HHS Public Access

Author manuscript

Mucosal Immunol. Author manuscript; available in PMC 2013 March 01.

Published in final edited form as:

Mucosal Immunol. 2012 September ; 5(5): 472–479. doi:10.1038/mi.2012.40.

Leptin and Mucosal Immunity

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Abstract

Enhanced susceptibility to infection has long been recognized in children with congenital deficiency of leptin or its receptor. Studies in mice have demonstrated that leptin deficiency affects both the innate and acquired immune systems. Here we review recent studies that demonstrate the impact on immunity of a common non-synonomous polymorphism of the leptin receptor. In a Bangladesh cohort of children, the presence of two copies of the ancestral Q223 allele was significantly associated with resistance to amebiasis. Children and mice with at least one copy of the leptin receptor 223R mutation were more susceptible to amebic colitis. Leptin signaling in the intestinal epithelium and downstream STAT3 and SHP2 signaling were required for protection in the murine model of amebic colitis. Murine models have also implicated leptin in protection from other infections including *M. tuberculosis, K. pneumoniae* and *S. pneumoniae*. Thus, the role of leptin signaling in infectious disease and specifically leptin-mediated protection of the intestinal epithelium will be the focus of this review.

Keywords

leptin; intestinal epithelial cells; infectious diseases; *Entamoeba histolytica*

Introduction

At the crossroads between nutrition and immunity lies the adipocytokine leptin. Leptin and its receptor, LepR, are found throughout the central nervous system and the periphery [1-4]. Leptin is produced primarily by adipocytes, but is also produced by a number of other cell types, including gastric and colonic epithelial cells, and T-cells, especially during acute inflammation [5-8]. In the central nervous system, leptin controls the appetite by signaling satiety, leading to a reduction in food intake while also increasing energy expenditure [1, 2]. First characterized for its effect on metabolism, leptin is now recognized as an important modulator of both the innate and adaptive immune systems [5, 9-12]. Recently, we and others

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The Authors have no conflicting interests to report.

have observed leptin to be a critical factor in mediating resistance to microbial pathogens [13-19].

Congenital Human Leptin and Leptin Receptor Deficiency

Congenital leptin and leptin receptor deficiencies predominantly affect a handful of highly consanguineous families [20-25]. Leptin deficiency is due to carriage of homozygous mutations such as Δ133G, which result in a truncated leptin molecule that lacks biologic activity [22-24]. Similarly, leptin receptor deficiency is often caused by homozygous mutations that affect receptor processing and function (discussed below) [20, 21, 26]. Within affected families, homozygotes for catastrophic mutations in either leptin or leptin receptor present with similar phenotypes, providing further evidence to the exclusivity of the ligand/receptor interaction. Affected individuals are normal weight at birth, but then rapidly display hyperphagia and early-onset obesity in late infancy (prior to the first birthday) [20, 23]. Along with obesity, these individuals possess characteristic phenotypes such as hypogonadism, severely delayed onset of puberty (menses beginning in the third decade), hypothyroidism and immune dysfunction [20, 22-24]. Interestingly, heterozygotes for mutations are not affected in any appreciable way studied, and are of normal weight [20, 23, 24].

Congenital Leptin and Leptin Receptor Deficiency and Immune Dysfunction

One of the first reports implicating leptin as critical for proper immune function in humans was the observation that family members of leptin deficient probands with early onset obesity were ~25 times less likely than normal weight children to survive childhood. Recurrent infections ultimately led to the death of 7 out of 11 obese children, whereas no children of normal weight from the same family died of childhood diseases [24]. Similarly, obese children in families with homozygous LepR deficiency had greater incidences of respiratory infections, which lead to the deaths of two children [21]. Physiologic fluctuations in leptin have also been observed in association with changes in immune status. Increases of leptin concentrations following weight gain in previously malnourished children were linked to Th1 mediated responses [27]. In another example, drops in leptin levels during fasting in rheumatoid arthritis patients correlated with a decrease in the activation of CD4 T-cells [28].

