Leptin Signaling in Intestinal Epithelium Mediates Resistance to Enteric Infection by *Entamoeba histolytica*

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Abstract

Leptin is an adipocytokine that links nutrition to immunity. Previous observation that a genetic polymorphism in the leptin receptor affected susceptibility to *Entamoeba histolytica* infection led to the hypothesis that leptin signaling plays a protective role during intestinal amebic infection. Here we show that mice lacking the functional leptin receptor developed devastating mucosal destruction after *E. histolytica* infection. Bone marrow chimera experiments demonstrated that leptin receptor expressed on hematopoietic cells was not sufficient to confer resistance. Similarly peripheral knockout of the leptin receptor rendered animals susceptible, indicating that central expression of the leptin receptor was not sufficient to confer protection. The site of leptin action was localized to the gut via an intestinal epithelium-specific deletion of the leptin receptor, which rendered mice susceptible to infection and mucosal destruction by the parasite. Mutation of tyrosine 985 or 1138 in the intracellular domain of the leptin receptor, which mediates signaling through the SHP2/ERK and STAT3 pathways respectively, demonstrated that both were important for mucosal protection. We conclude that leptin-mediated resistance to amebiasis is via its actions on intestinal epithelium rather than hematopoietic cells or the brain, and requires leptin receptor signaling through both the STAT3 and SHP2/ERK pathways.

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Introduction

Malnutrition represents a significant health problem in the developing world. Increasing evidence has supported an epidemiological association between susceptibility to infection and malnutrition 1–5. Since 1999 we have been conducting a prospective case control study to examine the genetic and environmental factors that can increase the risk of diarrheal diseases in Dhaka, Bangladesh. We found that malnourished individuals were more likely to acquire *Entamoeba histolytica*-associated diarrheal illness 4, 5. Given that malnourished children had lower leptin levels than the well-nourished ones 6, we asked whether the increased susceptibility to amebiasis in malnourished individuals could be partly explained by the diminished function of leptin, an adipocyte-derived hormone that acts as a link between nutrition and immunity 3, 7, 8. Supporting a link between leptin and amebiasis, we also found that susceptibility to amebic infection in the children was associated with an amino acid substitution (Q223R) in the extracellular domain of the leptin receptor (Duggal, Guo & Petri, unpublished).

Leptin plays a pleiotropic role in mammalian physiology. Leptin acts centrally to regulate food intake and energy expenditure through its functional long form receptor (OB-Rb; LRb) in the hypothalamus 9, 10. In the periphery, leptin promotes the survival and function of a variety of immune cells 11–19 and acts as a pro-inflammatory cytokine which orchestrates the inflammatory response towards a Th1 phenotype while suppressing Th2 and regulatory T cell functions 20, 21.

This is of particular interest in the setting of *E. histolytica* infection as a Th1 response (specifically, IFN-γ) is a critical component of protective immunity against amebiasis 22–24. An additional potential protective role of leptin against amebiasis is in mucosal immunity, as the leptin receptor is also expressed in the intestinal mucosa 25. Indeed, leptin helps maintain intestinal mucosal intergrity 26, stimulates mucin secretion upon mucosal damage, 27 and modulates intestinal barrier function via its pro-proliferative and anti-apoptotic effects 28, 29.

The leptin receptor belongs to the type I cytokine receptor family 30. Leptin binding to LRb results in the activation and tyrosine phosphorylation of Janus kinase 2 (JAK2), which induces subsequent phosphorylation of conserved tyrosine residues in the intracellular tail of LRb. Each phosphorylated tyrosine residue mediates distinct signals as shown in Fig. 1. Phosphorylated Tyr1138 recruits STAT3, which upon phosphorylation mediates the transcription of Suppressor of Cytokine Signaling (SOCS3) as well as positive effectors of leptin action. Phosphorylated Tyr985 recruits the SH2-containing tyrosine phosphatase (SHP-2) and mediates activation of MAPK/ERK; during prolonged stimulation, phosphorylated Tyr985 also binds SOCS3 and mediates feedback inhibition of LRb signaling 31–33. In addition to the signal pathways coupled to tyrosine residues, some signals emanate directly from Jak2, including the insulin receptor substrate protein (IRS), PI3K/Akt pathway, and a minor component of the ERK pathway 31, 32. It has been reported that PI3K/Akt or SHP2/MAPK/ERK signaling contributes to LRb-mediated anti-apoptotic and pro-proliferative effects 17, 28, 34. LRb Tyr1138-STAT3 and Tyr985-SHP2 signaling in the hypothalamus is crucial for the regulation of neuroendocrine functions such as food

intake, energy balance and reproduction 31–33. How these pathways impact the actions of leptin in the periphery and in mucosal immunity remains under investigated.

In this study, we examined the role of leptin in host resistance to *E. histolytica* infection, and sought to understand the mechanisms by which this adipocytokine confers mucosal protection. By using a series of genetically-engineered mice including those with site-specific disruption/deletion of leptin receptor, we investigated the site of leptin action and the signaling pathways that are important for leptin-mediated protection.

Results

Leptin signaling is important in mucosal resistance to intestinal amebiasis

To test the role of leptin-signaling in host resistance to amebiasis, mice genetically deficient in leptin (B6.V-*Lep^ob^/j*, ob/ob) and in the functional leptin receptor (B6.Cg-m *Lepr^db^/j*, db/db) were tested for susceptibility to intestinal amebiasis. Compared to the highly resistant C57BL/6 wild-type mice, ob/ob and db/db mice were significantly more susceptible to the establishment of *E. histolytica* infection at 48 hours as determined by both cecal amebic antigen and cultural positivity (presented as infection rate) (Fig. 2 **a**). Interestingly, the db/db mice displayed an unusual rapid death phenotype 16 hr after *E. histolytica* infection: up to 50% of the db/db mice were found dead or euthanized before the scheduled termination time (48 hr) due to a moribund state. The mortality rate of db/db mice was significantly higher than that of the wild-type mice (p <0.01 at 48 hr), and approaching significance compared with ob/ob mice that exhibited intermediate level of mortality (p=0.07) (Fig. 2 **b**). In contrast, the early mortality phenotype was not seen in db/db mice sham-challenged with conditioned ameba culture media (Fig. 2 **b**). The pathology of the infected ceca reflected the more severe course of amebiasis in both ob/ob and db/db mice: whereas infected wild type mice exhibited normal mucosa hyperplasia (Fig. 2 **c**, left), the ceca from ob/ob and db/db mice revealed near complete loss of the epithelium, and substantial tissue disruption associated with invading trophozoites (Fig. 2 **c**, middle and right). The sham-infected db/db mice displayed normal-looking cecal histology (data not shown). We concluded that the lack of leptin or functional leptin receptor increased susceptibility to intestinal amebiasis in the first 48 h after infection.

