canonical Th2 cytokines, IL-4 or IL-13, both of which signal through their common receptor, IL-4Rα.

IL-4 upregulates hResistin expression

To examine whether IL-4 can induce hResistin expression, we isolated and cultured h*Retn*Tg^{+} bone marrow derived macrophages (BMMac) and stimulated these cells for 16 hours at 37ºC with recombinant IL-4. After 16 hours, supernatant was removed and cells were lysed to extract RNA. hResistin expression was significantly increased following IL-4 stimulation (40ng/mL)

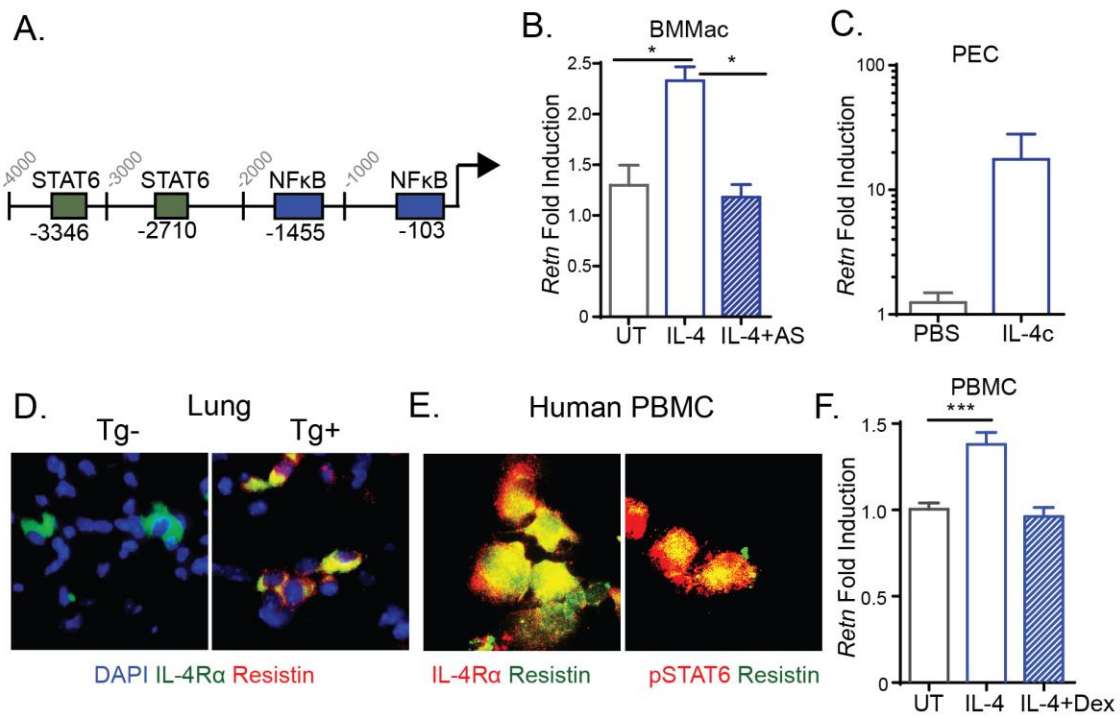

Figure 16: IL-4 and STAT6 signaling induce hResistin Expression. (A) (C) MatInspector analysis of the regulatory region upstream of the h*Retn* promoter. Matrix similarity ≥0.85. (D-E) h*Retn* mRNA was quantified *in vitro* by IL-4 treatment of BMMacs ± STAT6 inhibitor (AS) or *in vivo* by injection of IL-4c. (F-G) Co-expression of h*Retn* and IL-4Rα or pSTAT6 was examined by IF of *Nb*-infected lung tissue or human PBMC. (H) IL-4 induced h*Retn* was validated in human PBMC. Data (mean±SEM) are representative of 2 separate experiments (n=4).

(Figure 16B). To examine whether IL-4 also increases hResistin expression *in vivo*, we used IL-4 complex (IL-4c), which extends the bioactivity of IL-4 *in vivo* [181]. Briefly, recombinant IL-4 and anti-IL-4 antibody were mixed at a 2:1 molar ratio and injected intraperitoneally into h*Retn*Tg^{+} mice on day 0 and day 2. At day 4, peritoneal cells were recovered and lysed to examine hResistin expression by real-time PCR. Compared to h*Retn*Tg^{+} mice treated with isotype control, IL-4c treated mice had a 10-fold increase in hResistin expression (Figure 16C), confirming that IL-4 does regulate hResistin expression.

Human resistin expression is STAT6 dependent

Following IL-4 or IL-13 binding to IL-4Rα, STAT6 is phosphorylated, dimerizes and translocates to the nucleus, where it induces production of AAMac genes. To test whether hResistin expression is mediated by STAT6, h*Retn*Tg^{+} BMMacs were treated with IL-4 ± AS1517499, a STAT6 inhibitor, and incubated for 16 hours at 37ºC. Following incubation, cells were recovered and RNA extracted for real-time PCR. Cells stimulated with IL-4 expressed more hResistin, but this was completely abrogated in cells treated with IL-4+AS1517499 (Figure 16B). This suggests that hResistin expression is dependent on STAT6 phosphorylation and activity. Supportive of this, immunofluorescence of *Nb*-infected lung cells revealed co-expression of IL-4Rα and hResistin (Figure 16D). To confirm that this also occurs in human cells, we stimulated PBMC with recombinant IL-4, cytospun cells and did immunofluorescence staining. This

revealed co-expression of hResistin with IL-4Rα and pSTAT6 (Figure 16E). Furthermore, when PBMC are stimulated with IL-4 for 16 hours at 37ºC and recovered for real-time PCR, we show that IL-4 can induce hResistin expression, but this was abrogated by the immunosuppressant dexamethasone (Figure 16F).

Discussion

Although TLR4 signaling is essential for LPS-mediated hResistin expression *in vitro*, it is unclear what mechanisms regulate hResistin transcription in helminth infection. When TLR4$^{-/-}$/h*Retn*Tg^{+} mice were infected with *Nb*, hResistin expression was similar to TLR4$^{+/+}$/h*Retn*Tg^{+} mice. This suggests that hResistin expression is not dependent on TLR4 during helmninth infection. This removes the possibility that hResistin expression is stimulated by symbiotic bacteria introduced into the host by the helminth or translocated bacteria as a result of tissue damage. This may also remove some helminth antigens that bind to and signal through TLR4, such as the *Acanthocheilonema viteae* antigen, ES-62 [59], *F. hepatica* fatty acid binding protein [60], or *Onchocerca volvulus* activation associated protein-1 [182].

While previous studies have shown that NF-κB and C/EBP as transcriptional regulators of hResistin, our data is the first to support IL-4 and STAT6 as regulators of hResistin expression. IL-4 and STAT6 signaling are essential for AAMac polarization, RELMα production and proper immunity against helminths. Our data confirms that hResistin is regulated by the same mechanisms that regulate murine RELMα, and likely explains why hResistin and murine RELMα have similar expression patterns and are made by the same cell types. However, further studies would need to confirm that IL-4/STAT6 are essential for hResistin

expression during helminth infection. This could be accomplished with the use of IL-4$^{-/-}$ or STAT6$^{-/-}$ mice.

IL-4/STAT6 signaling could also explain the increased expression of hResistin in other Th2 settings, including asthma or wound healing. Indeed, in schoolchildren with atopic asthma, hResistin expression is increased in the serum [183]. In addition, hResistin expression is elevated in patients with chronic obstructive pulmonary disease (COPD) [184]. hResistin is also increased in blunt chest trauma and acute lung injury [185], where it may increase the severity of lung injury though neutrophil extracellular trap formation [121]. In helminth infection, hResistin expression is detrimental to the host, but future studies would need to examine whether hResistin is beneficial or detrimental to the host in other Th2 settings.

Aside from IL-4 and STAT6 signaling, helminth antigen can also induce hResistin expression in human PBMC [34]. However, the mechanism or specific helminth antigens that induces hResistin expression are unknown. To date, there are no well characterized *Nb* excretory or secretory proteins and any potential receptors for *Nb*, but other helminth ES products have been identified and do regulate the host's immune response. Furthermore, other helminths actually generate and releases exosomes that contain proteins (*F. hepatica*) or microRNAs (*H. polygyrus*, *Litomosomoides sigmodontis*) to counteract the host's immune response [186, 187]. In addition, helminth tegument contains chitin, a biopolymer

that is known to induce AAMac gene transcription. Thus, there are many potential pathways for hResistin transcriptional regulation directly induced by helminth antigens.

CHAPTER FOUR – Human Resistin Protects Against Endotoxic Shock by Blocking LPS-TLR4 Interaction and Promoting Anti-Inflammatory Cytokines

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Abstract

Helminths trigger multiple immunomodulatory pathways that can protect from sepsis. Human resistin is an immune cell-derived protein that is highly elevated in helminth infection and sepsis. However, the function of hResistin in sepsis, or if resistin influences helminth protection against sepsis, are unknown. Employing hResistin-expressing transgenic mice (h*Retn*Tg^{+}), or recombinant hResistin, we identify a therapeutic function for hResistin in lipopolysaccharide (LPS)-induced septic shock. hResistin also enhanced helminth-induced immunomodulation, with increased survival of *Nippostrongylus brasiliensis (Nb)*-infected h*Retn*Tg^{+} mice following a fatal LPS dose compared to naïve mice or *Nb*-infected h*Retn*Tg^{-} mice. Employing immunoprecipitation assays, h*RETN*Tg^{+}*Tlr4*$^{-/-}$ mice, and human immune cell cultures, we demonstrate that hResistin binds the LPS receptor Toll-like Receptor 4 (TLR4) and triggers a switch from pro-inflammatory to anti-inflammatory signaling. Together, our studies identify a critical role for hResistin in blocking LPS function with important clinical significance in helminth-induced immunomodulation and sepsis.

Introduction

In the United States, 750,000 people are diagnosed with sepsis each year, with a mortality rate of 30% [188]. Sepsis pathogenesis is exacerbated by the inflammatory response to the pathogen-associated molecular patterns ligand lipopolysaccharide (LPS), a main component of gram negative bacterial cell walls. LPS binds to toll-like receptor 4 (TLR4) and induces an NF-κB dependent inflammatory cascade resulting in excessive production of TNFα and IL-6. These proinflammatory cytokines are initially beneficial in bacterial killing, but eventually damage the host's cells and tissues. For instance, excessive production of TNFα causes endothelial cell injury, leading to vascular permeability, low blood pressure, and organ failure [189]. Recent studies have targeted this innate inflammatory pathway as a potential treatment of sepsis. *Tlr4*$^{-/-}$ mice or mice treated with anti-TLR4 antibodies were resistant to *E.coli*-induced sepsis [190]. Nonetheless, clinical trials with anti-TLR4 antibodies or TLR4 antagonists have not been successful [191-193], and treatment for sepsis is currently limited to antibiotics and supportive care. Epidemiology studies show that sepsis can result from other infections, including gram-positive bacteria, viruses or fungi, which also stimulate an excessive inflammatory response [188]. Given the lack of specific treatments for sepsis and the high incidence of sepsis in multiple infections, it is critical to identify new regulatory pathways that mitigate the sepsis pathogenesis.

Helminth infections trigger multiple immunomodulatory pathways that protect against inflammatory diseases including inflammatory bowel disease and sepsis [194, 195]. Helminths can cause debilitating symptoms including anemia, intestinal blockage and malnutrition, therefore, identifying the specific pathways that are protective against inflammatory diseases is necessary to avoid the pathogenic consequences of helminth infection. Recent studies have shown that chronic infection with filarial nematode *Litomosoides sigmodontis* protected mice from fatal sepsis through the TLR2-dependent activation of macrophages and the inhibition of proinflammatory cytokines [75]. Additionally, helminth antigens such as *Acanthocheilonema viteae* ES-62 and *Fasciola hepatica* fatty acid binding protein, bind to TLR4 and reduce proinflammatory cytokine production [59, 60].

hResistin, a member of the resistin-like molecule family of secreted proteins, is expressed in many inflammatory diseases, such as diabetes [84], atherosclerosis [108] and rheumatoid arthritis [152]. More recently, elevated hResistin expression has been reported in infectious settings, including helminth, bacterial and viral infection [120, 121], and in sepsis [113]. Several studies have shown that LPS promotes high level expression hResistin *in vitro* and *in vivo* [122, 124, 153]. Functionally, hResistin increased the production of proinflammatory cytokines and formation of neutrophil extracellular traps in the lungs and exacerbated acute LPS-induced lung injury [121]. In clinical reports, increased circulating resistin has been correlated with the severity of sepsis leading to the suggestion of resistin as a diagnostic marker of sepsis [110, 113-116]. However,

mechanistic studies investigating the function of hResistin in sepsis have not been performed.