Leptin replacement therapy of leptin deficient individuals provided the first direct evidence for a role of leptin in promoting Th1 mediated immunity. In a replacement study by Farooqi et al, prior to initiation of treatment, two patients were found to have reduced CD4 T-cell populations (thus a reduced CD4/CD8 ratio), hyporesponsive T-cells, severely reduced IFN-γ expression, and reduced levels of cytokines such as IL-10 and IL-4, while TGF-β was increased. Upon long-term leptin replacement therapy, CD4 numbers, IFN-γ expression and T cell function were recovered, while TGF-β decreased to normal levels [12]. A similar immunophenotype and “recovery” following 48 months of leptin replacement therapy was observed in a young girl. Furthermore, the improved immune function coincided with decreased incidents of (previously) recurring urinary tract infections and perineal dermatitis. The authors also observed improvement of asthma symptoms (no asthma related hospital visits since 12 months of treatment) [22]. Of note, similar immune dysfunction was observed in LepR deficient individuals as well [21].

The observations that leptin mediates CD4 T-cell proliferation and acts as a pro-inflammatory cytokine have been further corroborated and expanded upon using *in vitro* analysis of human PBMCs and murine models of leptin and leptin receptor deficiency (*ob/ob* and *db/db* mice, respectively). Briefly, leptin has been found to modulate the innate and adaptive responses by repressing proliferation of regulatory T-cells, modulating T-cell and monocyte function, enhancing phagocytosis, and coordinating cytokine expression during infection [9-11, 25, 29-31]. Thus, it is easy to hypothesize that leptin plays an active role during infectious disease.

Leptin Receptor Signaling

There are several identified isoforms of LepR that are divided into short, secreted or long forms. The long form of the receptor, LepRb, contains all canonical signaling sites, while the short forms lack much of the intracellular domain and capacity to signal [3, 4, 32, 33]. LepRb is expressed in numerous cell types including neurons, intestinal epithelial cells (IECs) and immune cells, such as macrophages, T cells, natural killers cells and polymorphonuclear cells (PMNs) [6, 7, 13, 14, 25, 29, 31, 32, 34-36]. Upon leptin binding to the extracellular ligand binding domain of the homodimeric LepRb, JAK2 becomes activated and phosphorylates three tyrosine residues on LepRb (Tyr 985, 1077, and 1138). These phosphorylated tyrosines are in turn recognized by STAT3, STAT5, and SHP2, as shown in Figure 1. Following phosphorylation of STAT3 and STAT5 by JAK2 there is initiation of multiple downstream signaling events, as depicted in Figure 1. pSTAT3 induces expression of SOCS3 that in turn binds to Tyr 985 and attenuates LepRb signaling, thus providing feedback inhibition of STAT3 activation [3, 4, 33, 37-41]. Along with SOCS3, leptin activated pSTAT3 induces cell-type dependent expression of genes important in metabolism and/or inflammation such as pro-opiomelanocortin (POMC), and IL-6 [3, 4, 42, 43]. It has recently been shown that ablation of Tyr 1138-STAT3 signaling, and subsequent deregulation of SOCS3 mediated attenuation, lead to aberrant/enhanced signaling in other pathways downstream of LepRb [44].

SNPs and Their Role in LepR Fuction

Complete leptin resistance has been attributed to various frameshift, nonsense, and missense mutations within LepR [21, 26]. Four non-synonymous SNPs (A409E, R612H, W664R, and H684P) in the extracellular domain of *LepR* have been characterized by Kimber et al in order to better understand their consequences on LepR signaling [26]. *In vitro* STAT3 reporter assays using cell lines transfected with the mutant receptors showed that three mutant LepRs lacked the ability to respond following leptin binding (A409E, W664R, H684P) while one (R612H) displayed significant but reduced activity. The authors further reported that the decreased activity could be attributed to drastically reduced levels of mutant LepR expression on the cell surface in the case of W664R, H684P and R612H. A409E had surface expression levels similar to wild-type LepR, however due to location within the immunoglobulin-like domain it was postulated to attenuate receptor activation following leptin binding [26].

