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Leptin Metabolically Licenses T Cells for Activation To Link Nutrition and Immunity

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Immune responses are highly energy-dependent processes. Activated T cells increase glucose uptake and aerobic glycolysis to survive and function. Malnutrition and starvation limit nutrients and are associated with immune deficiency and increased susceptibility to infection. Although it is clear that immunity is suppressed in times of nutrient stress, mechanisms that link systemic nutrition to T cell function are poorly understood. We show in this study that fasting leads to persistent defects in T cell activation and metabolism, as T cells from fasted animals had low glucose uptake and decreased ability to produce inflammatory cytokines, even when stimulated in nutrient-rich media. To explore the mechanism of this long-lasting T cell metabolic defect, we examined leptin, an adipokine reduced in fasting that regulates systemic metabolism and promotes effector T cell function. We show that leptin is essential for activated T cells to upregulate glucose uptake and metabolism. This effect was cell intrinsic and specific to activated effector T cells, as naive T cells and regulatory T cells did not require leptin for metabolic regulation. Importantly, either leptin addition to cultured T cells from fasted animals or leptin injections to fasting animals was sufficient to rescue both T cell metabolic and functional defects. Leptin-mediated metabolic regulation was critical, as transgenic expression of the glucose transporter Glut1 rescued cytokine production of T cells from fasted mice. Together, these data demonstrate that induction of T cell metabolism upon activation is dependent on systemic nutritional status, and leptin links adipocytes to metabolically license activated T cells in states of nutritional sufficiency. ***The Journal of Immunology*****, 2014, 192: 136–144.**

Nutritional status is well known to regulate immune function, as obesity is associated with increased inflammation, whereas malnutrition is associated with immune deficiency and increased susceptibility to infection (1–3). Although the links between nutrition and adaptive immunity remain poorly understood, systemic energy balance between the demands of the immune system and other life-critical systems, such as cardiovascular, respiratory, and neurologic, must be maintained and prioritized. Immune responses can consume significant nutrients. Although resting T cells use an oxidative metabolism primarily for ATP generation, effector T cell activation sharply increases the demand for macromolecule biosynthesis (1). To meet this need, activated effector T cells dramatically increase glucose uptake and metabolism to activate a program of aerobic glycolysis reminiscent of cancer cells (4, 5). It has recently been demonstrated that regulatory pathways controlling T cell metabolism are intimately linked to T cell function (4, 6, 7). Increased expression of the glucose transporter Glut1 is sufficient to increase T cell cytokine production and proliferation (5). Moreover, activated effector T cells rely on glucose availability, glucose uptake, and aerobic glycolysis to survive and function properly (5, 8). How T cell metabolic demands are regulated by systemic nutritional status, however, is not clear.

The adipokine leptin may play a key role to balance energy expenditure and nutritional status in the immune system. Leptin is secreted in proportion to adipocyte mass and is best known for its role in regulating body weight and energy expenditure via signaling in the hypothalamus, where full-length leptin receptors (LepRs) are highly expressed (9, 10). However, leptin is also a critical regulator of immunity and functions as a proinflammatory cytokine (11, 12). Leptin deficiency in both mouse and human results in immune defects characterized by decreased total T cell number, decreased $CD4^+$ T cell number, and a skewing away from a Th1 and toward a Th2 phenotype, resulting in protection against certain forms of autoimmunity and increased susceptibility to intracellular infections (13–16). Both the metabolic and immune defects in leptin deficiency are reversed following treatment with recombinant leptin protein (17–19); however, the mechanisms of leptin regulation of immunity and T cell function are uncertain (20, 21).