Identification of the target site on which leptin acts to protect

Leptin exerts its pleiotropic effects via leptin receptors expressed on many cell types. So the next step towards understanding the role of leptin in mucosal immunity was to identify the relative importance of leptin receptors expressed on intestinal epithelial cells, immune cells, and the central nervous system. To first address whether immune cells are a major target for leptin action, we generated reciprocal bone marrow chimeras between wild type C57BL/6 (B6) and db/db mice. Specifically, the hematopoietic systems of recipient B6 or db/db mice were ablated by irradiation prior to transplant of bone marrow from db/db or B6 donors respectively. Efficient reconstitution of db/db→B6 and B6→db/db chimeras was confirmed by flow cytometric analysis of PBMCs stained with Thy 1.1 and 1.2 (Fig. 3 **a**). Nine weeks post-transfer, mice were challenged with *E. histolytica* trophozoites. At 48 hr post-challenge, B6→db/db chimeras recapitulated the susceptible phenotype of db/db → db/db mice with

high amebic antigen (Fig. 3 **b**), high culture-positivity rate (Fig. 3 **c**) and high mortality (Fig. 3 **d**). Likewise, db/db → B6 chimeras exhibited the resistance as seen in B6→B6 group (Fig. 3 **b-d**), suggesting that leptin's action on bone marrow-derived immune cells is not sufficient to mediate mucosal resistance to *E. histolytica*.

To delineate the contribution of central versus peripheral leptin signaling to mucosal immunity, we generated mice in which leptin receptor signaling was selectively disrupted in peripheral tissues. Specifically, we crossed mice whose LRb exon 17 is flanked by two loxP sequences (Lepr^flox/flox mice) with transgenic mice expressing a tamoxifen-inducible Cre recombinase (ROSA26^Cre-ERT2/+) mice), to produce Lepr^flox/flox, ROSA26^Cre-ERT2/+) (Cre) mice and Lepr^flox/flox (non-Cre) mice. As exon 17 encodes the Jak2 binding site that is indispensable for downstream LRb signal transduction, deletion of exon 17 results in a disrupted/dysfunctional LRb 35. Because tamoxifen (Tam) has a poor permeability across the blood-brain barrier, Tam treatment of Cre but not control non-Cre mice selectively disrupts leptin receptor signaling in peripheral tissues. A previous report demonstrated that the Tam-induced deletion process was complete shortly after 5-d Tam treatment, and that the pattern and extent of deletion remained essentially unchanged after that 36. The extent of Tam-induced deletion of exon 17 in the brain and cecum was examined at the time when *E. histolytica*-infected mice were sacrificed (day 4-post infection, 22 days after Tam treatment) by PCR and gel electrophoresis, using primers flanking the two loxP sites (Fig. 4 **a**). Extensive deletion of exon 17 was detected in the cecum of Tam-Cre mice (Fig. 4 **a**, "Tam-Cre", "Cec" lanes). No deletion was detected in the brain of Tam-Cre mice (Fig. 4 **a**, "Tam-Cre", "Br" lanes), and no deletion occurred in either brain or cecum of untreated Cre or Tam-treated non-Cre mice (Fig. 4 **a**, "Cre" and "Tam-non-Cre"). To test the susceptibility of Tam-Cre mice to intestinal amebiasis, Tam-treated Cre and non-Cre mice were challenged with *E. histolytica*. Increased susceptibility was seen in the Tam-treated Cre mice compared with the non-Cre controls as determined by culture positivity and amebic antigen (Fig. 4 **b**). Furthermore, disease severity was also increased in Tam-Cre mice as measured by substantial mucosal destruction and inflammation (Fig. 4 **c**, top) while the *E. histolytica*-infected controls mainly exhibited submucosal edema or modest disruption of mucosal architecture with mild infiltration (Fig. 4 **c**, bottom). No histological difference was seen between the uninfected Cre and non-Cre animals before or after tamoxifen treatment (data not shown). These data indicate that leptin receptor signaling in the brain is not sufficient to provide protection against intestinal amebiasis.

To determine whether intestinal epithelium was a direct target of leptin for the mucosal resistance to amebiasis, mice selectively lacking functional leptin receptors in intestinal epithelial cells were generated. Specifically, we crossed mice harboring floxed leptin receptor alleles (Lepr^flox/flox) with mice expressing Cre under the control of the villin promoter (Vil-Cre) 37, generating Lepr^flox/flox, Vil-Cre (Vil-Cre KO) and Lepr^flox/flox (flox/flox control) mice. PCR was performed with genomic DNA from the cecum, liver or the intestine-associated adipose tissue to confirm the tissue specificity of Cre recombination. The deleted allele was detectable in the cecum of Vil-Cre KO but not flox/flox mice. Furthermore, no deletion was detected in non-intestinal tissues of the Vil-Cre KO mice (Fig. 5 **a**). Deletion of leptin receptor was further confirmed by cecal immunohistochemistry: the

flox/flox control mice exhibited intense staining for LR at basolateral and apical membranes of intestinal epithelial cells (Fig. 5 **b** top and middle), while the Vil-Cre KO cecum only revealed background staining at the intestinal lining (Fig. 5 **b** bottom). Vil-Cre KO mice and the non-cre flox/flox were then challenged with *E. histolytica* and sacrificed 4 days later. As shown in Fig. 5 **c**, mice homozygously deleted for *lepr* in the intestine exhibited a statistically higher infection rate than the flox/flox controls as determined by cecal amebic antigen and cultural positivity (7 out of 12 *vs* 1 out of 9, p< 0.05). An extensive mucosal erosion and epithelial loss were observed in the cecum of infected Vil-Cre KO mice, in contrast with the relatively normal histology of the control cecum (Fig. 5 **d**). These results indicated that leptin receptor expressed on the intestinal epithelium was a critical target site for leptin-mediated protection against amebiasis.

Identification of the key leptin receptor signaling pathways for leptin-mediated protection

To examine the contributions of downstream LRb signaling pathways to leptin's protective effect in the setting of *E. histolytica* infection, the relative susceptibility of mice engineered to express mutations in key tyrosine residues (Tyr1138 and Tyr985) in the LRb intracellular tail was evaluated. Mice carrying a serine replacement of Tyr1138 in the leptin receptor (S1138) have disrupted STAT3 signaling, and mice carrying a leucine substitution at Tyr985 (L985) have disrupted SHP-2/MAPK/ERK signaling and SOCS-3-mediated feedback inhibition. Both tyrosine substitutions rendered mice more susceptible to intestinal amebiasis according to the cecal antigen and cultural positivity, although none of the variants presented the early mortality phenotype seen for the db/db mouse (Fig. 6 **a**, **b**). Interestingly, the histopathology differed in these two variants upon infection: the majority of the infected S1138 mice showed a disrupted intestinal mucosa with ulcerative/necrotic lesions in the cecum (Fig. 6 **c**). In contrast, infected L985 mice mainly presented with mucosal hyperplasia and little mucosal destruction (Fig. 6 **d**). Invading trophozoites could be found in some infected S1138 mice along the lamina propria and basement membrane (Fig. 6 **e**, **f** arrows). The naïve S1138 and L985 mice both exhibited normal intestinal morphology without detectable differences (data not shown). These data indicate that both the STAT3 and SHP-2/MAPK/ERK signaling pathways contribute to leptin's protective effects.

Discussion

Our results show for the first time that the adipocytokine leptin plays a protective role in mucosal resistance to amebic infection, and does so primarily via leptin receptors expressed on intestinal epithelial cells. Furthermore, both the Y1138/STAT3 and Y985/SHP2/ERK signal transduction pathways of LRb are required for leptin-mediated protection.