Exome Sequencing Project Identified LepR SNPs

Given the complexity of the LepR signaling cascade and the observation that mutations outside of the binding domain can drastically attenuate signaling, it is easy to imagine that numerous other SNPs or mutations may exist which modulate the signaling capacity of LepR. The NHLBI Exome Sequencing Project (ESP) has proved to be an excellent tool in identifying naturally occurring SNPs and predicting their potential impact on protein function [45]. ESP consists of Exome sequence data from 5,379 Americans, subdivided by ethnic origin (European American and African American). The Exome Variant Server (EVS), supported by the ESP, identified 55 non-synonomous SNPs in *LepR*; of these 32 were predicted to be probably or possibly damaging, while the remaining 23 were predicted to be benign. 161 individuals (3%) were found to be homozygous for potentially damaging mutations (Asn656Lys being the most common, n=159) [45].

Of the four reported non-synonomous polymorphisms associated with congenital LepR deficiency, only one, R612H, was identified in heterozygous carriage through the Exome project, however this is likely due to ethnic differences in the populations sampled [26, 45]. Due to the sizable number of SNPs that are predicted to be potentially damaging to LepR, and the identification of 161 homozygotes, it seems plausible that attenuated LepR signaling and/or partial leptin resistance, if not complete resistance, occurs outside of highly in-bred families. Moreover, SNPs predicted to have benign effects on LepR signaling may actually significantly modulate signaling. We have shown that the most common LepR SNP, Q223R (RR n=1301, QR n=2631, QQ n=1447), predicted by EVS to be a benign change, is significantly associated with susceptibility to amebiasis (discussed below) [13, 41]. Therefore, even slight modifications within LepR have profound effects that may determine the progression, severity, and outcome of disease.

Leptin is a Critical Mediator of Resistance to Amebiasis

We have found that the Q223R mutation in LepR is associated with the parasitic pathogen, *Entamoeba histolytica* [13, 41]. *E. histolytica* is the causative agent of amebic dysentery, amebic diarrhea, and amebic liver abscesses and causes an estimated 100,000 deaths yearly [19, 46]. Upon ingestion of cysts found in contaminated water supplies, the parasite undergoes excystation in the intestine where it can then colonize or cause invasive disease [47-50]. While *E. histolytica* causes severe morbidity and mortality in endemic areas, infection is often self-limiting or asymptomatic [19, 49, 51]. Due to the dichotomy that exists between infectious outcomes, we postulated that host factors were involved in mediating disease severity.

We have previously investigated the association of enteric protozoan-related diarrheal illness with the nutritional status and growth of preschool children in Dhaka, Bangladesh. The cohort consisted of 221 children aged 2-5 years who were followed prospectively for diarrheal illness over 3 years. Along with incidences of diarrhea, other parameters such as weight and height were measured. Children with *E. histolytica*-associated diarrhea were 2.9 times more likely to be underweight and 4.7 times more likely to be stunted [19, 52]. In agreement with the literature and the role of leptin in metabolism, we found that the malnourished children of the Bangladesh cohort had significantly lower levels of serum

leptin [13]. The second piece of evidence linking leptin to amebiasis was immunohistochemistry of biopsies from patients with acute amebic colitis that showed LepR expression in the intestinal epithelium during acute infection (Figure 2). Because amebae invade through the intestine by inducing apoptosis of IECs, killing might be blocked by the known anti-apoptotic and pro-proliferative functions of leptin. We hypothesized that the low levels of leptin could account for the increased susceptibility of malnourished children to amebiasis. In order to test our hypothesis, further studies in both human and mice were undertaken (discussed below).

Through a nine-year prospective study of *E. histolytica* infection in a cohort of children (enrolled at preschool age), we found increased susceptibility associated with the Q223R SNP in LepR. Interestingly, the Q223R polymorphism is located in the cytokine receptor homology domain 1 of LepR, outside of the known binding domain. Children with at least one arginine allele (223R) were nearly four times more likely to have an *E. histolytica* infection compared with those homozygous for the glutamine allele (223Q) [13]. The Q223R association persisted after accounting for malnutrition, sex, and age, and was also found to be significantly associated with adult amebic liver abscesses[13]. While observations made in individuals with congenital leptin deficiency or complete leptin resistance suggested that leptin signaling could have a potential role in infectious diseases, these studies were the first to show a direct association of a leptin receptor mutation with human infection.