The LepR is a member of the class I cytokine receptor family and is upregulated on T cells following activation (22, 23). Signaling via the LepR results in increased PI3K/Akt activity, Jak2/STAT3 activation, and MAPK signaling (24–27). Leptin has also been found to activate mammalian target of rapamycin (mTOR) complex 1 in regulatory T cells (Treg) and correlate with hyporesponsiveness and decreased proliferation of Treg (28). Many of these signaling molecules, particularly PI3K/Akt and mTOR complex 1, have been implicated in the regulation of T cell metabolism (1). Previous studies suggest that leptin exerts effects on T cell number and function both by direct signaling through LepRs expressed on the T cell and indirectly through influences on

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Abbreviations used in this article: $db^{-/-}$, leptin receptor–deficient; Glut1 Tg, transgenic expression of Glut1; LepR, leptin receptor; *LepR-fl*, leptin receptor floxed; mTOR, mammalian target of rapamycin; Treg, regulatory T cell; wt, wild-type.

the T cell environment (29–33). Direct leptin signaling may enhance the production of Th1 type cells, promoting inflammation, stimulating lymphocyte proliferation, and protecting against lymphocyte apoptosis (11, 32, 34). No role for leptin in T cell metabolism, however, has been reported.

In this study, we show that leptin is essential to link T cell metabolism to nutritional status and balance energy expenditure and immunity. Fasting-induced hypoleptinemia led to persistent T cell metabolic and activation defects. We found leptin was required for activated effector, but not Treg, cells to upregulate the glucose transporter Glut1 to support glucose uptake and metabolism required for proliferation and inflammatory cytokine production. Defects in glucose metabolism and function of activated peripheral T cells from fasted mice were rescued by leptin given either in vitro to isolated T cells in culture or in vivo to fasted animals. Importantly, direct rescue of glucose uptake with a Glut1 transgene also overcame functional defects induced by fasting and hypoleptinemia. These results show that leptin provides a crucial metabolic licensing signal to allow activated T cells to increase glucose metabolism, thus linking nutritional status, cellular metabolism, and immunity.

Materials and Methods

Mice

C57BL/6J, LepR mutant ($db^{-/-}$), LepR floxed (*LepR-fl*), Lck-Cre, CD4-Cre, and Rag1 knockout mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Lck-Cre–transgenic mice were crossed with *LepR-fl* mice to generate *LepR-fl/fl Lck-Cre*$^+$ mutants, *LepR-fl/*$^+$ *Lck-Cre*$^+$ heterozygotes, and *LepR*$^{+/+}$*Lck-Cre*$^+$ controls. CD4-Cre–transgenic mice were crossed with *LepR-fl* mice to generate *LepR-fl/fl CD4-Cre*$^+$ mutants and *LepR*$^{+/+}$*CD4-Cre*$^+$ controls. Glut1 transgenic (Glut1 Tg) mice were previously described (5). Mice were bred and housed under specific pathogen-free conditions at Duke University Medical Center. All studies were performed under protocols approved by the Institutional Animal Care and Use Committee. For fasting studies, mice were deprived of food for up to 48 h, but had full access to water. Where indicated, recombinant leptin (R&D Systems, Minneapolis, MN) was injected at a concentration of 1 μg/g body weight, twice daily for 48 h while fasting; PBS of equal volume was injected into control mice. Experiments were performed using sex-matched mice between 8 and 24 wk of age.