The role of leptin in promoting host resistance to infection has been demonstrated in several studies using leptin- and leptin receptor-deficient mice 38–40. Prior studies implicated the pro-inflammatory effect of leptin on cell-mediated immunity as being important for control of infection. However, leptin is a pleiotropic hormone that regulates the neuroendocrine axis centrally and immune/epithelial homeostasis peripherally 3, 7, 8. While the pro-inflammatory cellular response may contribute to the clearance of the infection in some circumstances, the effects of leptin on prevention of epithelial apoptosis and promoting

tissue repair might be required for mucosal defense against pathogens like *E. histolytica*. In our study, leptin appeared to be an essential factor for host resistance to intestinal amebiasis, as supported by the enhanced susceptibility in ob/ob and db/db mice. Compared to the highly resistant wild type B6 controls, an increase in mortality rate was observed in db/db and ob/ob mice after *E. histolytica* infection, and the mice exhibited profound mucosal destruction in the intestine. Interestingly, although no statistical significance was reached, the receptor deficient db/db mice appeared to be more susceptible to both infection and mortality compared to the leptin deficient ob/ob mice. This finding suggested that the leptin receptor may signal to some extent in the absence of leptin, or the existence of additional ligands other than leptin that might partially compensate the leptin deficiency in ob/ob mice. A similar pattern has been observed for Ciliary Neurotropic Factor (CNTF), where mice lacking the CNTF receptor, rather than mice lacking CNTF, exhibited profound motor neuron deficits at birth 41. Our data, together with the previous observations that ob/ob mice have enhanced sensitivity to endotoxin-induced lethality 42 and organ failure 43, 44, suggest that leptin may be involved in the acute phase response to systemic inflammation and mucosal injury.

The connection between leptin deficiency and disease susceptibility is likely to be complex. The functional leptin receptor (LRb or OB-Rb) is expressed on many immune cell types. Leptin has been shown, for instance, to modulate T cell proliferation, promote Th1 responses, and protect a variety of cell types from apoptosis 3, 8, 11–13, 45. These observations led to the speculation that lack of effects of leptin on immune cells might account for the impaired resistance to amebic infection observed in ob/ob and db/db mice. In order to determine the relative contribution of leptin effects on the immune versus non-immune system, we examined the susceptibility of bone marrow chimeras (BMCs) between db/db and wild-type B6 mice. Both the infection and mortality rates were similar in db/db mice reconstituted with either db/db or wildtype bone marrow, indicating that leptin actions on radiosensitive hematopoietic cells are not sufficient to confer protection. Accordingly, LRb expressed on radioresistant cells, such as neurons or peripheral non-immune cells, may play an essential role in protection.

LRb-expressing neurons in the brain have the potential to regulate susceptibility to infection directly or indirectly. With regard to the former, leptin could act centrally by regulating the activity of nerves projecting from the CNS to immune organs or the intestine for example. Alternatively, leptin's actions could be an indirect secondary effect of the multiple neuroendocrine and metabolic abnormalities that result from lack of central LRb function. For example, lack of central leptin function in ob/ob and db/db mice increases activation of the hypothalamic-pituitary-adrenal axis, leading to increased glucocorticoid and decreased thyroid and growth hormone levels which could indirectly affect immune and intestinal functions important in amebic infection 46. To address the contributions of central leptin signaling to leptin-mediated protection, Tam-Cre mice with an intact leptin receptor present in the brain but disrupted in periphery were generated and tested for their susceptibility to amebiasis. An increase in both susceptibility and disease severity were observed in Tam-Cre mice compared with non-cre littermates, indicating that intact central leptin signaling alone is not sufficient to protect the host from mucosal infection, further pointing to the

importance of intact LRb signaling in the periphery for protection. LRb expression has been detected in the gut epithelium 25, 47, together with the fact that mucosal destruction in db/db mice happens within 24hrs, suggesting that leptin might be acting locally on intestinal epithelial cells to mediate protection. This hypothesis was finally verified by infecting mice harboring a targeted deletion of *lepr* in intestinal epithelial cells (Vil-Cre KO), and showing that they exhibited significantly higher susceptibility and a greater level of mucosal destruction compared to their littermate controls. Taken together, these data demonstrate that leptin-mediated protection from amebiasis is mediated primarily through a direct action on the epithelial cells of the intestinal mucosa.

Some insight into the mechanism by which leptin mediates mucosal protection has been gained from our genetic studies that identified Q223R, a common genetic polymorphism in the leptin receptor gene, to affect the susceptibility to amebiasis (Duggal, Guo & Petri, unpublished). Children with the R allele showed significantly higher susceptibility than subjects homozygous for the Q allele, and this association was further verified in transgenic mice showing that the R allele carriers were more susceptible to amebic infection along with a greater level of epithelial apoptosis and reduced Akt phosphorylation in the gut. The leptin receptor has anti-apoptotic effects via its activation of the PI3K/Akt pathways 17, 28, 29, and *E. histolytica* is known to kill host cells by inducing caspase 3 activation 48, 49. We therefore hypothesize that the ancestral leptin receptor with the favorable Q allele protects the intestinal epithelium from *E. histolytica* induced apoptosis by activating the PI3K/Akt pathway.

Increased susceptibility to amebiasis was also observed in both S1138 and L985 variants. The S1138 variant has impaired LRb-STAT3 signaling and disrupted expression of a variety of STAT3-inducible genes; the L985 mutant on the other hand, has enhanced STAT3 activation due to the loss of inhibitory signaling. It has been shown that the hypothalamic signaling of leptin through STAT3 plays a critical role in regulation of neuroendocrine functions 31, 50, which explains the obesity and infertility phenotype of the S1138 mouse. However, how the LRb-STAT3 signaling might function in periphery remains unclear. Previous studies have shown that the IL-6/gp130/STAT3 signaling axis is important in maintaining intestinal homeostasis as 1) Mucosal inflammatory cells responding to IL-6/ IL-6R trans-signaling contribute to the development of colitis in a STAT3-dependent pathway 51, 52; and 2) Enterocyte-specific STAT3 activation promotes epithelial survival and proliferation 53, 54. As an IL-6 receptor family member, leptin receptor is presumably acting on the intestinal mucosa in a similar pattern. The enhanced susceptibility of S1138 mice to intestinal amebiasis indicated the importance of LRb-STAT3 signaling in leptin-mediated mucosal defense, but the downstream events accounting for the protection remain unknown. Studies are underway to identify the candidate effectors that are inducible by LRb-STAT3 and involved in the local mucosal defense (i.e. anti-apoptosis, pro-proliferation and anti-microbial peptide production).

The blocked inhibitory signaling in the L985 variant has been reported to result in enhanced STAT3 activation and accordingly a "gain-of-function" leptin-hypersensitivity phenotype characterized by low body weight, low circulating leptin, and resistance to diet-induced obesity 32, 33. It has been demonstrated that the strength and duration of STAT3 activity

determines if it is anti- or pro-apoptotic, and enhanced STAT3 signaling due to loss of SOCS3 could induce apoptosis via repressing the PI3K/Akt pathway and reducing Bcl-2 and Cyclin D1 expression 55. This finding could partly explain why we saw an increased susceptibility to amebiasis in the L985 mice with disrupted SOCS3 binding and enhanced STAT3 signaling. Another important pathway through LRb Y985 is SHP2-GRB-MAPK/ERK, whose functional effect in vivo has not been well defined. The uncoupled SHP2 binding to the L985 mutant disrupts MAPK/ERK activation, which has been considered as another pro-survival signal (in addition to PI3K/Akt) and is implicated in the survival of various types of epithelial cells 56–58. Thus the increased susceptibility in L985 mice may be via attenuated MAPK/ERK signaling due to the L985 mutation. Note that the cecal histopathology was different for the L985 and S1138 infected mice (Fig. 6 **c-f**), suggesting the divergent effects of leptin action in control of epithelial resistance.