Site and mechanism of leptin receptor signaling in E. histolytica Infection

Murine infection models were utilized in order to delineate the mechanism of leptin-mediated resistance to *E. histolytica*. Our initial studies (in the C57BL/6 background) demonstrated that leptin deficient (*ob/ob*) and LepRb deficient (*db/db*) mice were highly susceptible to infection with *E. histolytica*, whereas wild-type C57BL/6 were resistant [13, 14]. Similar to human studies, we found that mice either homozygous or heterozygous for the 223R allele of LepR were significantly more susceptible to amebic infection. Infected 223R animals exhibited greater pathology with increases in IEC apoptosis and mucosal destruction [13]. These studies provided rationale for further use of murine models to delve into the mechanisms behind the leptin effect.

Bone marrow chimeras were created from wild-type and *db/db* mice in order to define the cellular compartment affected by leptin. Surprisingly, these mice demonstrated that LepRb signaling in bone marrow derived cells was not responsible for the susceptibility observed in *db/db* mice. As LepRb is expressed in IECs during infection, mice with IEC specific deletion of LepRb (vil-cre LepR) were created and found to be significantly more susceptible to infection with *E. histolytica* when compared to wild-type littermate controls [13, 14]. Thus, the primary cell type responsible for leptin-mediated resistance was identified as IECs.

The study of the downstream signaling pathways important for LepRb-mediated resistance identified that signaling through both LepRb/Tyr 985 and 1138 was critical for protection from *E. histolytica* challenge in the murine model. While both mice exhibited increased susceptibility to *E. histolytica* challenge, dramatic differences in phenotype were observed. Specifically, greater mucosal damage occurred in the S1138 mice, while the L985 mice

exhibited minimal mucosal damage with higher levels of mucosal hyperplasia [14]. These observations suggest that leptin signaling activates more than one pathway to promote resistance to *E. histolytica*. To delve further into the molecular events following activation of LepRb, *in vitro* studies utilizing knock-in mutants of leptin receptor tyrosines at 985, 1077, and 1138 were performed. These experiments showed that STAT3 signaling via Tyr 1138 was essential for leptin mediated cellular resistance to *E. histolytica* killing *in vitro*. Interestingly, these studies have also shown that the 223R LepRb is significantly impaired in activation of STAT3 following leptin binding [41]. Taken together these studies demonstrated that leptin-mediated protection against *E. histolytica* required signaling through (at least) Tyrs 985 and 1138.

Leptin and other models of infectious disease

Utilizing murine models, leptin has been found to modulate disease during infection with other important human pathogens. Studies in *ob/ob* and *db/db* mice have shown that leptin is important for a successful host response against *Mycobacterium tuberculosis* [15]. A recent study by Lemos et al, found that *db/db* mice infected with *M. tuberculosis* exhibited a dysregulated immune response and subsequently, impaired bacterial containment. The cellular compartment responsible for the susceptible phenotype was outside of bone marrow derived cells, which led them to hypothesize that leptin was acting at other sites, one of which could be the pulmonary epithelium, as alveolar type I and II epithelial and bronchial epithelial cells have been found to express LepRb [15, 44].

While there is now evidence for leptin signaling in IECs providing protection from amebic infection, leptin signaling in immune cells may also be important in other infectious diseases. Interestingly, Mancuso et al found that acute leptin depletion via fasting of wild-type animals for 48 hours lead to an increase in *S. pneumoniae* load [18]. Using an *ex vivo* murine infection model, Moore et al found that neutrophils from leptin deficient mice were significantly attenuated for opsonophagocytosis of *K. pneumoniae*, an effect that could be reversed upon exogenous leptin administration (both *in vivo* and *in vitro*) [16]. Similarly, alveolar macrophages from *ob/ob* mice were impaired in their ability to phagocytose *S. pneumoniae* and PMNs displayed a reduction in killing. Again, administration of leptin restored proper effector functions in these cells [17]. Alveolar macrophages express LepRb, and are capable of activating STAT3 following stimulation with leptin. Abrogation of STAT3 signaling of LepRb by mutation of the Tyr 1138 led to protection from *S. pneumoniae*, an effect that was linked to increased production of leukotrienes in alveolar macrophages [44]. These studies and others indicate the potential for leptin signaling in both epithelial cells and immune cells in response to infection.