Here, we use transgenic mice that express hResistin (h*Retn*Tg^{+}) to study the function of hResistin in a mouse model of sepsis. LPS injection resulted in significantly increased circulating hResistin in the h*Retn*Tg^{+} mice, which were critically protected against fatal LPS-induced inflammation compared to littermate control h*Retn*Tg^{-} mice. Further, therapeutic treatment with recombinant hResistin protected C57BL/6 mice against LPS-induced mortality. We tested if hResistin contributed to helminth-induced immunomodulation, and observed that hResistin enhanced the protective effects of *Nippostrongylus brasiliensis* infection in LPS-induced endotoxic shock. Mechanistically, hResistin inhibited LPS-induced neutrophilia, and promoted a shift from a pro-inflammatory signaling (e.g. TNFα, NF-κB) to an anti-inflammatory pathway (e.g. IL-10, STAT3). hResistin has been proposed to bind TLR4, however, more recent studies have questioned this interaction [132]. Using immunoprecipitation assays, *Tlr4*$^{-/-}$ mice and human immune cell assays, we provide direct evidence that hResistin binds TLR4 and prevents subsequent LPS function. To test the effect of hResistin on TLR4 signaling *in vivo*, we generated h*Retn*Tg^{+} mice on a *Tlr4*$^{-/-}$ background, and observed that the anti-inflammatory effects of hResistin were TLR4-dependent. Together, our studies identify a previously unrecognized role for hResistin in blocking LPS function and promoting anti-inflammatory pathways with important clinical implications for helminth-induced immunomodulation and sepsis.

Materials and Methods

Mice

Resistin transgenic mice were generated as previously described [124]. Briefly, the human resistin gene, along with 21,300 bp upstream and 4,248 bp downstream of the human resistin start site, were engineered through a bacterial artificial chromosome (BAC). *Nippostrongylus brasiliensis* life cycle was maintained in Sprague-Dawley rats purchased from Harlan Laboratories, as previously described [143]. Mice were anesthetized with isoflurane and injected subcutaneously with 500 L3 larvae. Eggs in the feces of infected mice were counted using a McMaster counting chamber on days 6-9 following infection. On day 14 post Nb infection, mice were injected with 12mg/kg (females) or 20mg/kg (males) LPS followed by serum collection by retro-orbital bleeding 6 hours after that. For LPS-induced sepsis model, mice were injected with 0.05mg/kg LPS (Sigma), then with 12mg/kg LPS 12 hours later. Mice were monitored at least twice a day and euthanized according to humane endpoints. Body temperature was measured by rectal thermometer (Braintree Scientific). All animals in the experiment were age-matched (6-8 weeks old), gender-matched and housed in a specific pathogen free facility.

Vascular Permeability Assay

Evan's blue dye is a common method to measure vascular permeability because Evan's blue binds to serum albumin. Under homeostatic conditions,

endothelial cells do not permit the extravasation of albumin and Evan's blue into the tissue. When the vasculature is vulnerable, as in endotoxic shock, albumin and Evan's blue can leave the blood vessel and become incorporated into the tissue. Mice were anesthetized by isoflurane and injected by retro-orbital intravenous injection with 200µL of 0.5% Evan's Blue dye in PBS (Sigma). After 10 minutes, mice were euthanized by CO_2 and perfused with 20mL PBS. Tissue was then excised, weighed and placed in a tube with 500µL N,N-dimethylformamide (Sigma). After 24 hour incubation of tissue in a 55ºC heatblock, extracted Evan's blue was measured at 610nm. This was compared to a standard curve and calculated based on weight of the tissue.

Human PBMC

Human buffy coat was purchased from Zen-bio. Buffy coat was overlaid on top of Histopaque-1077 and spun at 700g at 25˚C with no brake and PBMCs were recovered from the interphase. PBMCs were then plated on a 96-well plate and stimulated with mammalian-derived human resistin (1ug/mL, Life Span Biosciences) or media for 24 hours. Supernatant was removed for cytokine analysis, followed by addition of LPS (100ng/mL, Invivogen) for the next 24 hours. Supernatant was then recovered after LPS stimulation and cytokines were quantified by ELISA.

Binding Assay and Flow Cytometry

Lung tissue was diced, incubated in 30µg/mL DNAse I and 1mg/mL collagenase/dispase for 25 minutes in 37˚C shaking incubator and passed through a 70 µm cell strainer to generate single cell suspension. ACK lysis was used to remove red blood cells. Dissociated lung cells were washed with FACS buffer and incubated for 1 hour at 4˚C with 0.5µg recombinant resistin (Peprotech) or PBS for control. Cells were then washed twice in FACS buffer, incubated with Fc block (10ug/mL αCD16/32, 5'), stained with biotinylated αhResistin (Peprotech, 30'), followed by detection with BV605-conjugated streptavidin (BD Biosciences, 30'), F4/80, SiglecF, CD4, Ly6C, CD11b, CD11c, Ly6G, MHCII and CD3. For competitive binding assay, cells were first incubated with 0.5µg recombinant hResistin for 30 minutes at 4˚C. Cells were then washed in FACS buffer, then incubated with 0.5µg LPS and incubated for 30 minutes at 4ºC. Cells were washed in FACS buffer, incubated with Fc block, and stained with BV605-conjugated streptavidin and other primary antibodies (F4/80, SiglecF, CD4, CD11b, CD11c, CD3, MHCII, Ly6G and Ly6C). For human PBMC binding assay, the same protocol was used with CD14, CD16, CD3, CD66b, CD19, CD11b and HLA-DR anti-human antibodies.

Following LPS injections, peritoneal cavity was washed and PECs recovered in 5mL PBS. Cells were then washed in FACS buffer, incubated with Fc block, and stained with the following antibodies: F4/80, SiglecF, CD4, CD115,

CD11b, CD11c, CD3, MHCII, Ly6G and Ly6C. All samples were run on the BD LSRII and analyzed on FlowJo (v10).

Cytokine Quantification

For sandwich ELISA, Greiner 96-well high binding plates were coated with primary antibody to the cytokine (Peprotech, eBioscience) overnight at 4ºC. After blocking the plates with 5% NCS in PBS for 1 hr, sera or cell culture supernatant was added at various dilutions and incubated at room temperature for 2 hr. Detection of cytokine was done with biotinylated antibody for 1 hr. Following incubation with streptavidin-peroxidase (Jackson Immunobiology) for 1 hour, the peroxidase substrate TMB (BD) was added followed by 2N H2SO4 as a substrate stop. Optical density (OD) was captured at 450 nm. Samples were compared to serial-fold dilution of recombinant protein. For luminex, inflammatory cytokine kits (eBioscience) were run according to manufacturer's instructions and quantified on Luminex MagPix (Luminex Corp.). For cytokine bead array, cytokine kits from BD Bioscience were run according to manufacturer's instructions on BD LSRII and analyzed using FCAP Array Software (BD Biosciences).

Pull-down Assay

His-tagged MBP, human TLR4 and human CAP1 were added to nickel-nitrilotriacetic acid-agarose (Invitrogen) in binding buffer (50mM NaH_2P0_4, 500mM NaCl, 10mM Imidazole, pH 8.0) and mixed end-over-end for 1 hour at 4°C. Supernatant was removed after spinning at 1000g, 3min and human Resistin from

E. coli (Peprotech) or human Resistin from 293T cells (LifeSpan Biosciences) was added in binding buffer, and mixed end–over-end for 1 hour at 4°C. Mixture was spun at 1000g for 3min, and supernatant was removed. The beads were then washed with wash buffer (50mM NaH_2P0_4, 500mM NaCl, 20mM Imidazole, pH 8.0) and the protein were eluted with elution buffer (50mM NaH_2P0_4, 500mM NaCl, 250mM Imidazole, pH 6.0). His-MBP, His-human TLR4 and His-CAP1 were detected by His antibody (Abcam), human Resistin was detected by Resistin antibody (from Lazar lab) on western blot.

Western Blot

Peritoneal cells were lysed in RIPA buffer (150mM Sodium chloride, 1%Triton X-100, 1% Sodium deoxycholate, 0.1% Sodium dodecyl sulfate, 50mM Tris-HCl and 2mM EDTA). Proteins were boiled in loading buffer (Bioland Scientific LLC), denatured then separated with SDS-PAGE gels, transferred to PVDF membranes (Millipore), and blocked with 5% BSA (Sigma) or 5% milk by standard protocol. The phosphorylation level or proteins were detected with the following antibody: anti-pSTAT3, anti-pTBK1, anti-IκBα, anti-βactin (Cell signaling Technology), then incubated with anti-rabbit or mouse HRP-conjugated IgG (Cell signaling Technology). Finally, proteins were examined by ECL (Pierce Chemical Co.) and exposed with X-ray film.

Structural Predictions

The structural analysis of the TLR4-resistin interactions was performed with the structure of human TLR4-MD2-LPS complex (PDB code: 3FXI) and human resistin built by homology modeling. The sequence of human resistin was downloaded from the universal protein sequence (Uniprot, Entry no. Q9HD89). Then, this sequence was used in SWISS-Model Server for homology modeling to find a structural template. This server found mouse resistin (PDB code: 1RFX) as the template with maximum sequence identity of 57.61%. Therefore, mouse resistin 3D structure was used to build the structural model for human resistin using the server. The trimer of human resistin was used to form the human TLR4-resistin complex by manually docking the resistin trimer to the TLR4 dimer from the TLR4-MD2-LPS complex based on shape-fitting. Similarly, the mouse TLR4-resistin complex was built by docking mouse resistin (PDB code: 1RFX) to the mouse TLR4-MD2-LPS complex (PDB code: 3VQ2). PDBePISA server was used to confirm the feasibility of all the models and detailed protein-protein interactions. Pymol software was used for all modeling manipulation.

Statistical Analysis

All statistics were generated on GraphPad Prism using where appropriate log rank test, student t-test, one-way ANOVA or two-way ANOVA. *, $p \leq 0.05$, **, $p \leq 0.01$, ***, $p \leq 0.001$, ****, $p \leq 0.0001$.

Ethics Statement

All protocols for animal use and euthanasia were approved by the University of California, Riverside Institutional Animal Care and Use Committee (https://or.ucr.edu/ori/committees/iacuc.aspx; protocol A-20120023B and A-20150028E) and were in accordance with the National Institutes of Health Guidelines. Animal studies are in accordance with the provisions established by the Animal Welfare Act and the Public Health Servies (PHS) Policy on the Humane Care and Use of Laboratory Animals. Human buffy coat (~60mL of concentrated leukocytes and some erythrocytes) were collected from healthy donors with signed informed consent by Zen-bio, Inc. Isolation of PBMC and assays were performed at UCR with the approval of the University of California, Riverside Institutional Review Board (HS-13-134 and HS-14-048).

Results

Resistin protects against LPS-induced septic shock in h*Retn*Tg^{+} mice

In the United States, approximately 750,000 people are diagnosed with sepsis each year, with 25-30% of these patients eventually succumbing to death. Given that hResistin is elevated in septic patients, we investigated the function of hResistin in sepsis. By using transgenic mice that express human resistin but are deficient in murine resistin (h*Retn*Tg^{+}), we can examine the immunomodulatory function of resistin *in vivo*. In these mice, the human resistin gene, along with its entire regulatory region, was inserted using a bacterial artificial chromosome. Previous characterization of these mice revealed that hResistin is expressed in these mice similarly to human. Human resistin expression is increased following LPS induction and helminth infection, where it is functional and leads to increased insulin resistance and exacerbated helminth infection, respectively [124, 196]. As a model of LPS-induced sepsis, we injected two doses of LPS 12 hours apart. First, mice were challenged with a low dose of LPS (0.05mg/kg) to induce hResistin expression and mimic common comorbidities associated with sepsis, such as diabetes, hypertension or other inflammatory diseases. This was followed by a second fatal dose of LPS (Figure 17A). At 6 and 18 hours post injection, serum was collected by retro-orbital bleeding for cytokine analysis, and mice were monitored for symptoms of endotoxic shock over the next 180 hours. Mice were euthanized based on humane end points. Following LPS injection, hResistin