Taken together, it is possible that multiple leptin signaling pathways are involved in protection against intestinal amebiasis, and the mechanisms by which 223R LRb fails to protect might not be the same for the S1138 and L985 receptors. Further study is necessary to clarify the relationship between STAT3/SOCS3, MAPK/ERK and PI3K/Akt pathways in leptin-mediated resistance to amebiasis.

In conclusion, the data presented here indicate that differential susceptibility to amebiasis can be explained at least in part by variable leptin receptor function; Leptin-mediated resistance is via its actions on intestinal epithelium rather than hematopoietic cells or the brain, and receptor signaling through STAT3 or SHP2/ERK are both involved in leptin-mediated resistance to *E. histolytica* infection. By showing the association of the adipocytokine leptin with susceptibility to a mucosal parasitic infection, and by demonstrating its site of action and signaling pathways, our work provides insights into the mechanisms by which malnutrition compromises the immune system.

Methods

Animals

Six-week-old male C57BL/6J wild-type (B6 WT), leptin-deficient (B6.V-Lep^ob/J, ob/ob) and functional leptin receptor-deficient (B6.Cg-m Lepr^db/J, db/db) mice were purchased from Jackson Laboratories (Bar Harbor, ME). Animals were maintained under specific-pathogen-free conditions at the University of Virginia. All protocols were approved by the Institutional Animal Care and Use Committee.

C57BL/6J mice carrying a replacement of the wild type LRbY1138 with a serine residue (S1138) were generated by Bates et al. as previously described 50. C57BL/6J mice carrying a LRb^985 Tyr→Leu substitution (L985) that replaces the wild type Tyr985 were generated by Björnholm et al as previously described 59. Interbreeding of heterozygous animals yielded wild-type, heterozygous and homozygous mice for this study.

The Tam-Cre mouse model with an intact LRb in the brain but a disrupted LRb in periphery was generated using a tamoxifen (Tam)-inducible Cre-LoxP system 36. Briefly, the *Lepr^flox/flox* mice with LRb exon 17 flanked by two *loxP* sequences 35 were crossed to

$ROSA26^{Cre-ERT2/+}$ transgenic mice, in which a Cre-ER^{T2} fusion gene was introduced into ROSA26 locus. The Cre recombinase ubiquitinously expressed in $ROSA26^{Cre-ERT2/+}$ mice remains inactive until it is bound to tamoxifen (Tam). $ROSA26^{Cre-ERT2/+}$ transgenic mice were backcrossed to $Lepr^{flox/flox}$ mice to produce $Lepr^{flox/flox}$; $ROSA26^{Cre-ERT2/+}$ (Cre mice) and $Lepr^{flox/flox}$ (non Cre mice), segregating in the FVB/NJ background. Target deletion of exon 17 in peripheral tissues was induced by ip administration of Tam (1 mg/d for 5 consecutive days) to 6–8 weeks old Cre mice (designated as Tam-Cre mice). As exon 17 encodes the Jak2 binding site that is indispensable for the downstream LRb signal transduction, deletion of exon 17 results in a disrupted/dysfunctional LRb 35. Because of the poor permeability of the blood-brain barrier to Tam and its metabolites, Cre-ER^{T2} fusion protein in the brain cells remained inactive and thus the brain $Lepr$ remained intact in Tam-treated Cre-Tam mice 36. Tam-treated non Cre littermates and Cre mice with no Tam treatment were used as controls.

Intestine-specific deletion of $Lepr$ was generated by two successive crosses between Vil-Cre transgenic mice (B6 background, The Jackson Laboratories) and $Lepr^{flox/flox}$ mice (kindly provided by J. Friendman). Note that the $Lepr^{flox/flox}$ mice used here had the first coding exon of $Lepr$ flanked by two $loxP$ sites 37. Since the first coding exon contains a signal sequence that is critical for the expression of all LR splicing variants 37, deletion of this exon by Vil-Cre-mediated recombination results in a null allele of LR in the intestine.

Animal model for intestinal amebic infection

The parasites used for the infection model and the method for intracecal inoculation have been described previously 60 23. After infection by *E. histolytica*, mice were monitored closely for general well-being; those who were judged to be in a moribund state (seemingly sick, severely dehydrated, and complete loss of activity) were euthanized before the scheduled termination time, while the rest were sacrificed at 48 hrs (for B6 wild type vs. db/db and ob/ob) or 3–4 days (for S1138, L985, Tam-Cre and Vil-Cre mice) post-challenge.

Upon sacrifice each cecum was longitudinally bisected. Half was placed in Hollande's fixative (Sigma-Aldrich), paraffin embedded, and processed for hematoxylin and eosin staining. The other half of cecum was rinsed with 500 μl PBS; about 200 μl of cecal rinse was assayed for amebic antigen using the *E. histolytica* II ELISA kit (Techlab, Blacksburg, VA) and 300 μl cultured in complete TYI-S-33 medium with supplemental antibiotics for 5 days at 37°C.

Bone marrow chimera

Male C57BL/6 wild type (Thy1.2), C57BL/6 (Thy1.1) and db/db (Thy1.2) mice were used for the bone marrow transplantation. Recipient mice received total body irradiation (1300 rad from a ^{137}Cs source) and were reconstituted i.v. within 4 h with 10^7 bone marrow cells prepared from donor femurs and tibias, and then allowed to engraft for 8 wk. During weeks 0–3 of engraftment, chimeric mice were given trimethoprim/sulfamethoxazole-supplemented water for prophylaxis from opportunistic infection. Four groups were analyzed in this study: db/db mice reconstituted with Thy1.1 B6 bone marrow cells; Thy1.1 B6 mice reconstituted with db/db bone marrow cells; wild type B6 mice reconstituted with

congenic Thy1.1 mouse bone marrow cells; and db/db mice reconstituted with db/db bone marrow cells. Engraftment of hematopoietic cells from donor origin was confirmed on peripheral blood leukocytes at 6 wk post-reconstitution, and on splenocytes and mesenteric lymph node (MLN) cells upon sacrifice, by FACS using conjugated Abs to Thy1.1 and Thy1.2 (BD Biosciences).

Immunohistochemical staining

Immunohistochemical staining for leptin receptor was performed with an ABC kit (Vector Laboratories) per the manufacturer's instruction. Briefly, paraffin-embedded cecal sections were dewaxed with xylene and hydrated through alcohol gradient. Endogenous peroxidases were inactivated by incubating the slides in 0.3% hydrogen peroxide in methanol. After antigen retrieval with the Na-Citrate buffer (10 mM, pH 6.5), the sections were blocked with 5% normal rabbit serum, then incubated with goat-anti-mouse leptin receptor antibody (R&D) for 1 hr at room temperature. Slides were then incubated with the biotinylated rabbit-anti-goat IgG for 30 min, followed by 30 min of incubation with ABC reagent. Freshly made DAB substrate solution was used to develop the sections and counterstaining was performed with hematoxylin (Vector Labs). After dehydration through alcohol gradient and clearing with xylene, slides were mounted with Cytoseal XYL (Richard-Allan Scientific).

Statistics

All results are representative of at least two independent experiments with similar results. Data were analyzed with GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA USA). Mann-Whitney test was performed to compare the means of two groups and Kruskal-Wallis test for comparison of three or more groups. Logrank test was used to compare the difference in survival. P value < 0.05 was considered significant.