Mechanisms of Leptin Mediated Protection in IECs

Down-stream effects of LepR signaling vary depending upon cell type. In IECs leptin signaling has been shown to have numerous effects that could inhibit or protect against host cell killing by *E. histolytica* (Figure 1). *E. histolytica* predominantly kills host cells via induction of host-cell apoptosis, and LepRb signaling significantly increases anti-apoptotic pathways [13, 14, 53-55]. We have recently shown that *in vitro* leptin signaling via STAT3 activation at Tyr 1138 leads to differential regulation of numerous apoptotic genes [41]. These

expression changes could account for the increased cellular resistance exhibited during challenge with *E. histolytica*. Leptin also induces IEC expression of antimicrobial peptides [56, 57]. Peterson et al demonstrated that the defensin RegI, which is produced by IECs and induces epithelial repair, could be another mechanism of leptin-mediated defense [37, 56]. Additional potential mechanisms by which leptin may protect the intestine from infection include: 1) Increased goblet cell number and mucus secretion [57, 58]; 2) leptin signaling through STAT5 which could potentially lead to the strengthening and maintenance of tight junctions, preventing parasitic invasion and dissemination [59]; and 3) change in the gut microbiome [60, 61].

In addition to intrinsic IEC defenses, LepRb signaling can lead to production and secretion of pro-inflammatory cytokines and chemokines such as IL-6, IL-1β, CXCL-1 and IL-8, which attract and cause infiltration of immune cells such as neutrophils and T-cells [42, 43, 62]. Furthermore, leptin stimulation may have a role in T-cell differentiation. For example leptin modulates IEC production of IL-6, which in turn activates STAT3 in naïve T-helper cells that subsequently leads to differentiation into Th-17 cells [42, 63-65].

Critical Role of STAT3

There is no doubt that leptin has many unique functions, especially within the neuroendorcrine axis. However, it appears that many of the functions attributed to leptin in the epithelium are similar or redundant to effects of other cytokines. For example, leptin has been shown to be a critical mediator of restitution following injury to the epithelium, likewise, IL-6, IL-27, and IL-22 have also been found to play roles in wound healing [66-71]. Furthermore, IL-31, like leptin, has been shown to induce IEC expression of cytokines (IL-8), while IL-22 can enhance expression of antimicrobial peptides (RegIII) [69, 72, 73]. The common denominator of all these effects is STAT3. Numerous cytokine receptors, summarized in Figure 3, activate STAT3 resulting in a myriad of downstream effects [66-80]. The importance and pleiomorphic role of STAT3 is illustrated by the autosomal-dominant hyper-IgE syndrome (AD-HIES, also known as Job's syndrome), which is caused by dominant negative mutations within *stat3*. AD-HIES presents with numerous sequelae both immune and somatic, but is primarily an immunodeficiency with recurrent pneumonias, boils, dermatitis, very little or no Th-17 T cells, and high levels of IgE [81-83]. In murine models, tissue-specific deletions have shown STAT3 to be important in cancer, immunity and wound-healing [71, 81-83]. Thus, similarities between down-stream effects of leptin and other cytokines may in part be due to STAT3 signaling.

With so much redundancy is the effect of leptin on epithelial cells unique?—

IL-22, a member of the IL-10 family of cytokines, acts upon epithelial cells to promote cellular defenses. As discussed above, IL-22 shares many redundant functions with leptin including induction of antimicrobial peptide production by epithelial cells, and promotion of wound healing. Also like leptin, IL-22 signaling in epithelial cells leads to expression of anti-apoptotic genes and increased cellular proliferation [66, 69, 84-87]. While leptin and IL-22 appear to have similar roles, the context in which they act is important. For example, leptin signaling during infection with *M. tuberculosis* was important for regulation of the immune response and bacterial containment [15]. In contrast, Wilson et al, recently found IL-22 to be

dispensable for an appropriate immune response to numerous pathogens including *M. tuberculosis* [86]. Furthermore, *ob/ob* mice lacking leptin are protected against DSS colitis, while IL-22$^{-/-}$ mice showed more severe pathology [42, 87]. Thus, the circumstances in which these cytokines act is critical and allow for a fine-tuned and seamless immune response.