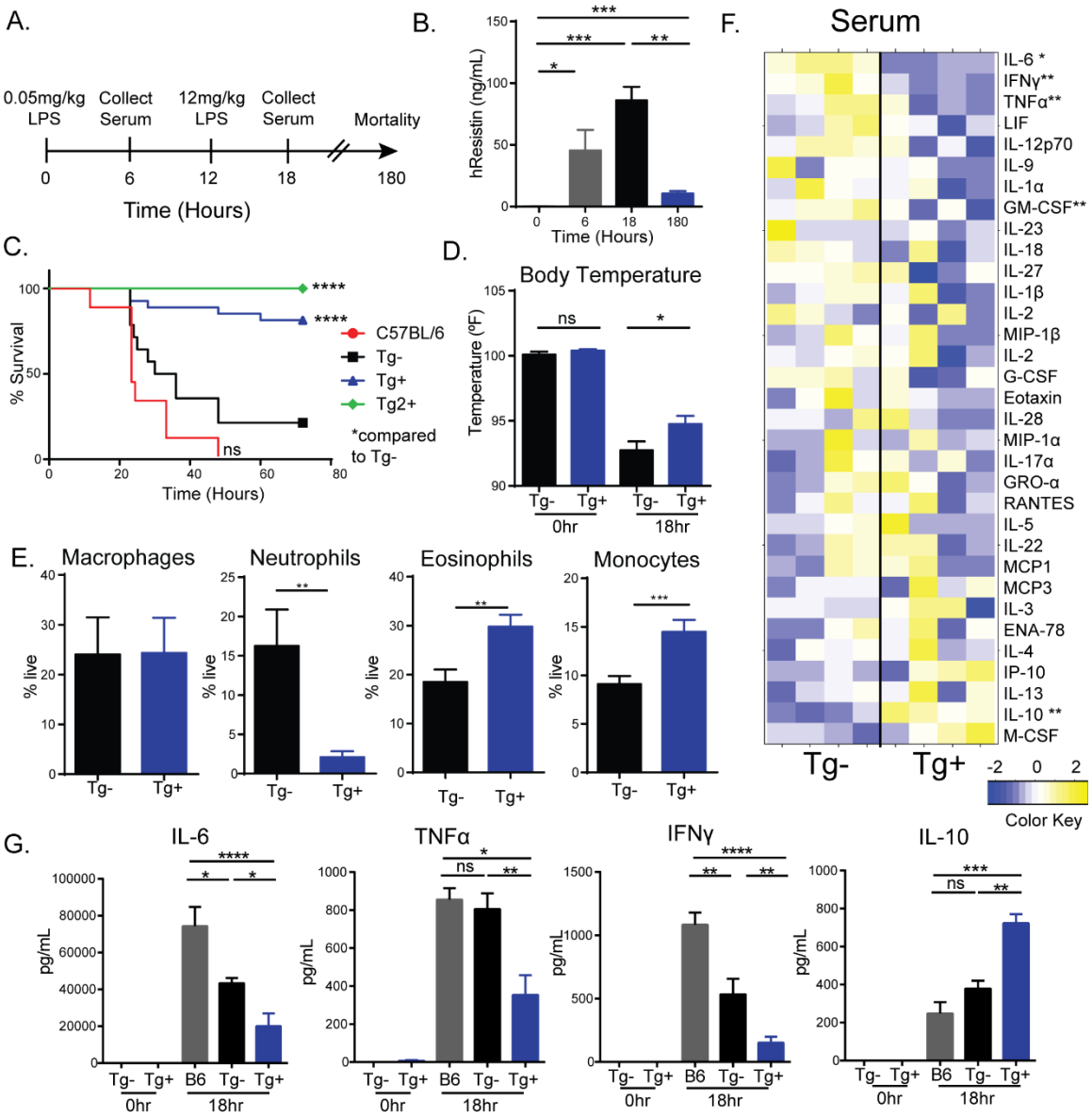

Figure 17: Human resistin protects against endotoxic shock. (A) Experimental design of LPS-induced sepsis model using h*Retn*Tg$^+$ and control h*Retn*Tg$^-$ mice. (B) Serum hResistin expression during LPS-induced sepsis is measured by ELISA. (C) Survival of C57BL/6, h*Retn*Tg$^-$ and h*Retn*Tg+ mice following LPS injections (n=13-17). (D) Body temperature of h*Retn*Tg$^-$ and h*Retn*Tg$^+$ mice at 0 hour and 18 hours post LPS injection. (E) PECs were recovered at 24 hours and analyzed by flow cytometry. (F) Heatmap of serum cytokines at 18 hours were measured by luminex. (G) Expression of circulating proinflammatory and anti-inflammatory cytokines at 0 hours and 18 hours. Data (mean±SEM, n=3 per group) are pooled from 3 separate experiments.

expression is quickly induced and prolonged, as it is significantly increased at just 6 hours and continues to be elevated even 180 hours post injection (Figure 17B), confirming previous reports of LPS-induced hResistin expression *in vivo* [124]. The expression of hResistin protected these mice from LPS-induced mortality, with only ~20% of these mice dying. This is in contrast with the ~80% of h*Retn*Tg^{-} mice succumbing to death and 100% mortality in C57BL/6 mice (Figure 17C). One symptom of endotoxic shock is the development of hypothermia, but h*Retn*Tg^{+} mice develop less hypothermia than h*Retn*Tg^{-} mice (Figure 17D), suggesting that hResistin expression lessened the physiologic symptoms of septic shock.

In the transgenic mice used in these experiments, the human resistin gene along with its regulatory region (~25000 bp total) was inserted randomly in the X chromosome. The hResistin gene was excised using *Not*I/*Bgl*II into pGL3-basic bacterial artificial chromosome and injected into fertilized C57BL/6 mouse oocytes [124]. h*Retn*Tg^{+} mice were identified by PCR and backcrossed onto mResistin$^{-/-}$ mice on a C57BL/6 background. Genetic analysis of the h*Retn*Tg^{+} mice revealed that hResistin was inserted in an intron of the X-linked IL-1 receptor accessory protein 2 (*il1rapl2*) gene. However, there are several reasons why insertion of h*Retn* into the *ilrapl2* gene should not affect our experiments. First, while resistin was inserted into this intron, it is >100,000 bp away from the closest exon, making it unlikely that it is interfering with expression of IL1RAPL2. Second, the h*Retn*Tg^{+} mice used in these experiments are heterozygous for human resistin, thus only one allele of *il1rapl2* would be affected. Finally, IL1RAPL2 is predominantly

expressed in the cerebral cortex, and not detectable in any other tissues [197]. Nevertheless, to ensure that any phenotype we see in the h*Retn*Tg^{+} mice is not due to disruption of *ilrapl2*, we duplicated our studies in a second transgenic line (h*Retn*Tg2^{+}), where resistin may be inserted in several possible positions (Table 5). Similar to our original transgenic line, the h*Retn*Tg2^{+} mice have an improved outcome of sepsis, with 100% survival.

To examine whether hResistin leads to any differences in cell populations, we collected PECs at 36 hours and analyzed cells by flow cytometry (Figure 17E). In un-challenged mice, macrophages, T cells and eosinophils make up the majority of the cells in the peritoneum, with negligible numbers of monocytes or neutrophils. Interestingly, there are more eosinophils in un-challenged h*Retn*Tg^{+} mice, suggesting an increase in Th2 immunity and an anti-inflammatory homeostatic state. Following LPS injection, monocytes, eosinophils and neutrophils are significantly increased in the peritoneal cavity, while T cell and macrophage populations decrease. Compared to h*Retn*Tg^{-} mice, h*Retn*Tg^{+} mice had more eosinophils and monocytes.

Luminex assays were used to quantify 33 cytokines in the serum of naïve and LPS-challenged mice at 18 hours (Table 6). Heatmap of all 33 cytokines revealed decreases in proinflammatory cytokines in h*Retn*Tg^{+} mice, such as TNFα, IL-6, IL-12, IL-1α and GM-CSF (Figure 17F), compared to h*Retn*Tg^{-} mice.

Chromosome	Position	Location
1	93446321	Intron of *Hdlbp*
1	165624851	Intron of *Mpzl1*
3	33231313	Non-coding region
5	57598562	Non-coding region
6	28049256	Intron of *Grm8*
7	51435070	Non-coding region
7	63552133	Intron of *Otud7a*
10	109378627	Non-coding region

Table 5: Possible insertion sites for h*Retn*Tg2^{+}.

	Naive		LPS-challenged		
Cytokine	**Tg-**	**Tg+**	**C57BL/6**	**Tg-**	**Tg+**
IFNγ	ND	ND	1236±179.4	459.6±98.59	152.4±46.84
IL-12p70	ND	ND	148.0±4.2	89.4±4.8	49.9±13.2
IL-13	ND	ND	38.3±3.4	30.7±3.3	17.5±8.4
IL-1β	4.8±1.5	5.5±2.7	176.0±24.5	143.5±34.8	112.8±48.0
IL-2	ND	ND	25.5±8.0	76.03±33.7	34.3±17.3
IL-4	2.1±1.7	2.2±1.1	24.3±1.1	18.0±2.2	14.4±4.0
IL-5	ND	ND	474.1±86.3	201.5±20.6	194.7±27.2
IL-6	11.2±6.6	17.0±9.8	78647±5830	42149±2913	23779±8731
TNFα	0.3±0.2	7.5±2.9	911.5±97.3	749.7±79.7	309.3±123.6
GM-CSF	3.5±3.0	6.6±6.6	157.3±6.0	132.8±5.3	74.03±15.2
IL-18	ND	ND	3658±136.2	15587±2520	11194±3065
IL-10	ND	ND	248.1±58.8	348.9±23.33	722.8±47.2
IL-17a	4.0±2.6	5.9±3.0	108.0±12.6	54.08±11.8	46.23±13.8
IL-22	5.9±5.9	ND	48239±3259	24175±6096	18114±6877
IL-23	ND	9.4±9.4	165.5±19.6	140.6±26.8	104.5±16.0
IL-27	7.3±1.0	10.05±1.8	404.6±17.0	137.2±33.7	63.4±14.5
IL-9	ND	ND	180.5±10.5	121.7±26.0	91.11±15.4
GRO-α	34.0±8.1	24.2±1.6	95373±6020	48714±10385	48984±11858
IP-10	6.1±0.3	7.2±1.6	2209±212.7	1171±177.6	1358±259.1
MCP-1	ND	ND	122252±11059	82231±13726	71782±20250
MCP-3	38.7±11.2	35.62±7.8	4226±99.8	3530±475.4	3571±469.3
MIP-1α	ND	ND	1123±97.7	248±76.1	153.9±30.1
MIP-1β	ND	ND	4253±310.6	1310±345.3	862.3±221.3
MIP-2	27.92±5.8	31.4±6.3	6932±585.3	3262±469.1	2352±793.7
RANTES	ND	ND	4376±133.9	2327±404.9	1622±382.4
Eotaxin	66.6±13.6	76.23±10.5	3879±98.1	2708±341.2	2113±398.9
IL-28	148.3±31.8	155.5±32.1	539.6±215.5	506.2±215.6	436.3±159.8
IL-3	3.4±0.6	3.9±0.5	3.8±0.2	3.2±0.5	3.6±0.4
G-CSF	ND	ND	3520±442.2	2717±386.4	2672±301.7
M-CSF	ND	ND	7.0±0.8	23.8±3.5	38.7±11.4
LIF	ND	ND	604.7±52.81	311.8±69.8	191.9±58.7
IL-1α	47.2±3.4	49.0±1.7	124.0±4.6	147.3±16.2	114.0±14.7
ENA-78	164.4±86.5	195.1±77.5	3713±360.2	3623±292.8	3054±667.8

Table 6: Cytokines regulated by hResistin during sepsis. C57BL/6, h*Retn*Tg^{-} and h*Retn*Tg^{+} mice were challenged with two doses of LPS i.p. and serum was collected at 18 hours. Cytokines (pg/mL) in the serum were measured by luminex.

On the other hand, anti-inflammatory and Th2 cytokines, IL-10, IL-13 and IL-4, are all increased in h*Retn*Tg^{+} mice. Following endotoxic shock, IL-6, TNFα, IFNγ and IL-10 are all significantly increased (Figure 17G). In comparison to h*Retn*Tg^{-} and C57BL/6 mice, h*Retn*Tg^{+} mice had a decrease in the proinflammatory cytokines, TNFα, IL-6 and IFNγ, suggesting that hResistin inhibits LPS-induced expression of these cytokines. In addition, there is also immune suppression, as we saw an increase in the anti-inflammatory cytokines, IL-10, IL-4 and IL-13 production in h*Retn*Tg^{+} mice. The slight protection found in h*Retn*Tg^{-} (~20% survival) compared to C57BL/6 mice (0% survival) is likely due to the decrease in IL-6 and IFNγ in h*Retn*Tg^{-} mice.

Altogether, this data suggests that hResistin protects against sepsis in two ways: first, by creating an anti-inflammatory environment by increasing IL-10 production and eosinophil numbers and second, by blocking the production of the proinflammatory cytokines directly responsible for septic shock. The increase of IL-10 may be due to the increase in eosinophil numbers, as eosinophils have been shown to be a significant source of IL-10 [198]. To our knowledge, this is the first report showing that hResistin reduces mortality during endotoxic shock and contrasts with previous reports that suggested that hResistin contributes to sepsis.

Recombinant hResistin can protect against endotoxemia

To confirm that hResistin directly reduces mortality in sepsis, C57BL/6 mice were treated with recombinant human resistin, recombinant mouse resistin or PBS

prior to LPS challenge and monitored for 180 hours for symptoms of shock (Figure 18A). While all of the C57BL/6 mice treated with PBS succumbed to LPS-induced sepsis, mice treated with recombinant human resistin were completely protected from sepsis (Figure 18B). On the other hand, murine resistin administration only protected 50% of the mice, although this difference was not significant compared to the PBS-treated group. This suggests that murine and human resistin have some overlapping functions in sepsis, but human resistin treatment completely protects against sepsis-induced mortality. In these studies, recombinant resistin was derived from bacteria (*E. coli*) and therefore may be contaminated with low levels of endotoxin. However, these results were not due to endotoxin tolerance, as human resistin protected against endotoxic shock compared to murine resistin, despite being generated with the same methodology and having similar levels of endotoxin contamination. Mice treated with hResistin were also protected from sepsis-associated symptoms, including hypothermia (Figure 18C) and vascular permeability of the lung (Figure 18D). Increase in vascular permeability, or leakiness, is a contributing factor for sepsis-induced organ injury or failure. In this assay, Evan's blue dye is injected intravenous into mice, and high vascular permeability results in more leakage of Evan's blue into the tissue. Since hResistin treated mice have less permeable vasculature, hResistin reduces lung injury caused by hypoperfusion.