Acknowledgments

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colitis-associated premalignant cancer in a murine model. J. Immunol. 2010; 184:1543–1551. [PubMed: 20042582]

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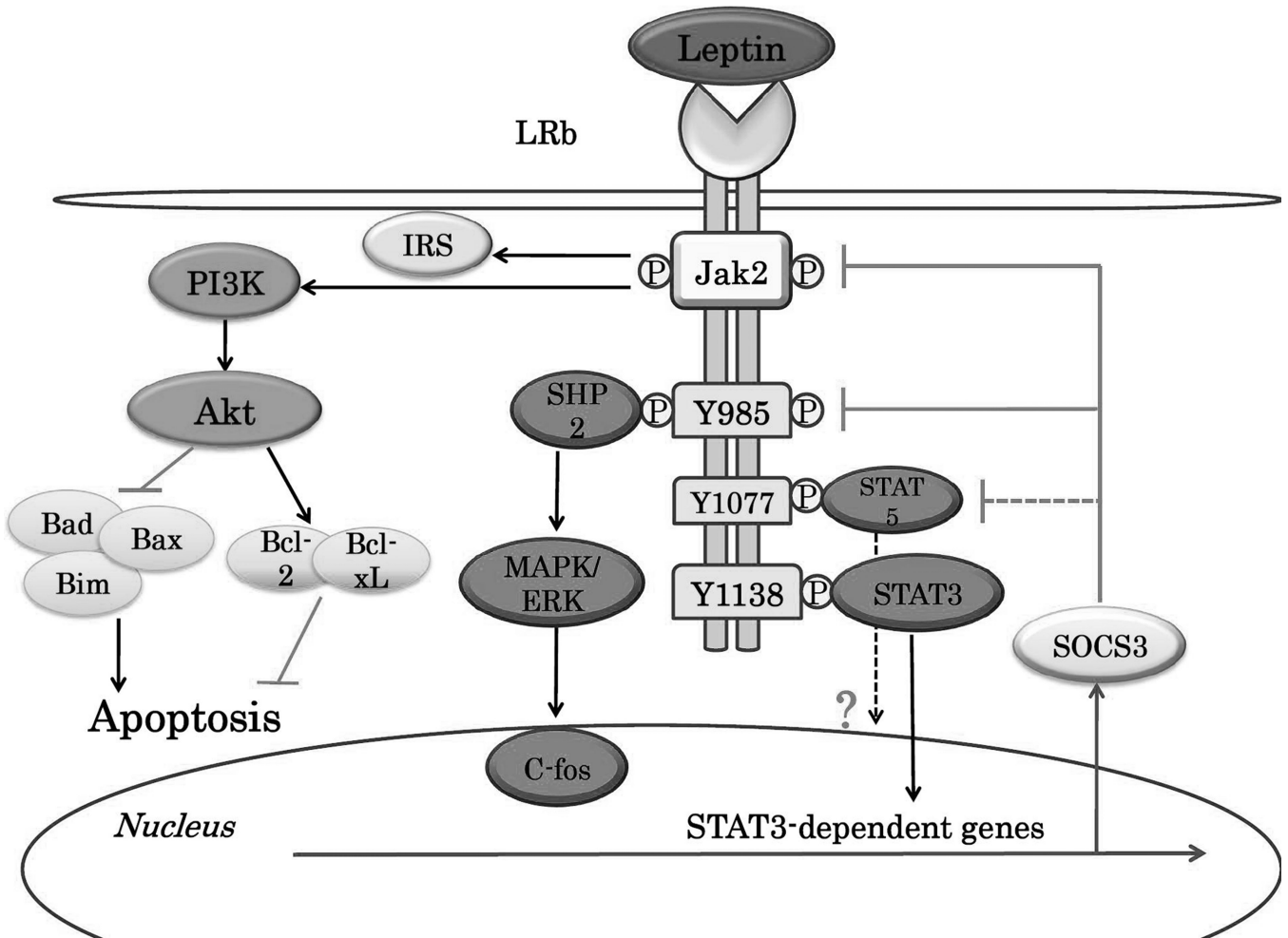
Figure 1*. Long form leptin receptor (LRb) signaling
Leptin binding to the long form LRb results in the activation and tyrosine phosphorylation of Janus kinase 2 (JAK2), which induces subsequent phosphorylation of downstream tyrosine residues (Tyr985, Tyr1077 and Tyr1138) in the intracellular tail of LRb. Phosphorylated Tyr1138 binds and mediates the phosphorylation-dependent activation of signal transducer and activator of transcription 3 (STAT3), which activates transcription of suppressor of cytokine signaling 3 (SOCS3) and other positive effectors of leptin action. Phosphorylated Tyr985 recruits the SH2-containing tyrosine phosphatase SHP-2, which activates the signaling pathway that culminates in extracellular signal-regulated kinase (ERK) activation. During prolonged stimulation, phosphorylated Tyr985 binds SOCS3 to mediate feedback inhibition of LRb signaling. Phosphorylated Tyr1077 recruits STAT5 to induce STAT5-dependent transcription activation; however, its functional effect is less clear. Signals mediated via phosphorylated Jak2 include insulin receptor substrate protein (IRS) and the PI3K/Akt pathway. The PI3K/Akt signaling is important for the LRb-mediated anti-apoptotic and pro-proliferative effects via down-regulating pro-apoptotic (eg. Bad, Bim, Bax and p53) while up-regulating anti-apoptotic/survival factors (eg. Bcl-2, Bcl-XL and Cyclin D).
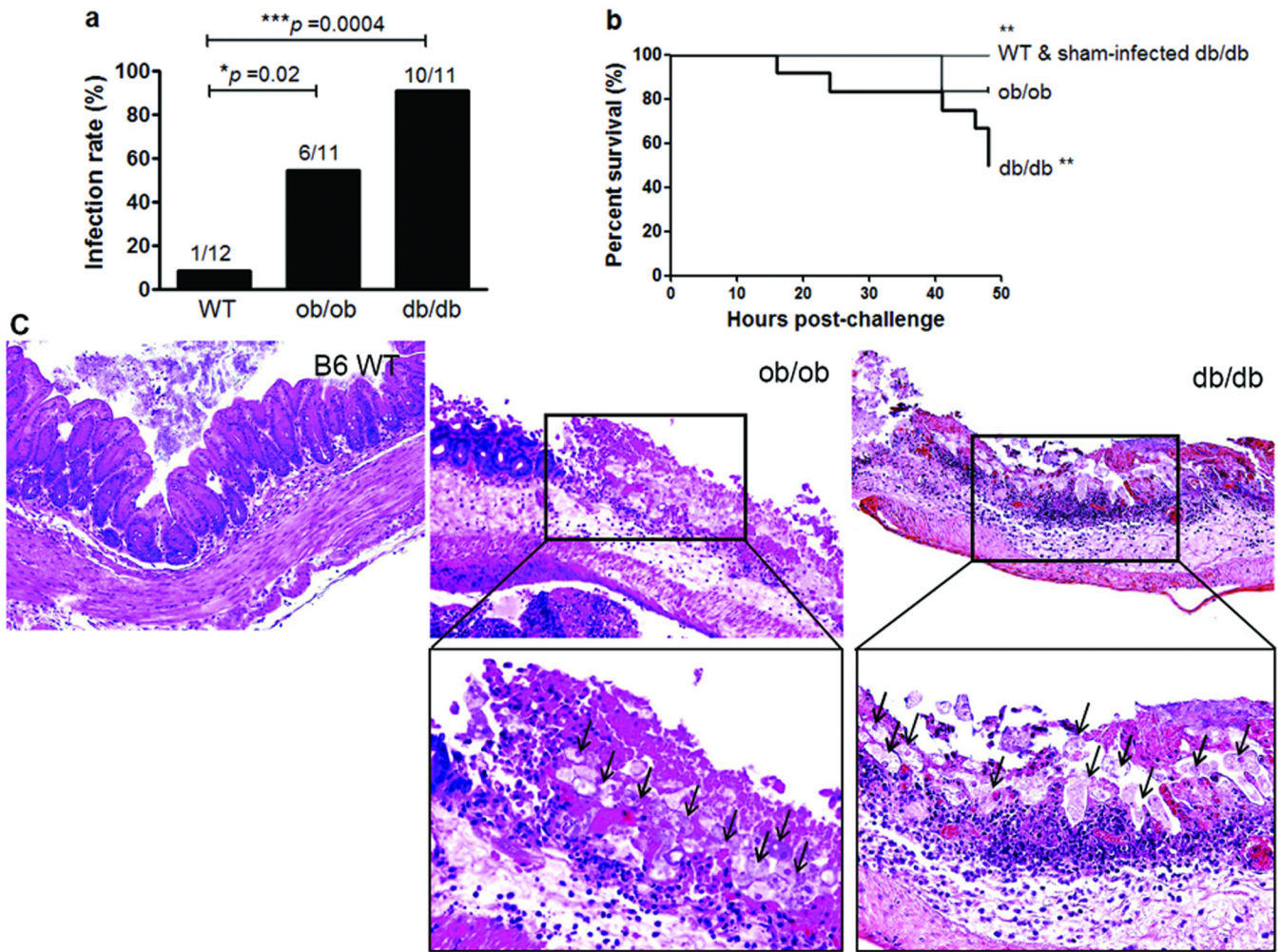*Figure adapted from Febbraio MA, *J Clin Invest*. 2007; 117(4): 841–849.