Summary

As a signal for starvation, leptin plays an essential role in the regulation of metabolic functions [1, 2]. Moreover, leptin has been shown to be a critical immunomodulator in both humans and murine models [5, 9-12]. Observational evidence for the importance of leptin in infectious disease was first described due to leptin and LepR deficient individuals having numerous recurrent infections and high amounts of mortality due to childhood diseases [21,24]. Our laboratory has demonstrated an association of human disease with leptin, via the Q223R polymorphism in LepR [13]. Furthermore, using murine infection models we have found that leptin signaling in the intestinal epithelium is a mediator of protection against *E. histolytica* [14]. Similarly, recent studies of infectious lung diseases have shown a role for leptin in protection against *M. tuberculosis*, *K. pneumoniae* and *S. pneumoniae* [15-18, 44]. Interestingly, there appears to be a dichotomy in leptin protection, wherein the leptin effect has been found to be significant both within and outside of the immune compartment.

Beyond those affected with congenital leptin deficiency and complete leptin resistance, SNPs in *LepR* can have dramatic effects on leptin signaling, as we have shown an association of the Q223R polymorphism with amebiasis [13, 41]. Due to the large number of SNPs identified through the NHLBI Human Exome Sequence Project it is easy to envision how carriage of SNPs can modify or attenuate LepR signaling thus potentially affecting host resistance to a myriad of significant diseases such as amebiasis, tuberculosis and bacterial pneumonias [45]. Factors other than genetics, such as nutritional status affect leptin levels. Both obesity and malnutrition are pandemics associated with immunodeficiencies that lead to increased susceptibility to infectious disease [88-90]. Interestingly, both obesity and malnutrition are associated with aberrant leptin levels, obesity causing chronically elevated levels, while malnutrition results in significantly diminished leptin levels. Given the significant impact leptin signaling has on both immune and epithelial cells, the increased rates of infection observed in obese and malnourished individuals suggests that dysregulated leptin signaling may be a critical contributor to the altered immune state in these conditions. Thus, further research into the cellular targets of leptin and the downstream effector functions mediated through LepR are critical in understanding how leptin functions outside of the neuroendocrine axis and affects host susceptibility to disease.

Acknowledgements

We would like to thank the Petri lab members and Lantz Mackey for critical reading of this manuscript. We apologize to those whose excellent work was not able to be included in this review due to space limitations. This work was supported by funding through NIH grant R01 AI026649 (WAP). Nicole Mackey-Lawrence was supported by the Research Training in Digestive Diseases fellowship 2T32DK007769-11.

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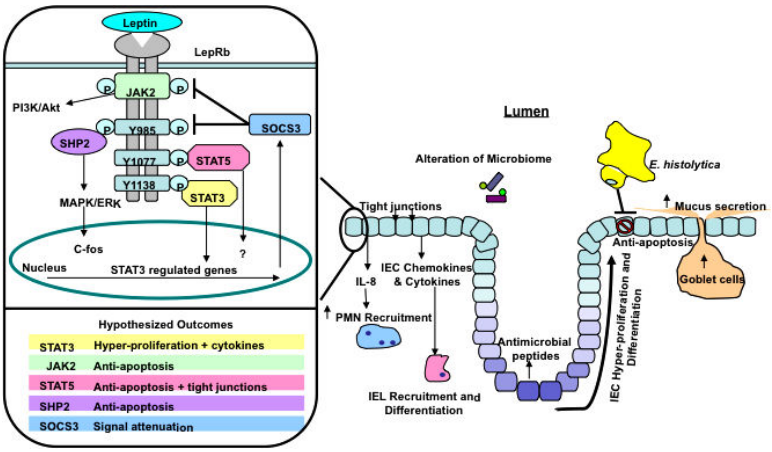

Figure 1. Intestinal Epithelial Cell (IEC) Leptin-mediated Signaling and Protection from Amebiasis

LepRb signaling and hypothesized IEC specific outcomes. Following LepRb mediated activation each signaling molecule is hypothesized to have different effects within IECs. JAK2⇒anti-apoptosis; Tyr985⇒SHP2⇒anti-apoptosis; Tyr985⇒SOCS3⇒signal attenuation; Tyr1077⇒STAT5⇒anti-apoptosis and tight junctions; Tyr1138⇒STAT3⇒hyperproliferation, cytokines and SOCS3. Also depicted are potential leptin-mediated IEC defenses against *E. histolytica*. These include increased goblet cell number and mucus production, induction of anti-apoptotic pathways, proliferation and differentiation of IECs, secretion of antimicrobial peptides, maintenance of tight junctions, expression of chemokines and cytokines, and alteration of the microbiome.