Cytokines were measured at 6 hours post LPS injection, which revealed a decrease in IL-6 and TNFα in mice treated with hResistin or mResistin (Figure

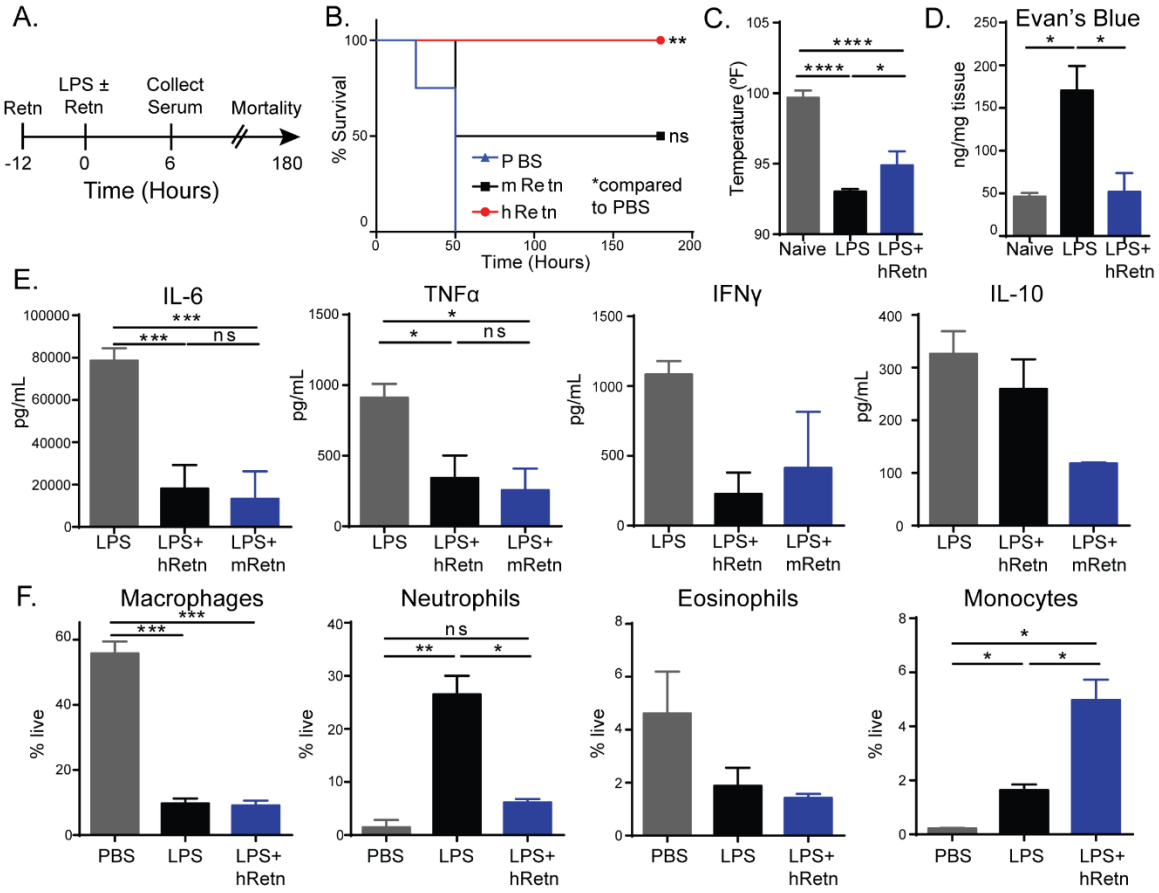

Figure 18: Therapeutic administration of hResistin during septic shock. (A) Experimental design of therapeutic resistin in sepsis model. (B) Survival of C57BL/6 mice treated with PBS or resistin prior to LPS injection. (C) Body temperature of mice at 0 hour and 6 hours post LPS injection. (D) Vascular permeability in the lung tissue was measured by Evan's blue intravenous injection. (E) Serum cytokines at 18 hours were measured by luminex. (F) PECs were recovered at 24 hours and analyzed by flow cytometry. Data (mean±SEM, n=5 per group) are representative of 2 separate experiments.

18E), again suggesting that the resistin homologs might have some overlapping functions. PECs were also recovered at 24 hours and analyzed by flow cytometry. Mice treated with hResistin reduced LPS-induced neutrophilia in the peritoneal cavity, but had increased inflammatory monocytes (Figure 18F). Since neutrophils contribute to sepsis progression through the production of reactive oxygen species and proinflammatory cytokines [199], the reduction of neutrophils and proinflammatory cytokines in hRetn-treated mice likely contributes to protection against endotoxic shock. These results could have major implications for novel treatment options for patients suffering from sepsis, as there are no treatment options for septic patients besides fluids and supportive care.

Nb-induced hResistin protects against sepsis

Given that filarial nematodes and soil-transmitted helminths are able to increase circulating hResistin expression [196], we hypothesized that helminth-induced hResistin could also protect against endotoxic shock. As a model for helminth infection, we use *Nippostrongylus brasiliensis* (*Nb*), a natural hookworm of rodents. *Nb* resides in the lungs for the first 48 hours before traversing to the small intestine to reproduce. Mice typically expel all worms by day 10 post infection. h*Retn*Tg^{+} and h*Retn*Tg^{-} Mice were injected with a fatal dose of LPS at day 14 post *Nb* infection and monitored for symptoms of septic shock for 48 hours (Figure 19A). Confirming our previous findings, h*Retn*Tg^{+} mice had increased egg burden at day 7 post *Nb* infection compared to h*Retn*Tg^{-} mice (Figure 19B).

Helminth-induced hResistin expression also led to complete protection from LPS-induced endotoxic shock, compared to LPS-only h*Retn*Tg^{+} mice or *Nb*+LPS h*Retn*Tg^{-} mice (Figure 19C). However, alternative methods of protection such as *Nb*-induced Treg infiltration and IL-10 expression partially protected h*Retn*Tg^{-} mice from septic shock (~50% of h*Retn*Tg^{-} *Nb*+LPS mice survive compared to 100% mortality in h*Retn*Tg^{-} LPS alone mice). To examine cytokine production, we performed a cytokine bead array on serum following *Nb* infection and LPS challenge. Both h*Retn*Tg^{-} and h*Retn*Tg^{+} mice express similar amounts of IL-10, MCP1 and IFNγ, however, h*Retn*Tg^{+} mice express less TNFα and IL-6 (Figure 19D). Although there were no significant differences in IL-10, this may be due to *Nb* inducing immense amounts of IL-10 that any IL-10 induced by resistin is negligible.

At 48 hours, peritoneal cells were recovered and cell populations were analyzed by flow cytometry. While there were no differences in macrophages or eosinophils, neutrophils and monocytes were increased in h*Retn*Tg^{+} mice (Figure 19E). Unlike the LPS only sepsis model, hResistin did not increase the frequency of eosinophils, possibly due to *Nb* infection already dramatically increasing eosinophil numbers. Peritoneal cells from *Nb*+LPS treated h*Retn*Tg^{-} and h*Retn*Tg^{+} mice were also flash frozen, lysed and analyzed by Western Blot to examine the activity of anti-inflammatory signaling pathways. We found an increase in IL-10 associated pathways, pSTAT3 and pTBK-1, with an increase in IκBα, an inhibitor of NF-κB signaling in h*Retn*Tg^{+} mice (Figure 19F). Thus, even though *Nb*-induced

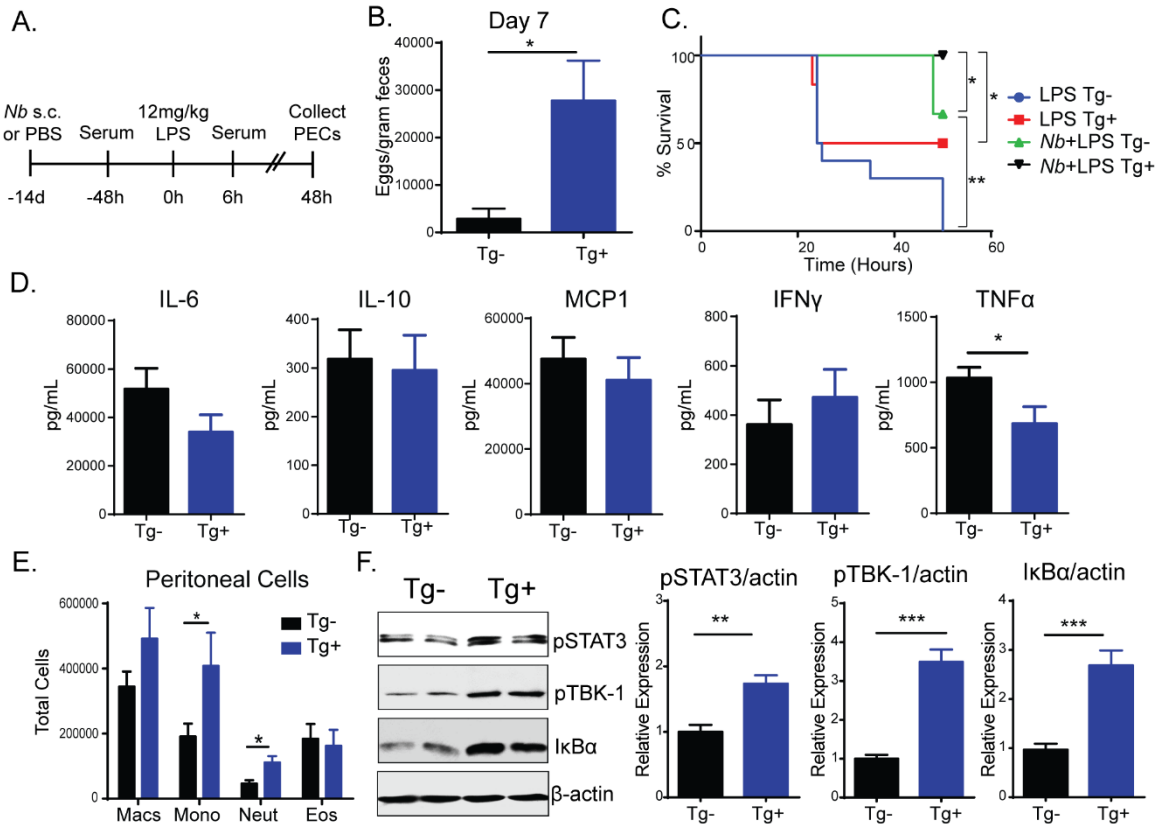

Figure 19: hResistin enhances helminth-mediated protection during endotoxic shock. (A) Experimental design of h*Retn*Tg^{-} and h*Retn*Tg^{+} mice infected with *Nb* followed by LPS challenge (C) Survival of h*Retn*Tg^{-} and h*Retn*Tg^{+} mice (n=6-10). (D) Serum cytokines were measured by cytokine bead array. (E-F) At 48 hours post LPS challenge, peritoneal cells were recovered and analyzed by flow cytometry (E) or Western blot (F). Data (mean±SEM, n=3 per group) are representative of 2 separate experiments.

hResistin expression leads to monocyte infiltration, anti-inflammatory signaling pathways are still activated to limit inflammation. This effect is through activation of IL-10 associated signaling pathways and decrease in proinflammatory pathways. Thus, hResistin is able to enhance the protective effect of helminths and Th2 immunity during endotoxic shock.

Human resistin binds to toll-like receptor 4

To examine how hResistin inhibits proinflammatory cytokines production during endotoxic shock, we investigated the receptor for hRestistin. There are currently two putative receptors for resistin, toll-like receptor 4 (TLR4) [133] and cyclic adenylate associated protein 1 (CAP1) [132]. Both studies used co-immunoprecipitation in human cell lines to show that hResistin promotes TNFα or IL-6 production through TLR4 or CAP1. However, it is unclear how hResistin interacts with these receptors *in vivo*. Since TLR4 signaling is crucial in progression of sepsis, we hypothesized that hResistin may be modulating inflammatory cytokines through TLR4.