Cell purification and culture

T cells were purified from spleen and mesenteric lymph nodes by negative selection (StemCell Technologies, Vancouver, BC, Canada), and cells were cultured in RPMI 1640 (Mediatech, Washington, D.C.) supplemented with or without 1 or 10% FBS (Gemini Bio-Products, West Sacramento, CA), L-glutamine (Invitrogen, Chicago, IL), penicillin-streptomycin (Invitrogen), HEPES buffer solution (Life Technologies, Carlsbad, CA), and 2-ME (Sigma-Aldrich, St. Louis, MO). T cell activation was induced by either culturing purified T cells on anti-CD3ε (clone 145-2C11) and anti-CD28 (clone 37.51)–coated plates (eBioscience, San Diego, CA) or coculturing with macrophages. For macrophage T cell cocultures, macrophages were derived from murine bone marrow and cultured in RPMI 1640 with addition of 3 ng/ml GM-CSF (eBioscience) for 7 d. Bone marrow cultures were washed, and macrophages were trypsinized, replated, and treated with LPS from *Escherichia coli* O127:B8 (Sigma-Aldrich) at 100 μg/ml for 1 h, followed by addition of freshly purified T cells. T cell macrophage cocultures were cultured with anti-CD3 (eBioscience) at 0.01 μg/ml for 1 d. Nonadherent cells were removed and replated for 1 h. Cells that remained nonadherent were >90% T cells and subject to further analysis. For Th17 and Treg subset generation, CD4$^+$ T cells were stimulated on irradiated splenic feeder cells (3000 rad) with 2.5 μg/ml anti-CD3 Ab at a ratio of 1:5. The Th17 subset was generated by culture with TGF-β (2 ng/ml; R&D Systems) and IL-6 (20 ng/ml; eBioscience); the Treg subset was generated by culture with TGF-β alone (3 ng/ml). On day 3 poststimulation, cells were split 1:2, washed, and replated with IL-2 alone for an additional 2 d. Where indicated, recombinant leptin (R&D Systems) was added into media at a concentration of 250 ng/ml (15.6 nM).

Flow cytometry and ELISA

Proliferation was determined by staining T cells with CFSE (Invitrogen) before culture and analyzed by dilution of fluorescent dye flow cytometrically. All flow cytometry was performed on an MACSQuant Analyzer (Miltenyi Biotec, Auburn, CA) and analyzed with FlowJo software (Tree Star, Ashland, OR). ELISAs were performed on supernatant from T cell cultures using rat Abs against murine IL-2 and IFN-γ (BD Pharmingen, San Jose, CA) and recombinant standards as previously described (5). Leptin ELISA was performed using Leptin Mouse Quantikine ELISA kit (R&D Systems) as per the manufacturer's instructions.

Metabolism assays

Assays for glucose uptake and glycolytic flux were performed as previously described (5, 6). Oxygen consumption rates and extracellular acidification rates were measured with an extracellular flux analyzer (Seahorse Bioscience, North Billerica, MA).

Quantitative real-time PCR and immunoblot analysis

Total mRNA was isolated from mouse T cells using TRIzol (Invitrogen), and cDNA was synthesized from RNA using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). Quantitative PCR was performed using iQ SYBR Green Supermix (Bio-Rad) and the following primers: *glut1* forward, 5′-AGC CCT GCT ACA GTG TAT-3′ and *glut1* reverse, 5′-AGG TCT CGG GTC ACA TC-3′; and *lepR* forward, 5′-GCT CTT CTG ATG TAT TTG GAA ATC-3′ and *lepR* reverse, 5′-ACC TGA TAT TGA AGC GGA AAT GG-3′. All samples were normalized to β_2-microglobulin mRNA levels. For immunoblotting, cells were lysed for 1 h on ice in 1% Triton X-100 and 0.1% SDS containing protease inhibitors (BD Pharmingen). Equivalent protein concentrations were loaded on SDS-PAGE gels (Bio-Rad) and probed with primary rabbit anti-Glut1 (Abcam, Cambridge, MA), followed by secondary anti-rabbit HRP (BD Pharmingen) and ECL (Thermo Scientific, Waltham, MA) for visualization.

Bone marrow transplants

Bone marrow was isolated from C57BL/6J and $db^{-/-}$ donor mice and injected via the tail vein into Rag1 knockout recipients that had been irradiated the day prior. Transplanted mice were maintained on antibiotic (Septra; Monarch Pharmaceuticals) water. Following 18 wk of reconstitution, recipient mice were sacrificed, and T cells were isolated from spleen and lymph nodes, as described above.

Statistical analysis

Data are presented as mean ± SD and were analyzed using a two-tailed Student *t* test. A confidence level of $p < 0.05$ was considered statistically significant for all data.