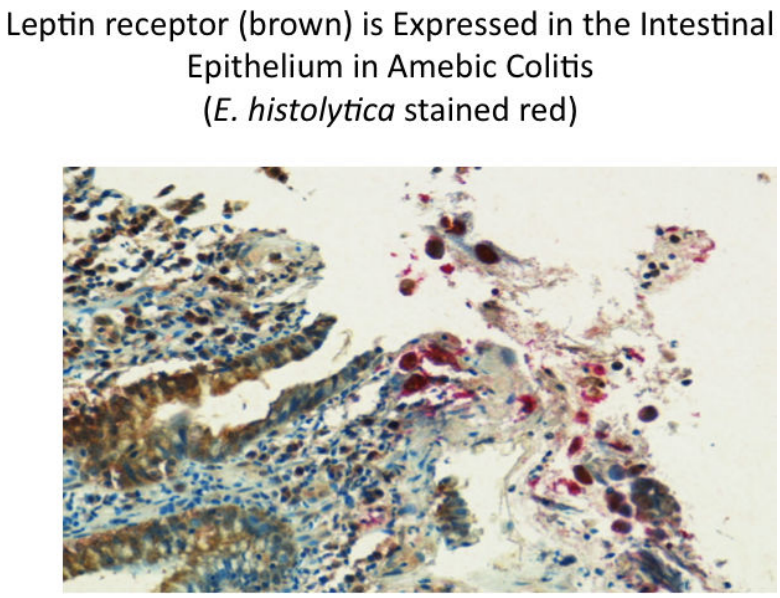

Figure 2. Leptin Receptor is Expressed in the Intestinal Epithelium During Amebic Colitis

Immunohistochemistry of a colonic biopsy from a patient with acute amebic colitis demonstrates LepR staining (brown) of intestinal epithelium and infiltrating white blood cells and invading *E. histolytica* trophozoites (red). Figure credit: Kristine M. Peterson, Robert Gilman, and William A. Petri Jr.

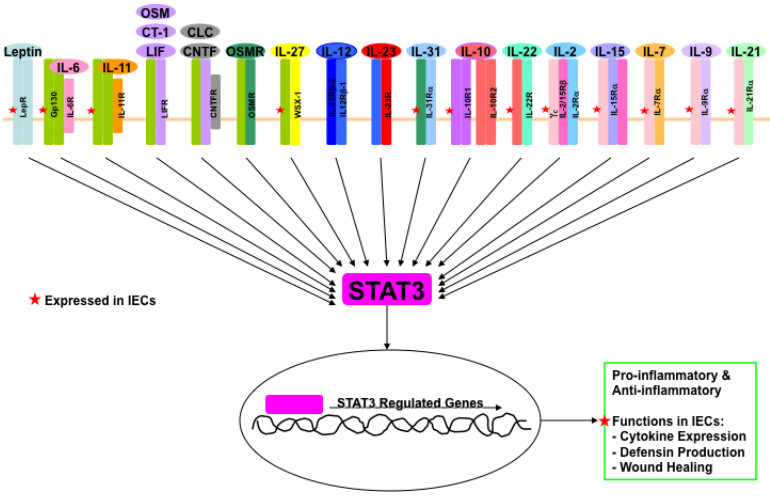

Figure 3. Cytokines That Activate STAT3

Schematic of cytokine receptors that activate STAT3. Cytokines included are leptin, IL-6, IL-11, OSM, CT-1, LIF, CLC, CNTF, OSMR, IL-27, IL-12, IL-23, IL-31, IL-10, IL-22, IL-2, IL-7, IL-9, IL-15 and IL-21. JAK-STAT pathway activation results in phosphorylation of STAT3 and translocation of pSTAT3 to the nucleus where it activates the transcription of target genes. Receptors noted by a red star are known to be expressed in IECs, and are predicted to induce cytokine and defensin (antimicrobial peptide) expression, and augment wound-healing.