In previously published data, we used RNA-seq of *Nb*-infected lung cells to show that hResistin increases proinflammatory cytokines and TLR signaling. We re-examined this dataset to quantify TLR4 and CAP1 expression during helminth infection [196]. In this dataset, TLR4 expression, but not CAP1 expression, is increased following *Nb* infection. Furthermore, when we compare h*Retn*Tg^{-} and h*Retn*Tg^{+} mice, only TLR4 expression is increased in h*Retn*Tg^{+} mice, suggesting

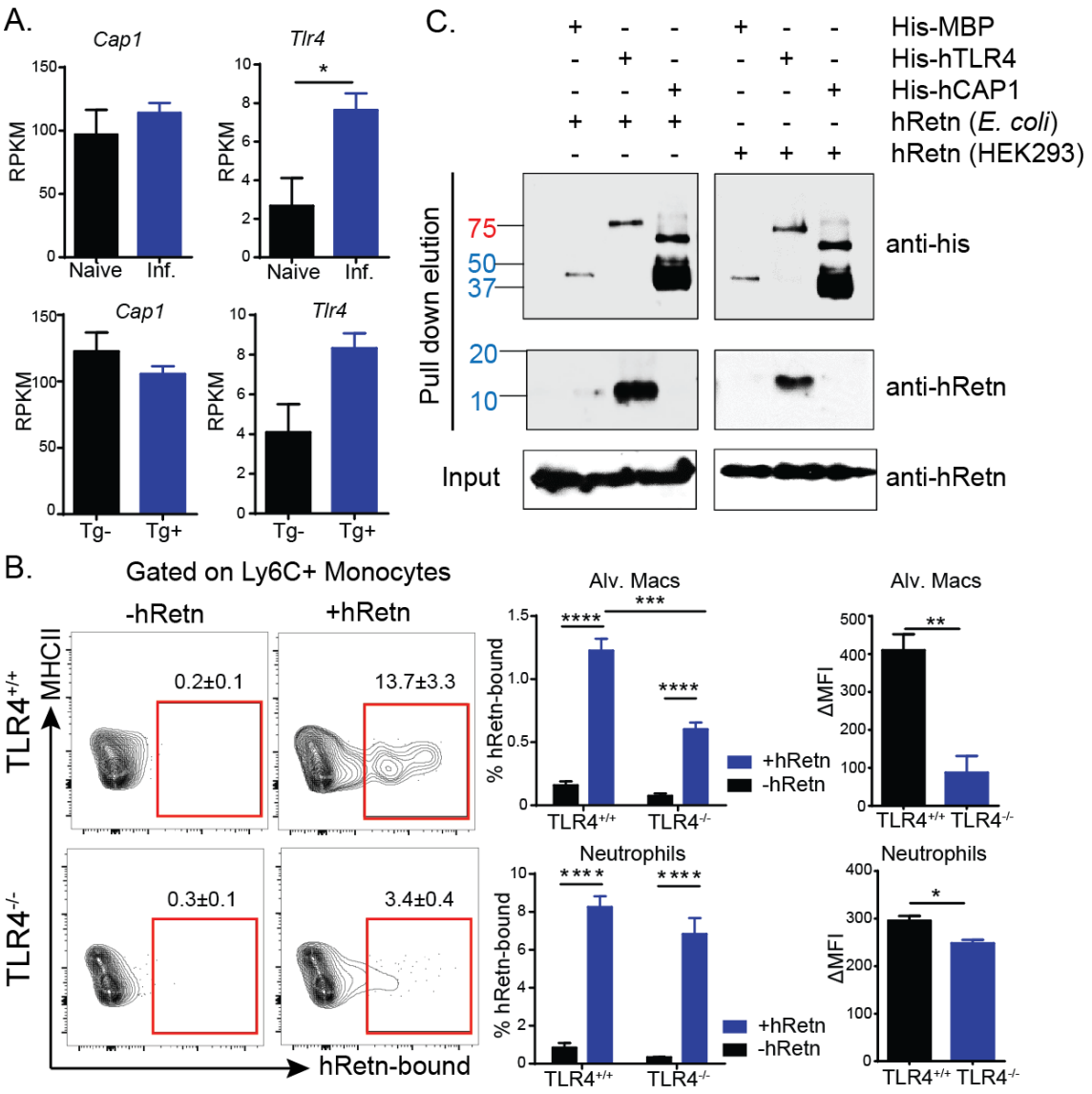

Figure 20: TLR4 is a receptor for hResistin. (A) CAP1 and TLR4 expression in *Nb*-infected lungs as measured by RNA-seq. (B) Cell binding assay with *Nb*-infected TLR4$^{+/+}$ and TLR4$^{-/-}$ lung cells. (C) Western blot of pull-down assay with his-tagged TLR4, his-CAP1, his-MBP and recombinant hResistin. Data (mean±SEM, n=3 per group) are representative of 3 separate experiments.

that TLR4 may be more functionally active in helminth infection (Figure 20A). To determine whether hResistin binds to TLR4, cell binding assays were performed using dissociated lung cells from *Nb*-infected $TLR4^{+/+}$ and $TLR4^{-/-}$ mice. We previously used this assay to show that hResistin binds to monocytes, alveolar macrophages and neutrophils. Briefly, lung cells were incubated with recombinant human resistin for one hour, stained with αhRetn antibody and analyzed by flow cytometry. These assays revealed that while ~13% of $TLR4^{+/+}$ $Ly6C^{+}$ monocytes can bind to hResistin, only 3% of $TLR4^{-/-}$ monocytes bind to hResistin (Figure 20B). On the other hand, $TLR4^{-/-}$ neutrophils are still able to bind to human resistin. We also examined mean fluorescent intensity of bound hResistin as a ratio of hResistin/PBS. TLR4 deficient alveolar macrophages bind significantly less hResistin, as they have significantly lower ΔMFI than $TLR4^{+/+}$ macrophages. Similarly, TLR4 deficient neutrophils do bind to less hResistin, although this difference is minor. Thus, while TLR4 is a receptor for human resistin, there may be other receptors for resistin.

With no commercially available CAP1 deficient mice, we examined direct protein-protein interaction of hResistin to TLR4 or CAP1 as an alternative to cell binding assays. To that end, a pull-down assay was performed with recombinant hResistin and his-tagged human TLR4 or his-CAP1. In this assay, we incubated hResistin with his-tagged hTLR4, his-tagged CAP1 or his-tagged maltose-binding protein (MBP) as control, followed by pull down of his-tagged protein using nickel-NTA agarose. Unbound hResistin is washed and the elution fraction containing

his-tagged protein and any bound resistin is analyzed by Western Blot. In the pull-down elution, Western blot using anti-his antibody confirmed that his-MBP, his-TLR4 and his-CAP1 are all pulled down by the nickel-NTA agarose beads. In addition, when blotting elution fraction with anti-hRetn antibody, a hResistin band is only detected in the elution with his-TLR4, not his-CAP1 or his-MBP (Figure 20C). To ensure that this interaction was not due to low level LPS contamination, we also performed this pull-down assay with mammalian cell-derived hResistin (HEK293 cells). Similar to *E. coli*-derived hResistin, mammalian-derived hResistin is only detected when pulled down with his-TLR4, and not his-MBP or his-CAP1. This data reveals that hResistin can directly bind to TLR4 and does not require other proteins to form a complex. While hResistin does not directly bind to CAP1, this data does not exclude the possibility that resistin might bind to CAP1 if other adaptor proteins are present.

Human resistin competes with LPS for binding to toll-like receptor 4

Based on the crystal structure of murine resistin and the amino acid sequence, we predicted the structure of human resistin. This revealed that human resistin (green) and murine resistin (cyan) have the same basic structure, with a coil-coil stem at the N terminal and a jelly-roll like head at the C-terminal, and form a trimer in physiological conditions (Figure 21A). This is consistent with the fact that murine and human resistin have ~60% sequence homology and that the conserved cysteine region in the C-terminal is essential for proper protein

structure. We then predicted the protein interaction between human resistin and TLR4, where the N-terminal of the human resistin hexamer (blue) binds to the same binding pocket of TLR4 (red) as MD-2 (white), the adaptor protein that mediates LPS binding to TLR4 (Figure 21B). Different hResistin chains (chains H, M, C) interact with different regions of TLR4 to make ionic interactions or hydrogen bonds (Figure 21B). Based on PDBePISA analysis, a total of eight hydrogen bonds or salt bridges are formed between TLR4 and hResistin at four interacting interfaces. At one of the interface, hResistin (chain M) makes two hydrogen bonds with TLR4: one between the carboxylate group of Glu30 and the guanidinium group of Arg355 of TLR4, the other hydrogen bond formed between the carboxylate of Glu34 with hydroxyl group of Tyr403 of TLR4 (Figure 21B, top left). At the second interface, the same hResistin chain (chain M) makes two more hydrogen bonds with TLR4: the guanidinium group of Arg65 making hydrogen bonds with Asn44 & Glu42 of TLR4 receptor (Figure 21B, bottom left). At the third interface, resistin (Chain H) makes three hydrogen bonds with TLR4: the guanidinium group of Arg65 from hResistin making hydrogen bonds with Asn268, Glu266 and Asp294 of TLR4 (Figure 21B, top right). At the fourth interface, hResistin (Chain H) makes one hydrogen bond with the TLR4: the guanidinium group of Arg65 making hydrogen bond with Gln484 of TLR4 (Figure 21B, bottom right).

Based on this structural prediction, we hypothesized that hResistin may compete with and physically block LPS/MD-2 from binding to TLR4 and reduce

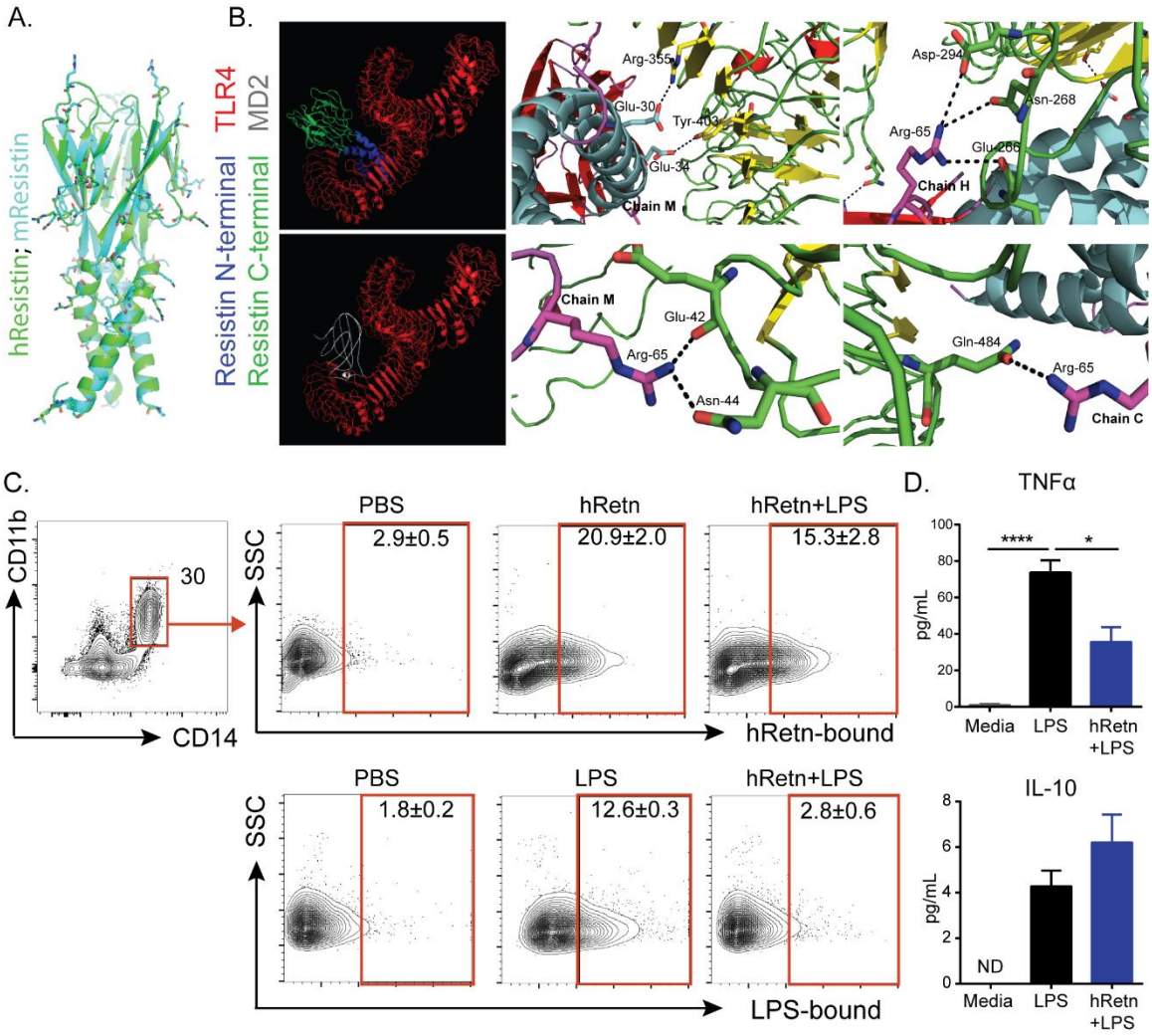

Figure 21: hResistin outcompetes LPS for binding to TLR4. (A) Prediction of human resistin (green) structure compared to murine resistin (cyan). (B) Structural modelling of resistin (blue and green) and TLR4 (red) reveals that resistin binds in the same binding pocket of MD2 (gray), the adaptor protein for LPS. Residues involved in hResistin (cyan and purple) and TLR4 (green and yellow) interaction. (C) Human PBMC were incubated with LPS in the presence or absence of human resistin and cell binding was measured by flow cytometry. (D) TNFα and IL-10 production by human PBMC that were stimulated with LPS ± hResistin. Data (mean±SEM, n=3-4) are representative of 4 separate experiments.

inflammation during endotoxic shock. To investigate this possibility, *in vitro* competitive binding assays were performed with human peripheral blood mononuclear cells (PBMCs). Human buffy coat was purchased commercially and PBMC were purified by density gradient. Briefly, blood was overlay on Histopaque-1077, spun for 20 minutes at 700g and PBMCs were recovered in the interphase. PBMCs were then subjected to competitive cell binding assay and hResistin or LPS binding was analyzed by flow cytometry. After gating on $CD14^+$ inflammatory monocytes, 20% of these monocytes can bind to hResistin, yet this percentage is not significantly reduced when incubating these cells with both hResistin and LPS (Figure 21C). On the other hand, ~13% of monocytes can bind to LPS-biotin when incubated alone, but this number is decreased to ~3% when PBMCs are incubated with hResistin and LPS. This revealed that hResistin inhibits binding of LPS to human PBMCs as well as murine cells. To examine whether hResistin affects downstream signaling of LPS, we cultured human PBMC with LPS in the presence or absence of human resistin. PBMCs treated with only LPS generated significantly more TNFα and less IL-10 than cells treated with hRetn+LPS, demonstrating a functional inhibition of LPS-induced inflammation (Figure 21D). These results were not an effect of endotoxin contamination, as the recombinant hResistin used in these studies was derived from HEK293 cells. Additionally, since LPS can activate both TLR4 and TLR2, we used ultrapure LPS derived from *Salmonella minnesota*, which only binds to and activates TLR4. Therefore, hResistin competes with LPS for binding to TLR4 and directly inhibits the production of proinflammatory

cytokines. These *in vitro* studies support the findings from our h*Retn*Tg^{+} mouse model, and validates that hResistin also protect against sepsis in human patients.