Figure 2. Deficiency in leptin signaling renders the mice more susceptible to intestinal infection by *E. histolytica*

(a) Mice genetically deficient in leptin (ob/ob) and in functional leptin receptor (db/db) were more susceptible to intestinal infection with *E. histolytica* as determined by cecal amebic antigen and cultural positivity (presented as infection rate); **(b)** An unusual moribund phenotype was observed in db/db and ob/ob mice that started within 24 h post challenge. The Kaplan-Meier survival curves depict time to death for mice challenged with *E. histolytica* in hours. The experiment ended after 48 hours. Logrank test for B6 versus db/db: p = 0.005 (no B6 WT or sham-challenged db/db mice died, 50% of infected db/db mice died); db/db versus ob/ob: p = 0.09 (17% of ob/ob mice died). For each group n = 12. **(c)** Mucosal hyperplasia was observed in the wild type mice (B6) that were infected (left), while ob/ob (middle) and db/db (right) mice had extensive epithelial loss and trophozoite invasion (arrows). Images are 100× except insets (bottom row) are 200×.
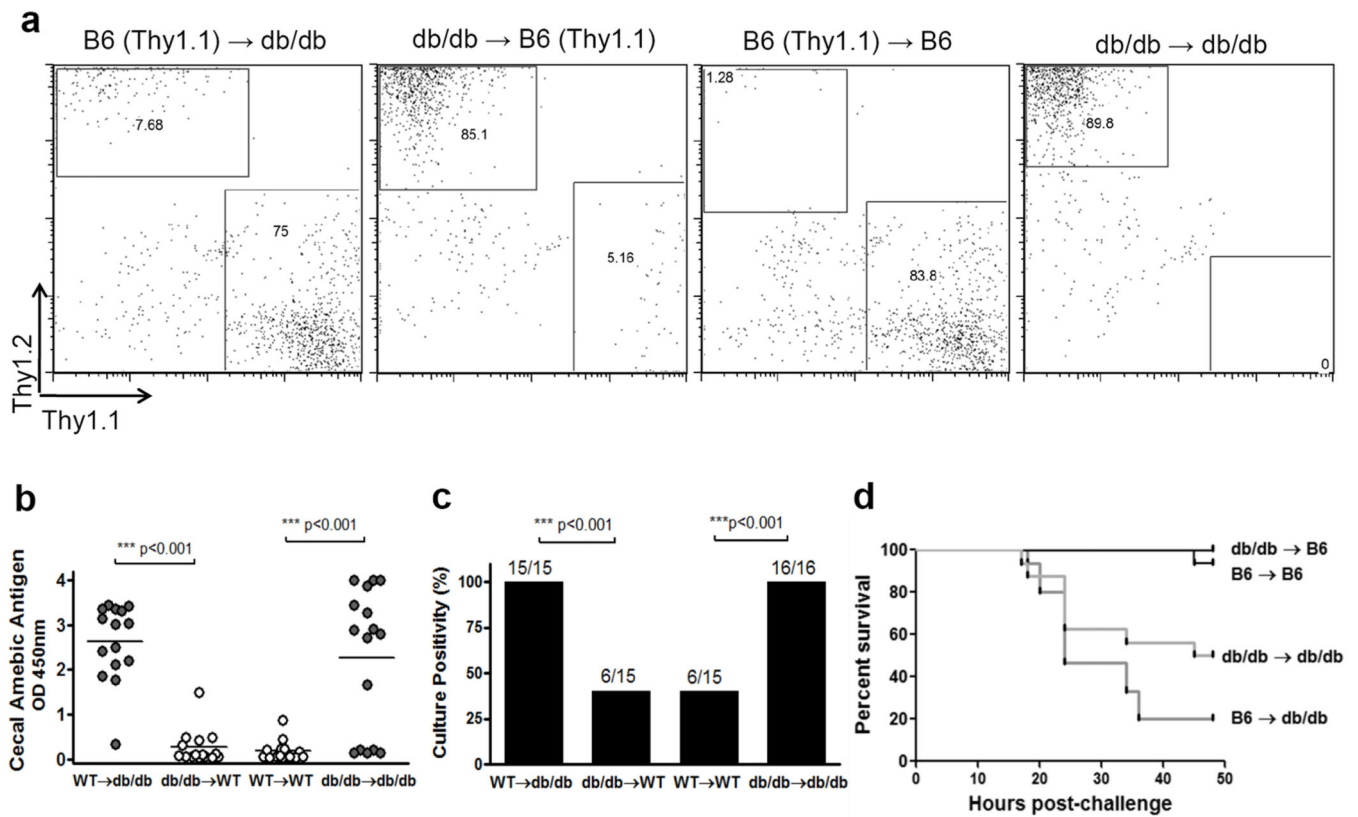
Figure 3. Leptin-mediated resistance to *E. histolytica* was not restored by transplant of a wild type marrow into db/db mice