Results

Fasting leads to persistent functional and metabolic T cell defects

Acute starvation and malnutrition can sharply reduce circulating leptin levels and are associated with immune defects (35–37). The mechanisms by which nutritional status is linked to energy-costly immune responses, however, are largely unknown. Effector T cells are highly dependent on glucose uptake and metabolism. To examine the role of fasting on T cell metabolism and function, wild-type (wt) C57BL/6J mice were fasted or fed ad libitum for 48 h. As previously described (37), fasting decreased serum leptin levels (Fig. 1A) and led to lower thymocyte and peripheral total T cell numbers (Supplemental Fig. 1). Remaining T cells were then isolated from control and fasted mice, cultured in complete media, and activated with Abs to CD3 and CD28 for metabolic and functional studies.

Despite culture in nutrient-rich media for 48 h, peripheral T cells isolated from fasted mice had persistent functional defects after stimulation. T cells from fasted mice had decreased ability to secrete IL-2 and IFN-γ compared with T cells isolated from fed controls stimulated under identical conditions (Fig. 1B, 1C). Moreover, whereas resting T cells from fasted or fed mice had equivalent levels of glucose uptake and glycolysis (Fig. 1D, 1E), activated peripheral T cells from fasted animals failed to fully increase glucose metabolism. Fasted T cells had an inability to upregulate glucose uptake (Fig. 1D) and glycolytic flux (Fig. 1E)

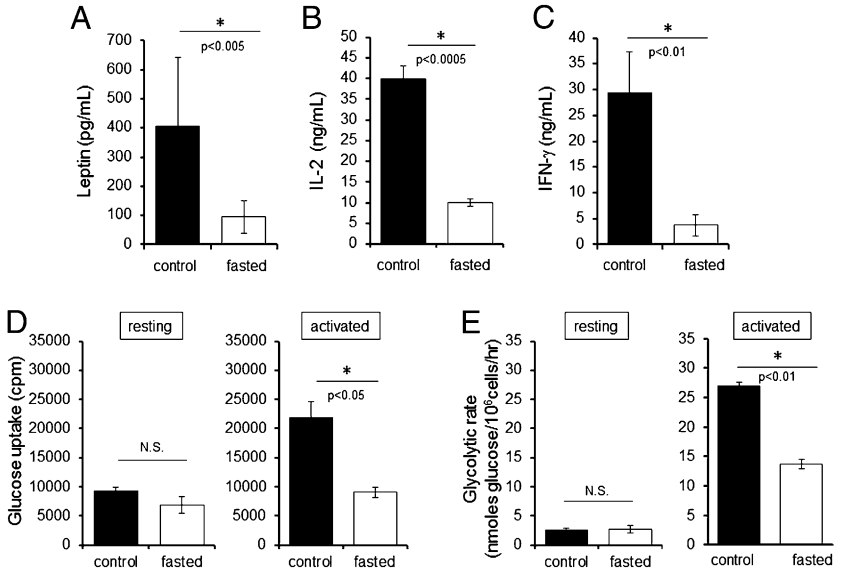

FIGURE 1. Fasting-induced hypoleptinema is associated with persistent functional and metabolic T cell defects. (**A**) wt C57BL/6J mice were either fasted for 48 h or allowed to feed ad libitum. All mice had full access to water. At the end of the fast, serum was collected from blood, and circulating leptin levels were measured by ELISA. Data are representative of five independent experiments, three mice per group. (**B** and **C**) Peripheral T cells from control and fasted C57BL/6J mice were then cultured in complete media with full nutrients and activated with Abs to CD3 and CD28 for 48 h, after which IL-2 and IFN-γ were measured in culture supernatants by ELISA. Data are representative of six independent experiments, three mice per group. (**D**) Glucose uptake was assessed by ^{3}[H]2-deoxyglucose uptake in both resting and activated peripheral T cells. Data are representative of two independent experiments for glucose uptake, three mice per group. (**E**) Glycolytic flux was determined by the generation of tritiated water by enolase in both resting and activated peripheral T cells. Data are representative of two independent experiments for glycolysis, three mice per group.

upon activation in complete media compared with T cells isolated from fed controls. These data show that fasting leads to reduced leptin levels and a persistent T cell defect in the ability to upregulate cellular metabolism during activation, even when nutrients are abundant. Nutrient availability itself, therefore, is not the critical regulatory element in this setting to allow metabolic reprogramming for T cell activation.