Resistin regulates anti-inflammatory signaling pathways through TLR4

To investigate whether hResistin signals through TLR4 under homeostatic conditions, we analyzed the activity of signaling molecules and transcription factors in naïve mice. Peritoneal cells were collected and pooled from h*Retn*Tg^{-}, h*Retn*Tg^{+}, TLR4$^{-/-}$/h*Retn*Tg^{-} and TLR4$^{-/-}$/h*Retn*Tg^{+} mice and signaling molecules were analyzed by Western Blot. In TLR4$^{+/+}$ cells, h*Retn*Tg^{+} mice had increased pSTAT3, pTBK-1 and IκBα compared to h*Retn*Tg^{-} mice (Figure 22A). These anti-inflammatory signaling molecules are induced by IL-10, regulate IL-10 production and inhibit NF-κB translocation to the nucleus and signaling, respectively. However, in TLR4 deficient mice, there were no significant differences in pSTAT3, pTBK-1 and IκBα between h*Retn*Tg^{+} and h*Retn*Tg^{-} mice. Peritoneal cells from naïve mice were also characterized by flow cytometry, which revealed an increase in monocytes in h*Retn*Tg^{+} mice, even in TLR4 deficient mice (Figure 22B). Eosinophil frequency was also higher in h*Retn*Tg^{+} mice and TLR4$^{-/-}$ mice, which may reflect the increase in anti-inflammatory signaling. This suggests that anti-inflammatory pathway activated by hResistin under homeostatic conditions is mediated through TLR4.

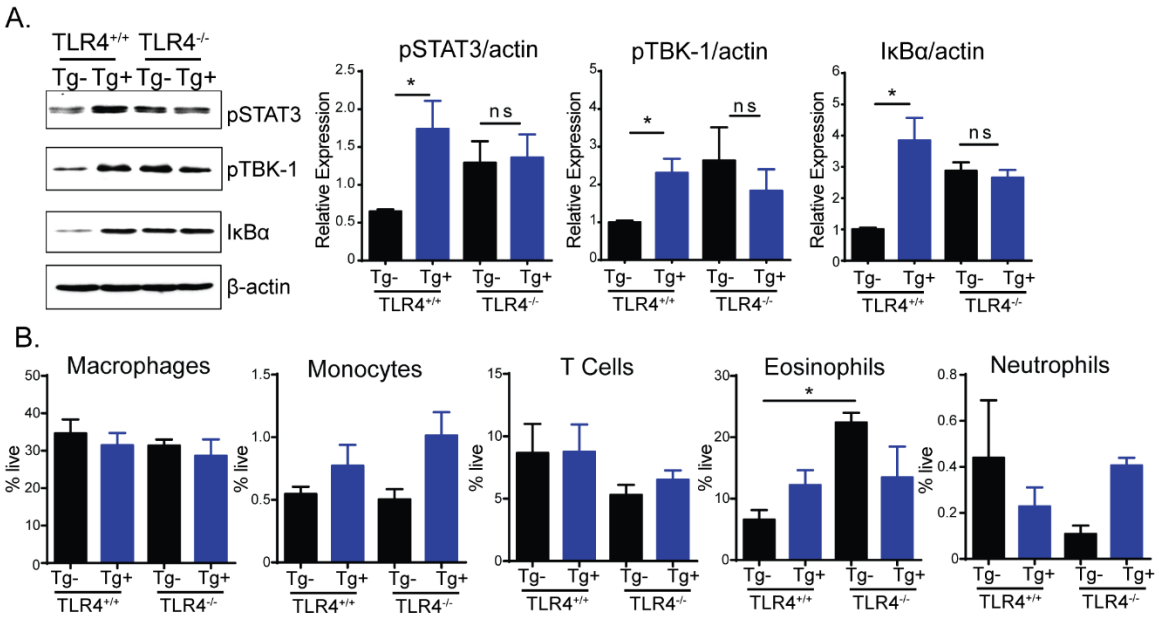

Figure 22: Resistin downstream signaling is TLR4-dependent. (A-B) Western Blot of signaling proteins (A) and flow cytometry (B) of PECs collected from naive TLR4$^{-/-}$/h*Retn*Tg^{+} and control mice. Data (mean±SEM, n=3-4 per group) are representative of pooled samples from 2 separate experiments.

Discussion

Despite major breakthroughs in the understanding of sepsis progression, sepsis still has a high mortality rate of 30%. This is due to a lack of effective treatment options, and many clinical trials have failed to reduce mortality in septic patients [191, 200]. Thus, alternative treatments for sepsis continues to be necessary. We have identified a novel mechanism for human resistin to protect against sepsis by blocking LPS-TLR4 interaction and excessive production of proinflammatory cytokines. While several clinical studies show that patients suffering from sepsis have elevated hResistin expression, it was initially believed that hResistin contributes to sepsis by promoting inflammation. This assumption was based on reports that LPS induces hResistin expression and that hResistin increases proinflammatory cytokines production *in vitro*. Our data confirm that hResistin expression is increased during endotoxic shock. However, rather than contributing to septic shock and increasing inflammatory cytokines, we propose that hResistin acts as a feedback mechanism to control the overreaction of the immune response to LPS-mediated TLR4 signaling. While patients with more severe clinical scores for sepsis have more circulating hResistin, this hResistin is necessary to try to decrease the inflammatory milieu caused by LPS. To examine the mechanisms behind hResistin-mediated protection during endotoxic shock, we investigated the receptor for hResistin.

There are currently two known receptors for hResistin, toll-like receptor 4 and cyclic adenylate associated protein 1. While our data supports the direct

interaction between TLR4 and hResistin, it is unclear what role CAP1 plays as the link between hResistin and LPS. Several pieces of our data suggest that CAP1 is unlikely to mediate protection during endotoxic shock. First, we were unable to detect any hResistin in the pull-down fraction with his-tagged CAP1, suggesting no direct interaction between hResistin and CAP1. Second, CAP1 is an intracellular receptor, thus hResistin would have to be endocytosed into the cytoplasm to interact with CAP1. In our cell binding assays, only surface-bound hResistin is detected, making it unlikely that CAP1 is the receptor in this case. Although other studies have identified TLR4 as a potential receptor for hResistin, this is the first to report that hResistin protects against sepsis through competition with LPS for TLR4. This competition between LPS and hResistin inhibits the production of inflammatory cytokines that is detrimental during sepsis. Perhaps most importantly, we show that recombinant hResistin could potentially be a novel therapy for patients suffering from sepsis.

Murine and human resistin have 60% sequence homology. Thus, it is no surprise that our pull-down assays revealed that both murine and human resistin can bind to TLR4. However, there is a discrepancy between how these proteins the protect against LPS. Although the presence of human resistin was beneficial to the host in sepsis, murine resistin did not reduce mortality during sepsis. This may occur because murine resistin expression in the blood is low and is not significantly increased following LPS injection. Only adipocytes secrete murine resistin, accounting for the relatively low expression found in the serum. While

murine resistin may be able to bind to TLR4, it is unclear whether it can outcompete LPS for binding to TLR4. In fact, in mRetn$^{-/-}$ mice, there is decreased IL-6 and IFNγ, suggesting that murine resistin does not decrease the inflammatory milieu found in sepsis.

Neutrophils are instrumental in controlling the initial bacterial or viral infection, but they can quickly contribute to sepsis through excessive production of proinflammatory cytokines. Indeed, many patients with sepsis have elevated numbers of neutrophils, and a prominent feature of acute respiratory distress syndrome (ARDS), lung injury caused by sepsis, is the influx of neutrophils in the lungs [201]. Once neutrophils have infiltrated the lungs, they contribute to organ failure and hypoperfusion through production of proteolytic enzymes and reactive oxygen species, which further increase vascular permeability. Furthermore, neutrophils damage tissue by formation of neutrophil extracellular traps (NETs) [202]. Given that mice treated with exogenous hRetn had intact vasculature in the lungs and a decrease in neutrophils, it is likely that hRetn prevents ARDS by limiting neutrophil infiltration into the lungs.

Production of IL-10 during sepsis is protective because it suppresses the production of TNFα and IL-6 [203, 204]. In IL-10 deficient mice, TNFα and IL-6 expression are exponentially greater and likely contributes to more rapid mortality in a cecal ligation and puncture model of polymicrobial sepsis [205]. Human resistin expression during endotoxic shock increases IL-10 production and activity of IL-10 associated signaling pathways, pSTAT3 and pTBK-1. STAT3 is activated

downstream of IL-10/IL-10R interaction and suppresses expression of proinflammatory cytokines. On the other hand, TLR4 activates two major signaling pathways, MyD88 and TRIF, where TRIF activates TBK1 and induces IL-10 production. Our data supports a model where hResistin binds to TLR4 and preferentially activates TBK1 in a TRIF dependent manner. Therefore, hResistin binding to TLR4 decreases inflammation in two ways, by inhibition of LPS-mediated inflammation and by increasing TBK1 activity and IL-10 production.

While helminth infection is associated with an increase in circulating LPS, there are numerous helminth-mediated immunoregulatory mechanisms in place to reduce LPS-associated inflammatory response. This includes Treg-produced IL-10 and helminth excretory/secretory products. For example, helminths infections are beneficial in sepsis by inducing IL-10 expression [75] and *Fasciola hepatica* secretes fatty acid binding proteins to reduce inflammatory responses [60]. Our data suggests that *Nippostrongylus brasiliensis* infection, through upregulation of the anti-inflammatory cytokine IL-10, is able to protect hosts from LPS-induced sepsis. Indeed, h*Retn*Tg^{-} mice are better protected from sepsis when infected with *Nb* compared to LPS alone. In addition to this, hRetn adds another layer of protection for the host to limit LPS-associated inflammation. Indeed *Nb*+LPS h*Retn*Tg^{+} mice fare much better than *Nb*+LPS h*Retn*Tg^{-} mice. The host has several pathways of helminth-induced protection during sepsis, including IL-10, eosinophil accumulation and now resistin. Furthermore, eosinophils, a marker of

Th2 immunity, is higher in septic patients who survived compared to patients who died [206].

The evolution of the immune response has received feedback from pathogens for millions of years. The infected host must balance the immune response carefully to limit damage caused by not only the pathogen, but also excessive inflammation. This balance is essential when the host is co-infected with a variety of pathogens, such as helminths and bacteria. In this study, we reveal that while hRetn exacerbates helminth infection, it protects the host from excessive inflammation caused by endotoxic shock. To our knowledge, this reveals a novel mechanism for humans to limit septic shock caused by an overactive type 1 immune response by blocking interaction between LPS and TLR4. In turn, this mechanism is exploited during helminth infection, as human resistin induced by helminth infection is able to protect the host from sepsis-induced death. Since the options for treatment against sepsis is currently insufficient, hRetn could provide a novel therapy against the proinflammatory milieu found during sepsis.

CHAPTER FIVE – CONCLUSION

Summary

Resistin expression is detrimental to the host during helminth infection

Previous studies identified $CD14^+$ blood monocytes as producers of hResistin following stimulation with *B. malayi* antigen. This prompted our investigation into the function of hResistin in helminth infection. In patients infected with filarial nematodes or soil-transmitted helminths, hResistin expression is upregulated in the serum. This increased expression of hResistin was associated with increased parasite burden in infected patients. To assess whether hResistin contributes directly to exacerbated helminth infection, we take advantage of transgenic mice where the human resistin gene, along with its entire regulatory region, was inserted in C57BL/6 mice using a bacterial artificial chromosome (h*Retn*Tg^+). In these mice, murine resistin was removed to limit any compensatory effects associated with the homologous protein. Following infection of these mice with the murine hookworm, *Nippostrongylus brasiliensis*, hResistin expression is upregulated by macrophages, monocytes and neutrophils. h*Retn*Tg^+ mice also had increased parasite burden, quantified by *Nb* eggs in the feces and adult worms in the small intestine. This data mirrored our results from the human patients infected with helminths, validating our use of the transgenic mice to model human helminth infections.