Recipient mice were reconstituted i.v. with bone marrow cells prepared from donor femur and tibia. Engraftment of donor hematopoietic cells were confirmed by FACS for Thy1.1 and Thy1.2 at 8 wk post-transfer on PBMCs **(a)** (n = 8/group, one representative mouse per group shown). At 9 wk post-transfer, mice were challenged intracecally with *E. histolytica* trophozoites and sacrificed after 48 hr for evaluation of infection according to cecal amebic antigen **(b)**, culture positivity **(c)**, and early mortality **(d)**. Logrank test for difference in survival: (db/db recipients *vs* WT recipients: p <0.0001; db/db to db/db vs WT to db/db: p =0.11. N = 16/group.
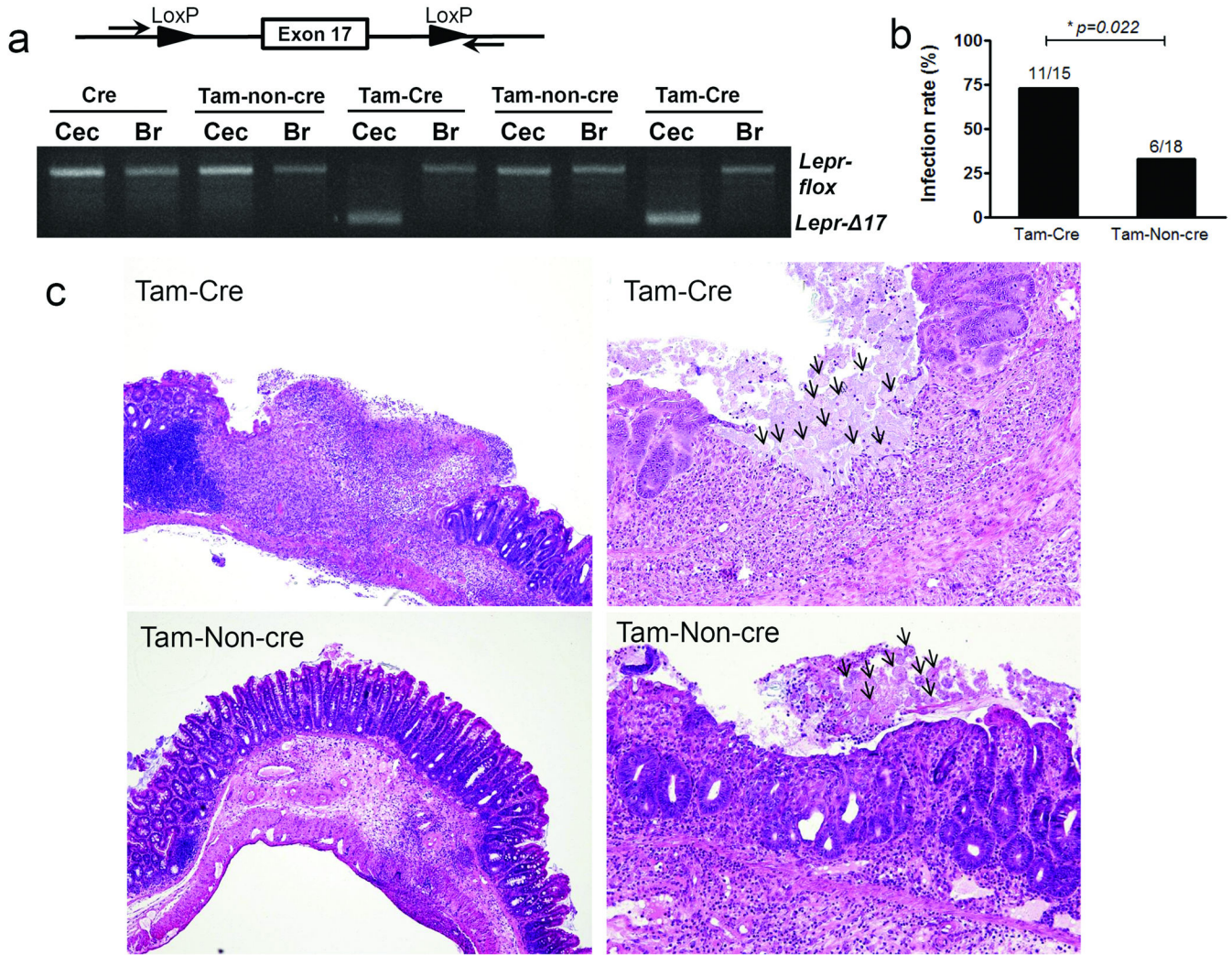
Figure 4. Peripheral disruption of leptin receptor resulted in increased susceptibility to *E. histolytica*