Leptin is required for activated T cell proliferation and inflammatory cytokine production

Given that hypoleptinemia was associated with fasting, we sought to determine if leptin could act to directly influence T cell function and metabolism. We first examined the ability of leptin to modulate inflammatory cytokine production in peripheral T cells. wt C57BL/6J CD4$^+$ T cells were purified and activated with Abs to CD3 and CD28 in serum-free media in the presence or absence of exogenous leptin. Full activation of T cells with anti-CD3 together with anti-CD28 costimulation resulted in a 2-fold increase in IL-2 and IFN-γ production in the absence of leptin. Although leptin had no effect on T cells stimulated only through anti-CD3, provision of exogenous leptin augmented inflammatory cytokine production of T cells fully activated with both anti-CD3 and anti-CD28 (Fig. 2A, 2B). These data are consistent with previous reports that LepR is induced after T cell activation (22) and promotes inflammatory cytokine production. In addition, these data demonstrate that leptin requires stimulation of the TCR together with costimulation.

CD4$^+$ T cells were next isolated from wt ($db^{+/+}$) and LepR-deficient ($db^{-/-}$) mice and activated in coculture with bone marrow–derived LPS-activated macrophages to provide costimulatory signals. Leptin is available from serum in culture media in these experiments. Activation of $db^{-/-}$ CD4$^+$ T cells resulted in decreased IFN-γ production as compared with $db^{+/+}$ CD4$^+$ T cells (Fig. 2C). Additionally, although activation of wt $db^{+/+}$ CD4$^+$ T cells resulted in robust proliferation, as indicated by dilution of CFSE, LepR-deficient $db^{-/-}$ CD4$^+$ T cells showed only modest proliferation (Fig. 2D). Thus, LepR signaling is required for normal proliferation in activated peripheral T cells in vitro. Altogether, these results demonstrate that leptin promotes T cell proliferation and inflammatory cytokine production after activation.

Leptin signaling is required for normal rates of glucose uptake, glycolysis, and mitochondrial respiration in activated T cells

Activated T cells rely on increased glucose metabolism to survive, proliferate, and produce cytokines; therefore, changes in T cell metabolism can directly impact T cell function and fate (5, 6, 38, 39). Given the ability of leptin to regulate both systemic