Resistin promotes inflammation and TLR signaling during helminth infection

To examine how hResistin leads to increased parasite burden, we took an unbiased approach and performed transcriptome analysis on whole lung RNA. Of the 981 differentially expressed genes (DEGs), many of were involved in the following Gene Ontology pathways: the immune response, TLR signaling and chemotaxis. hResistin increased production of the proinflammatory cytokines *Tnfa*, *Irf7*, *Ccl2* and *Tlr4*. An increase in *Ccl2*, an inflammatory monocyte chemokine, in the lungs was consistent with the formation of granulomas and increase of $Ly6C^+CD11b^+$ inflammatory monocytes in h*Retn*Tg^+ mice. Exogenous treatment of C57BL/6 mice with recombinant hResistin revealed that hResistin can directly recruit inflammatory monocytes. Furthermore, hResistin binds to monocytes and neutrophils to stimulate their expression of TNFα and TNFR2. In human patients infected with filarial nematodes and soil-transmitted helminths, hResistin expression also correlated with increased TNFα and CCL2 expression, confirming that hResistin also increases proinflammatory cytokines in helminth-infected patients. In addition, TNFα expression was positively correlated with increased parasite burden, suggesting that hResistin mediates exacerbated helminth infection through these proinflammatory cytokines.

On the other hand, hResistin had no significant effect on AAMac polarization or Th2 cytokines, IL-4, IL-5 or IL-13, when measuring these cytokines by RNA-sequencing, real-time PCR or ELISA. This was surprising given that

murine RELM proteins act as a negative feedback loop to limit excessive Th2-mediated chronic inflammation of the lungs [32, 33, 89]. Furthermore, murine resistin does not affect the outcome of helminth infection, as murine resistin deficient mice had a similar level of parasite burden as wild-type mice. It is possible that the disparate effects of murine and human resistin are due to the localization of these homologs; mResistin is expressed by adipocytes, while hResistin is expressed by monocytes/macrophages and neutrophils. Since *Nb* infects the lungs and small intestine of mice, adipocytes are not in contact with the helminths and do not increase their production of mResistin. Indeed, there were no increases in mResistin expression in the serum of C57BL/6 mice following *Nb* infection. Thus, despite the sequence homology between human resistin and its murine homologs, human resistin has a distinct function in helminth infection, and promotes a favorable environment for the helminths by increasing Th1 immunity.

Human resistin expression is regulated by IL-4 and STAT6

In chapter three, we sought to examine what helminth-derived factors are leading to upregulation of hResistin and identified a novel signaling pathway for hResistin expression. Previous studies have identified NF-κB and C/EBP as transcription factors that regulate hResistin transcription. During helminth infection, however, hResistin expression occurs even in TLR4 deficient mice, suggesting an alternative pathway that is independent of LPS. Analysis of the hResistin promoter for predicted transcription factor binding sites revealed two STAT6 binding sites,

which is downstream of IL-4Rα and regulates AAMac polarization. Treatment of mice with recombinant IL-4 revealed that hResistin expression can be stimulated by IL-4 *in vitro* and *in vivo*. This induction was dependent on STAT6, as there was no upregulation of hResistin in cells treated with IL-4 and STAT6 inhibitor. Using immunofluorescence, we also found that hResistin expression is co-expressed with IL-4Rα and pSTAT6. Finally, we were able to confirm that hResistin expression is dependent on IL-4 and STAT6 in human PBMC. This reveals that IL-4 and STAT6 phosphorylation likely plays a significant role in hResistin expression during helminth infection, although future studies would need to confirm this.

TLR4 is a receptor for hResistin

The increase in *Tlr4* and its downstream signaling pathways in h*Retn*Tg^{+} mice during helminth infection prompted our investigation into the hResistin receptor. Previous studies have identified two receptors for hResistin, TLR4 and CAP1, although it was unclear how hResistin signals through these receptors *in vivo* and during infectious diseases. Using a combination of cell binding assays and pull down assays, we demonstrate that hResistin can directly bind to TLR4, but not CAP1. Predictive structural analysis revealed that the N-terminal of hResistin binds to TLR4 in the same binding pocket as MD-2, the adaptor protein that enables LPS-TLR4 interaction and downstream signaling. This binding is facilitated by four residue interactions between the hResistin trimers and TLR4. In competitive binding assays, hResistin can inhibit binding of LPS to TLR4,

decreasing downstream signaling and subsequent TNFα production. Based on this data, we hypothesized that hResistin might reduce inflammation during sepsis by inhibition of LPS-TLR4 interaction.

Resistin protects the host against inflammatory cascade characteristic of septic shock

Bacterial infections can lead to sepsis when lipopolysaccharide (LPS), the endotoxic component of gram negative bacteria, binds to toll-like receptor 4, generating a cytokine storm characterized by IL-6 and TNFα. Although these cytokines combat infection, sepsis occurs when these cytokines cause collateral damage to the host's cells, low blood pressure and massive organ failure. Since sepsis is initiated by LPS-TLR4 interaction, we hypothesized that hResistin may protect the host during endotoxic shock by inhibiting LPS binding to TLR4. To investigate this possibility, we injected *E. coli*-derived LPS into h*Retn*Tg^{+} and h*Retn*Tg^{-} mice to model septic shock. Expression of circulating hResistin is quickly increased by 6 hours post LPS injection, where it protects the host from sepsis through downregulation of proinflammatory cytokines and increase in anti-inflammatory pathways, such as IL-10 and eosinophil recruitment. Thus, reduction of proinflammatory cytokines occurs in two TLR4-dependent ways. First, by inhibition of LPS-induced proinflammatory cytokine production and second, by direct stimulation of IL-10 production. This reduced cytokine production results in

diminished symptoms of septic shock, including hypothermia and vascular permeability.

Exogenous treatment of mice with recombinant hResistin also protected the host from endotoxic shock, suggesting that hResistin could be used as a therapy for septic patients. Again, this was associated with a decrease in proinflammatory cytokines and neutrophils, and a concurrent increase in eosinophils. Neutrophils are known to be key contributors to sepsis progression through production of proinflammatory cytokines and reactive oxygen species, which cause vascular permeability, low blood pressure and subsequent damage to the host's tissues [207]. On the other hand, eosinophils are significantly increased in sepsis [208] and are protective to the host in the cecal ligation and puncture model of sepsis [209, 210]. Given that hResistin is expressed in multiple helminth infections, we hypothesized that helminth-induced hResistin could enhance the protective effect of helminths during sepsis. In h*Retn*Tg^{+} mice infected with *Nb* prior to endotoxic shock, hResistin expression is significantly increased and protected the host from septic shock compared to *Nb*+LPS h*Retn*Tg^{-} mice or LPS alone h*Retn*Tg^{+} mice. This implies that increased hResistin expression in any situation is beneficial to the host during septic shock.

Resistin mediates inflammation in Th1 and Th2 environments

Although hResistin increases Th1 immunity in helminth infection, it paradoxically decreases the same proinflammatory cytokines during endotoxic

shock. While hResistin binding to TLR4 may induce some downstream signaling, it is to a lesser degree compared to LPS. This is evident when human PBMCs are stimulated with hResistin or LPS. Even when human PBMC are treated with more hResistin (1ug/mL) than LPS (100ng/mL), hResistin-treated cells produce 1000-fold less TNFα production compared to LPS (Figure 23). In a Th2 environment where LPS stimulation is minimal, hResistin is able to generate a mild inflammatory environment. Conversely, in the proinflammatory environment of sepsis, hResistin outcompetes LPS for binding to TLR4 and comparatively generates much less TNFα production. This mechanism is often exploited by helminths to protect against sepsis and excess inflammation. Helminth excretory/secretory proteins can modulate the host's immune response by inducing small amounts of proinflammatory cytokines, but leaving the host's immune cells unable to respond to LPS. Indeed, the well characterized filarial nematode secretory protein, ES-62, binds to TLR4 to induce low level TNFα in macrophages, but leaves these macrophages are unable to respond to subsequent stimulation with LPS [59]. Furthermore, *F. hepatica* fatty acid binding protein can bind to TLR4 and block activation and downstream signaling [60]. Based on this data, we propose that hResistin acts as another mechanism to enhance helminth-induced protection during septic shock.

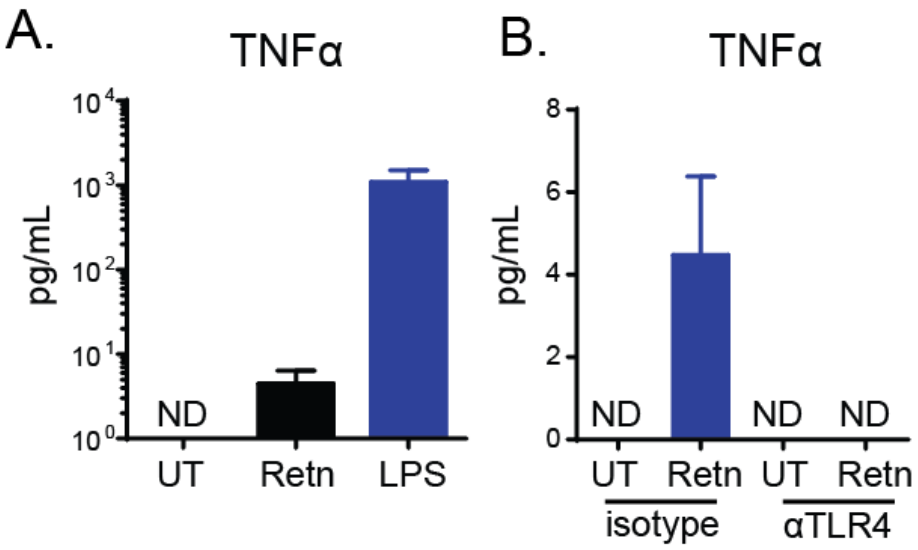

Figure 23: Resistin stimulates TNFα production through TLR4. (A) Human PBMC are stimulated with hRetn (1µg/mL) or LPS (100ng/mL) for 16 hours and TNFα production is quantified by ELISA. (B) Human PBMC are treated with αTLR4 neutralizing antibody (5µg/mL) or isotype control for 1 hour, then stimulated with hRetn (1µg/mL). Data (mean±SEM) are representative of 4 separate experiments (n=4).

Future Directions

How does hResistin bind to TLR4 or CAP1?

In multiple studies, both TLR4 and CAP1 have been identified as functional receptors using *in vitro* assays. Resistin can bind to both receptors and stimulate some proinflammatory cytokine production. Our pull-down assays confirm that hResistin can directly bind to TLR4, but not CAP1. Structural interaction predictions reveal that the N-terminal of hResistin trimers likely binds to TLR4 at four residue interactions. Further studies will need to be done to confirm that the N-terminal of hResistin does bind to TLR4 and that these specific residues of hResistin and TLR4 interact. Although we did not find direct interaction between hResistin and CAP1, this data did not exclude the possibility that hResistin may interact with CAP1 through an adaptor protein. Previous studies predicted that the C-terminal of hResistin would bind directly to the SH3 domain of CAP1 [132], although our pull-down assays were unable to duplicate these results. This may be complicated by the fact that CAP1 is an intracellular receptor and thus hResistin would have to be endocytosed or cross the cell membrane in order to interact with CAP1. One possibility is that hResistin is endocytosed after binding with TLR4, hResistin-CAP1 interaction may actually occur through TLR4 endocytosis and presentation of hResistin to CAP1.

While hResistin signals through TLR4 *in vitro*, it is unclear whether this also occurs *in vivo*, or whether resistin may have alternative pathways that could

compensate for a loss of TLR4. By crossing TLR4$^{-/-}$ mice with h*Retn*Tg+ mice, we can investigate the direct signaling pathway for hResistin in a multitude of diseases. Unfortunately, with CAP1$^{-/-}$ mice currently unavailable, these studies could not delineate whether hResistin signals though CAP1 *in vivo*.

Can hResistin act as a novel therapy for sepsis?