Lepr^flox/flox^/Cre (+) and Lepr^flox/flox^/Cre (−) mice were treated with tamoxifen for 5 days. **(a)** After tamoxifen treatment, genomic DNA was extracted from the brain (Br) and the cecum (Cec), and PCR was performed using primers recognizing the flanking sequences of the LoxP to examine the deletion of leptin receptor exon 17. **(b)** Tam-Cre mice with a peripheral disruption of the leptin receptor and control mice (Tam-Non-cre) with intact leptin receptor were intracecally infected with *E. histolytica*, and susceptibility to amebiasis was assessed by cecal amebic antigen and cultural positivity (presented as infection rate). **(c)** Representative histopathology of infected ceca from Tam-Cre (top) mice showed more severe destruction of mucosal architecture with large ulcerations and extensive infiltration compared to the infected Tam-non cre controls (bottom). Amebic trophozoites are indicated by arrows. Original magnifications: left 40×, right 100×.
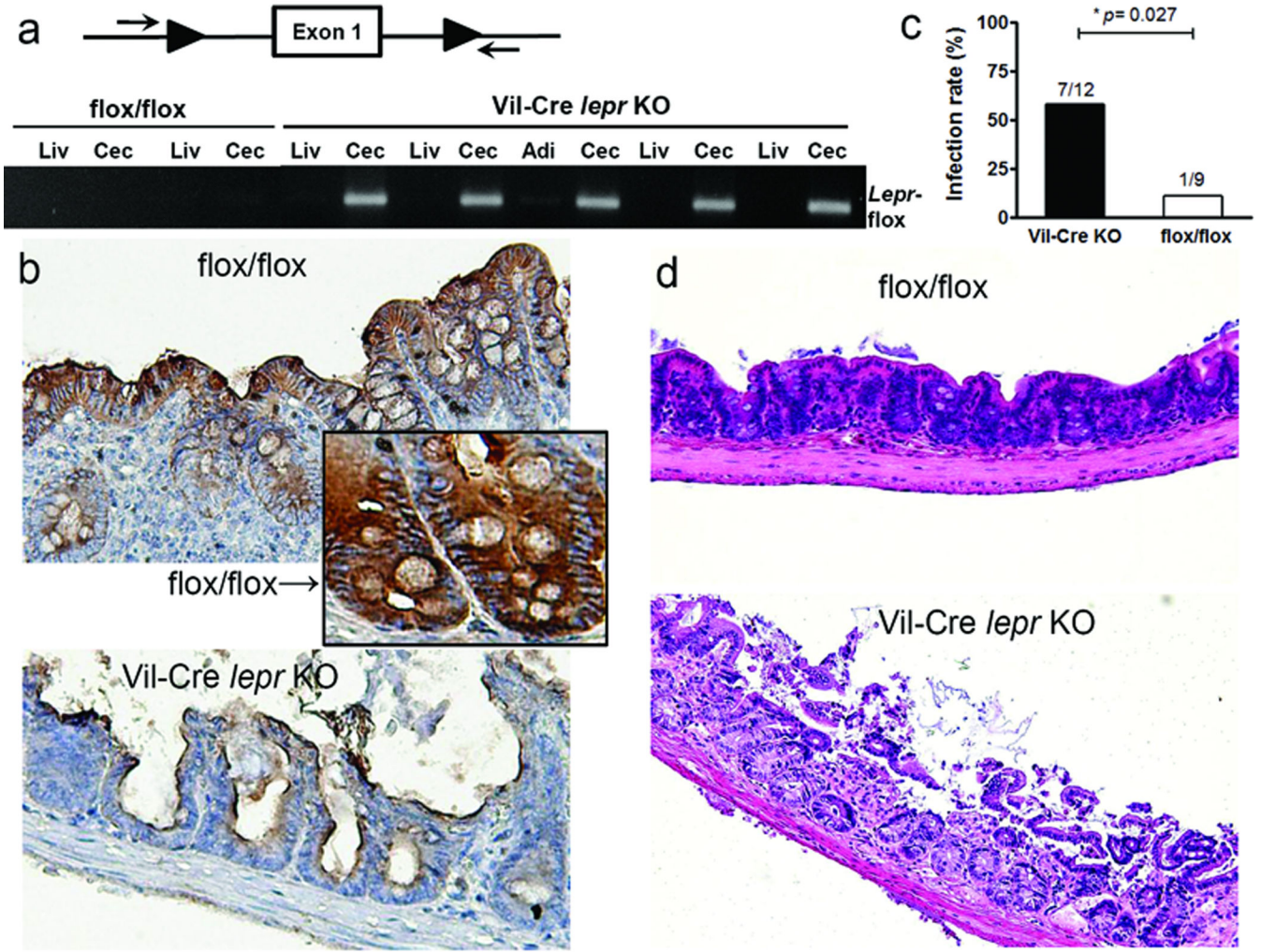
Figure 5. Intestine-specific deletion of leptin receptor rendered mice susceptible to amebiasis
Mice engineered with loxP sites flanking the first coding exon of the leptin receptor were mated with mice expressing the Vil-Cre recombinase. **(a)** Intestine-specific deletion of exon 1 was confirmed by PCR using primers flanking the LoxP sites (►). The primers amplified a single band from the *lepr* allele, while no product was generated from the control WT or floxed *lepr* allele. Genomic DNA was extracted from the cecum (Cec), liver (Liv) or the adipose tissue (Adi) associated with the intestine to examine the intestine-specific deletion of *lepr*. **(b)** Deletion of leptin receptor was further confirmed by its absence as demonstrated by immunohistochemistry in the Vil-Cre KO (bottom), but not the flox/flox control (top) cecum. Abundant basolateral staining of leptin receptor was seen in the flox/flox cecum (insert). Original magnifications: 200×; insert: 400×. **(c)** Vil-Cre KO mice and the non-cre flox/flox controls were intracecally challenged with *E. histolytica* and sacrificed 4 days after challenge. Intestinal infection was determined by culture positivity and cecal amebic antigen. **(d)** Representative cecal histopathology demonstrated substantial mucosal erosion and epithelial loss in the infected Vil-Cre KO mice but none of the flox/flox controls. Original magnifications: 100 ×.
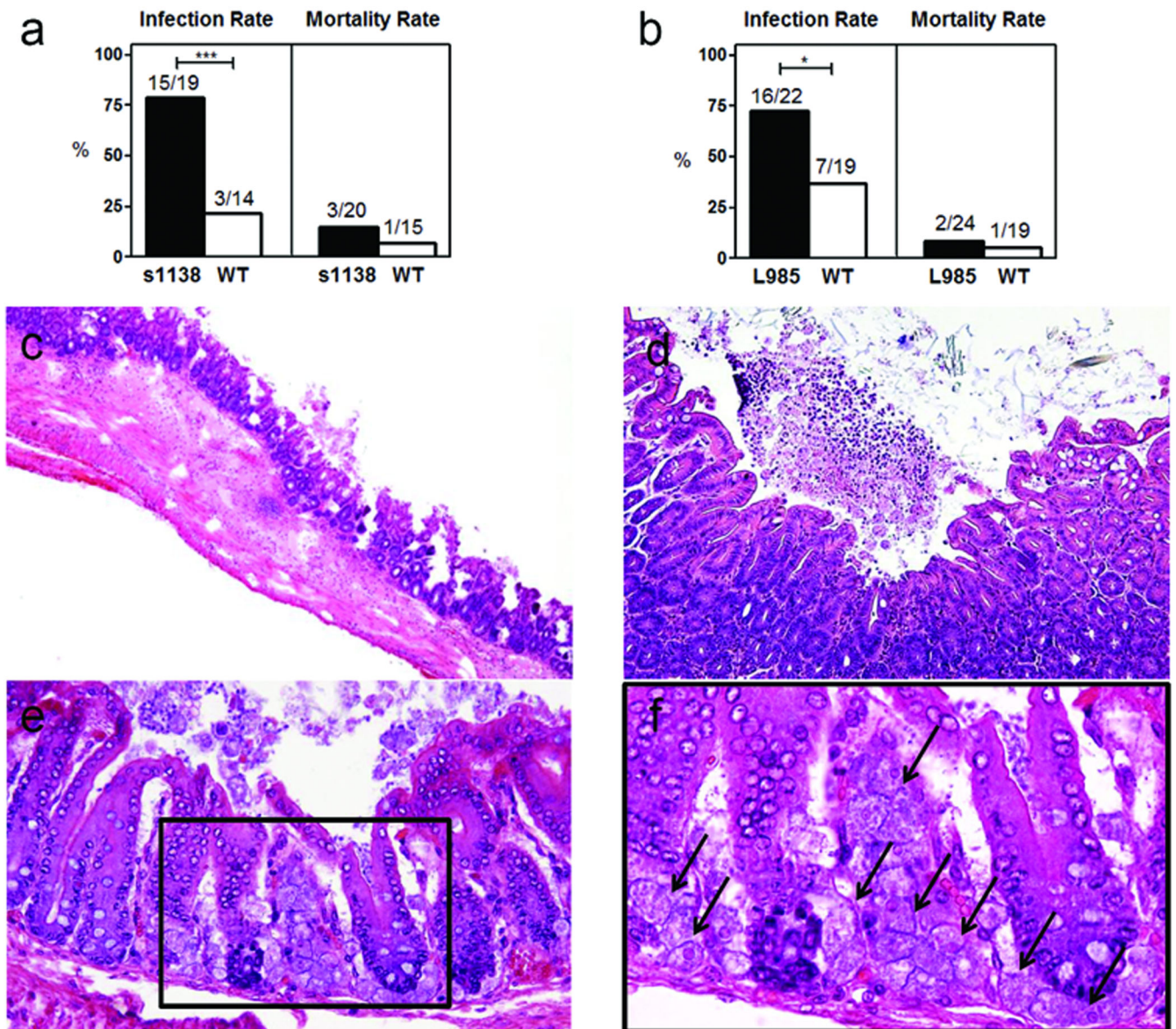
Figure 6. Signaling pathways through tyrosine 1138 and tyrosine 985 were both important in leptin-mediated protection

Mice with tyrosine residue substitutions, S1138 **(a)** or L985 **(b)** on the leptin receptor were more susceptible to intestinal infection with *E. histolytica* compared to wild type littermates, $p = 0.001$ and 0.02, respectively; Infection rate was determined by both cecal amebic antigen and cultural positivity. None of the variants had the rapid death phenotype as seen in the db/db mice. * $p<0.05$; *** $p<0.001$ compared to wild type littermates. Most of the infected S1138 mice showed tissue destruction including mucosal erosion, ulceration, and tissue necrosis **(c)**; Infected L985 mice were mainly manifested with mucosal hyperplasia with very little mucosal destruction **(d)**. Loss of epithelial integrity was seen in some S1138 mice with aggressively invading trophozoites (arrows) along the lamina propria and basement membrane **(e, f)**. Original magnification: C: 40×, D: 100×, E: 200×, F: 400×.