These studies revealed a clinically important role for hResistin in treatment of sepsis. Exogenous administration of recombinant hResistin protected the host from endotoxic shock. Based on this data, future studies should examine the generation of a stable *in vivo* form of hResistin that could mimic its function in sepsis. This could have direct impact on the millions of patients who are diagnosed with sepsis worldwide. Currently, patients diagnosed with sepsis are given a cocktail of antibiotics to clear bacterial infections and supportive care, such as regulation of low blood pressure. There are currently no FDA-approved treatment to inhibit the inflammatory milieu that leads to organ failure, and many clinical studies have failed to consistently reduce mortality in patients. Indeed, steroid administration in septic patients has been extremely ineffective, as 30% of patients still succumb to massive organ failure. Even monoclonal neutralizing antibodies to TLR4, while effective in mouse models of sepsis [190, 211], have been unsuccessful in clinical trials and do not reduce mortality [191]. This lack of effective treatments is due to the heterogeneity of the way sepsis is clinically presented, with a multitude of possible bacterial infections, bacterial growth rates

and dissemination, and differences in the host's own immune response. In addition, sepsis is difficult to diagnose without bacterial culture, which can take several days to confirm and delay appropriate treatment for these patients. Therefore, identification and generation of effective therapeutic drugs to treat sepsis and reduce inflammation is an important endeavor. Human resistin, through blocking LPS-TLR4 interaction and IL-10 induction, could provide the necessary anti-inflammatory signals to treat septic patients.

Does hResistin control bacterial infection?

While we show that hResistin protects against endotoxic shock by limiting proinflammatory cytokine production, it is unclear whether this decrease in antimicrobial cytokines can still limit growth of bacterial pathogens. These Th1 cytokines are important for initial control and clearance of bacteria. Human resistin expression, while protecting against endotoxic shock, may affect the host's ability to mount the initial Th1 response necessary to clear bacterial infections. Use of the cecal ligation and puncture (CLP) procedure could provide a better *in vivo* model of polymicrobial sepsis, compared to intraperitoneal injections of LPS. Since CLP involves a breach of barrier in the gastrointestinal tract, it closely mimics the dissemination of opportunistic pathogens in septic patients. This is physiologically relevant to the multitude of pathogens that typically invade when there is a breach in mucosal barrier. CLP in h*Retn*Tg^{+} and control h*Retn*Tg^{-} mice would reveal how

hResistin might balance the immune response to control the growth of bacteria and subsequent sepsis.

How does hResistin mediate anti-inflammatory cytokine production?

Many studies have shown that hResistin leads to proinflammatory cytokines through TLR4 and NF-κB signaling. However, we are the first to report that hResistin induces anti-inflammatory cytokines, specifically IL-10. TLR4 signaling is one pathway for IL-10 production, but rather than signal through the common adaptor protein MyD88, this signaling occurs through TRIF (Figure 24). Although both LPS and hResistin bind to TLR4, LPS may preferentially induce MyD88 and NF-κB signaling to produce proinflammatory cytokines, while hResistin may preferentially activate TRIF-dependent pathways to produce IL-10.

Furthermore, the source of these anti-inflammatory cytokines is unknown. The most common producers of IL-10 are AAMacs and regulatory T cells (T_{reg}), but eosinophils, monocytes and neutrophils can also can produce IL-10 in response to LPS stimulation. Given that hResistin binds to macrophages, monocytes and neutrophils, we hypothesize that hResistin-mediated IL-10 production occurs through these cells. In mice treated with recombinant hResistin, there was a decrease in neutrophils but an increase in monocytes, which could explain the increase in IL-10. Alternatively, in homeostatic conditions and following LPS injection, h*Retn*Tg^{+} mice have more eosinophils compared to h*Retn*Tg^{-} mice, which may contribute to the anti-inflammatory milieu. Although eosinophils do

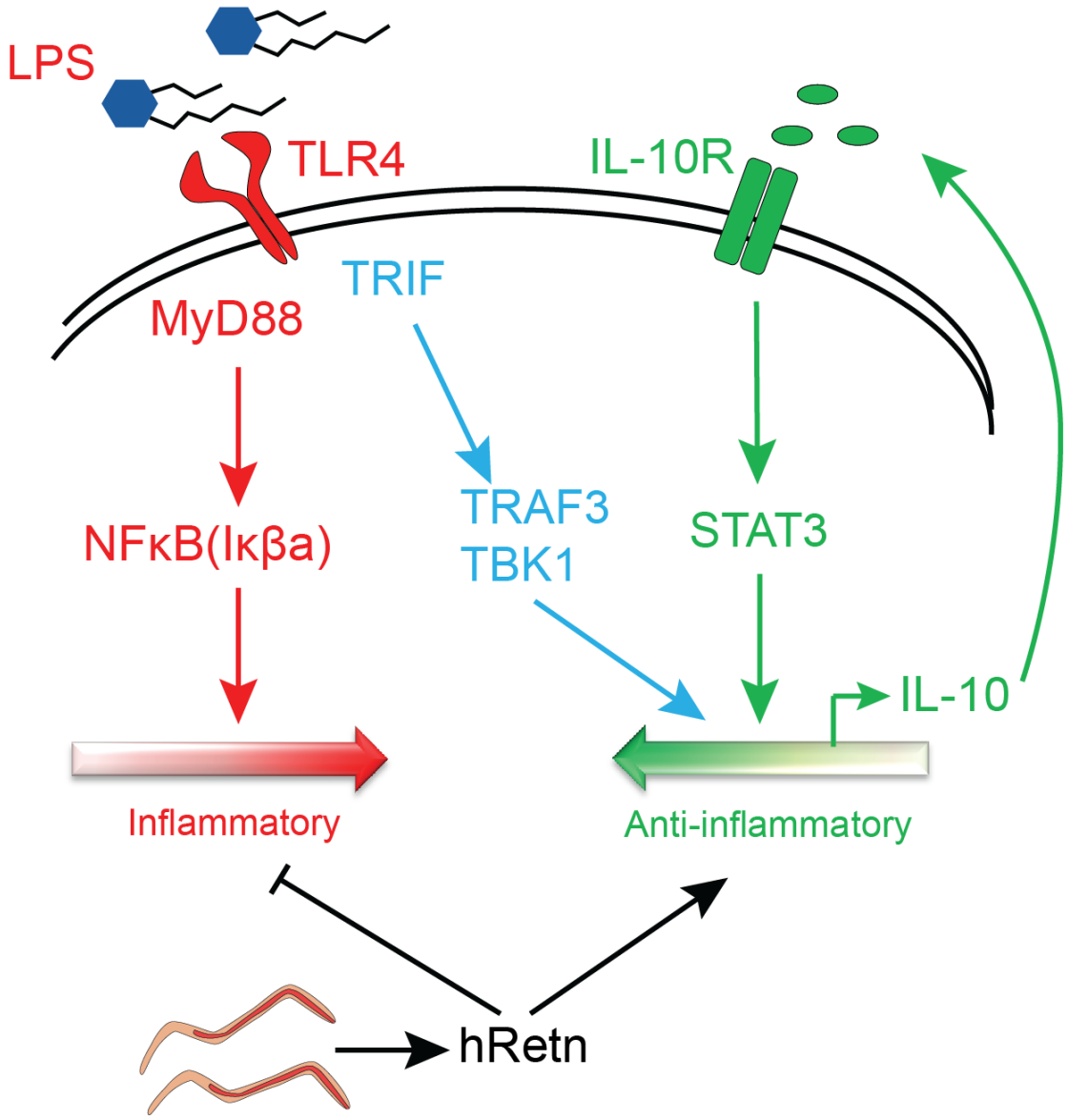

Figure 24: Signaling pathways during sepsis are regulated by hResistin.

express TLR4, we have found no evidence that hResistin directly binds to eosinophils. Thus, hResistin recruitment of eosinophils must be indirect, perhaps through upregulation of eosinophil chemokines, such as eotaxin, MCP3 or RANTES. Future studies should examine how these different cell populations contribute to hResistin-mediated IL-10 production in sepsis.

Are hResistin and mRELMα functional homologs?

Given that hResistin and mRELMα are both produced by macrophages in response to IL-4 and STAT6, we initially hypothesized that they may be functional homologs. During helminth infection, hResistin and mRELMα both lead to increased parasite burden, although these seem to be through different mechanisms. Human resistin increases worm and egg burden through upregulation of Th1 cytokines while mRELMα leads to susceptibility to helminth infections through downregulation of Th2 cytokines. Despite many studies investigating the role of mRELMα in helminth infection, many questions remain regarding the mechanism behind mRELMα-mediated susceptibility. For example, it is unknown what receptors bind to mRELMα, although initial studies reveal that mRELMα primarily binds to T cells while hResistin binds to monocytes, neutrophils and macrophage [196]. This data suggests that hResistin and mRELMα likely have different receptors and mRELMα does not signal through TLR4. Furthermore, it is also unclear what role mRELMα might have during sepsis and future studies should examine whether mRELMα also protects against endotoxic shock. This

could be examined with the use of mRELMα$^{-/-}$ mice or treatment of C57BL/6 mice with recombinant mRELMα. Thus, although hResistin and mRELMα have similar expression patterns and exacerbate helminth infections, additional evidence suggests that they may have distinct functions. Nevertheless, mRELMα$^{-/-}$/h*Retn*Tg^{+} mice should be generated to examine whether hResistin and mRELMα have overlapping functions in regulating immunity.

Conclusion

In this thesis, we investigated the role of hResistin in regulating immunity in both an anti-inflammatory setting (helminth infection) and a proinflammatory disease (sepsis). The results from these studies have revealed that while hResistin may exacerbate helminth infection, it also keeps the proinflammatory response during sepsis at bay (Figure 25). Indeed, helminth-induced hResistin reduces mortality during sepsis. Thus, it acts as a feedback mechanism that is permissive of non-fatal helminth infection, but protects the host against the more fatal inflammatory milieu indicative of bacterial infections and sepsis. This protection, however, occurs in a multitude of situations. For example, in a chronic low-grade inflammatory disease mimicked by a low dose LPS injection prior to a fatal dose, hResistin still protects the host by inhibiting LPS-TLR4 interaction and subsequent inflammatory cytokine production. This is essential when examining common comorbidities associated with sepsis, such as diabetes. This data challenges the current paradigm that hResistin might contribute to mortality during sepsis through upregulation of TNFα and IL-6. However, this paradigm was based on the assumption that hResistin was increased in septic patients and *in vitro* results that show that hResistin increases proinflammatory cytokine production. We propose a model where hResistin, while detrimental to the host during helminth infection, is protective during sepsis by outcompeting LPS for binding and signaling through TLR4.

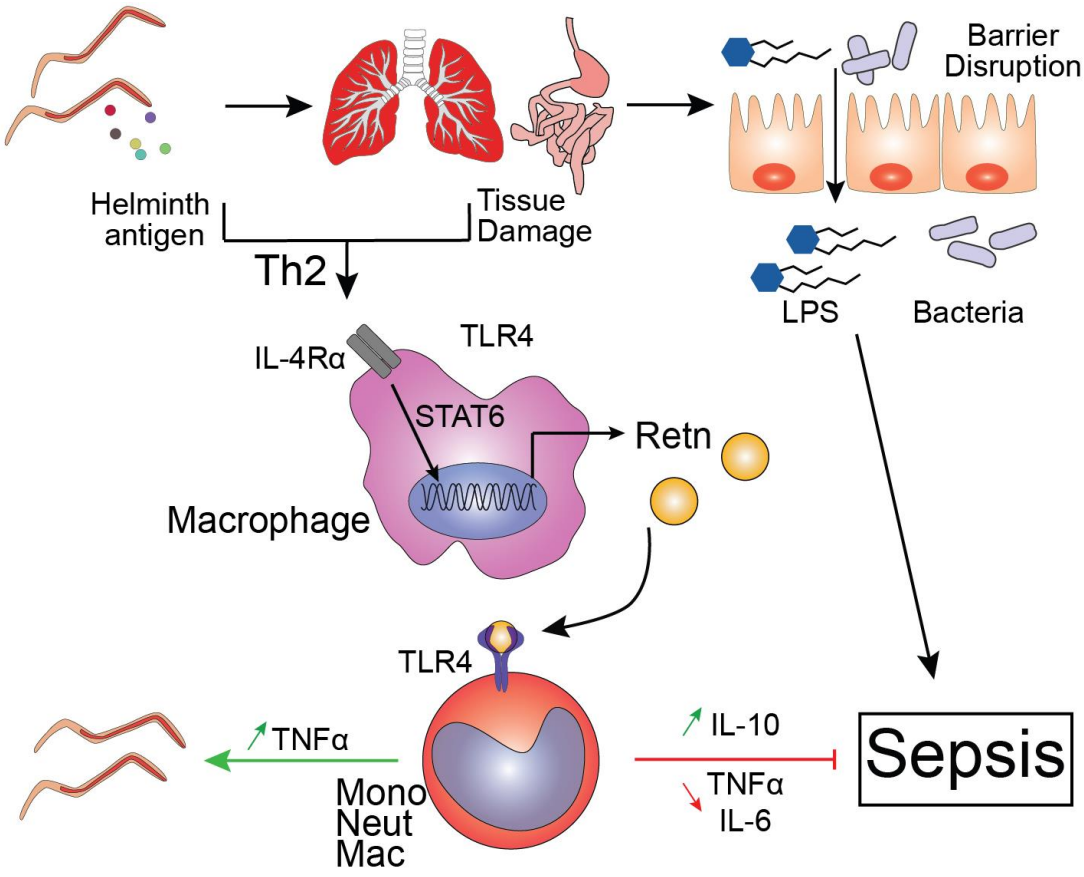

Figure 25: Resistin regulates immunity during helminth infection and sepsis.

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