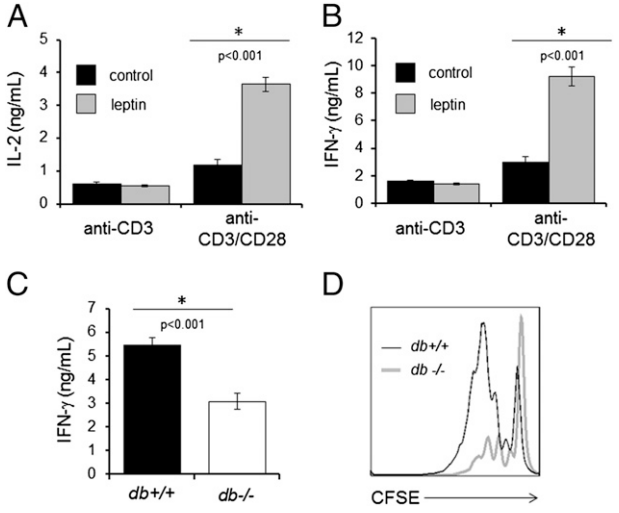

FIGURE 2. Leptin promotes activated T cell proliferation and inflammatory cytokine production. (**A** and **B**) CD4$^+$ T cells were isolated from wt C57BL/6J mice and stimulated with anti-CD3 alone or in the presence of anti-CD28 in serum-free media for 24 h. Cells were then treated with (gray bars) or without (black bars) 250 ng/ml leptin for an additional 24 h, after which supernatants were collected, and IL-2 (A) and IFN-γ (B) production in culture supernatants were measured by ELISA. Data are representative of two independent experiments. (**C**) CD4$^+$ T cells were isolated from wt ($db^{+/+}$, black bar) or LepR-deficient ($db^{-/-}$, white bar) mice and activated by coculture with wt bone marrow–derived macrophages activated with LPS plus low-dose anti-CD3 Ab for 48 h; production of IFN-γ in culture supernatants was measured by ELISA. Data are representative of three independent experiments. (**D**) CD4$^+$ T cells from wt ($db^{+/+}$, thin black line) or LepR-deficient ($db^{-/-}$, thick gray line) mice were activated with anti-CD3 and anti-CD28 Abs for 72 h. Proliferation was determined by staining T cells with CFSE prior to culture and analyzing dilution of the fluorescent dye flow cytometrically. Data are representative of two independent experiments.

metabolism (9) and T cell function, we hypothesized that leptin may directly control peripheral T cell glucose metabolism. This role for leptin has been shown in other metabolic cells, including muscle and adipose (40, 41). To test this potential mechanism of leptin action on T cells, $CD4^+$ T cells were isolated from wt ($db^{+/+}$) and LepR–deficient ($db^{-/-}$) mice, and glucose uptake was examined in both resting T cells and T cells activated with Abs to CD3 and CD28 in normal media with serum containing leptin. Although LepR deficiency ($db^{-/-}$) had no impact on glucose uptake of resting T cells, T cells stimulated with both anti-CD3 and anti-CD28 were dependent on LepR signaling to maximally upregulate glucose uptake following activation (Fig. 3A).

Downstream of glucose uptake, glucose is metabolized to pyruvate through glycolysis, and glucose-derived pyruvate is either converted to lactate for secretion or oxidized in the mitochondria. The glycolytic rate was equivalent in resting T cells from $db^{-/-}$ and $db^{+/+}$ mice (Fig. 3B). Consistent with reduced glucose uptake in activation, however, LepR-deficient $db^{-/-}$ activated T cells had decreased rates of glycolytic flux when determined by addition of [^{3}H]glucose to measure production of [^{3}H]H_2O by enolase, the penultimate glycolytic enzyme (42) (Fig. 3B). To examine the subsequent steps in glucose metabolism through conversion of pyruvate to lactate or through entry of pyruvate into the mitochondria for oxidative metabolism, we used extracellular flux analysis to examine media acidification as an indicator of lactate production and oxygen consumption to indicate mitochondrial metabolism. In both metabolic fates for glucose-derived metabolites, activated LepR-deficient $db^{-/-}$ $CD4^+$ T cells had reduced flux compared with wt $db^{+/+}$ T cells (Fig. 3C, 3D). LepR, therefore, is essential to maximally upregulate glucose metabolism during T cell activation.

Leptin regulates glucose metabolism in part by upregulation of Glut1

A family of glucose transporters mediates glucose uptake, and Glut1 is considered the primary glucose transporter expressed on the surface of activated T cells. The surface expression of Glut1 on T cells is highly regulated (43–45), and Glut1 can be a limiting component of glucose metabolism upon T cell activation (5). To test if failure to increase Glut1 expression contributed to the defective metabolic response of T cells that fail to receive leptin signals, $CD4^+$ T cells from $db^{-/-}$ and $db^{+/+}$ mice were activated in the presence or absence of anti-CD3 and anti-CD28 for 48 h, and Glut1 expression was measured. Leptin is available from serum in culture media in these experiments. LepR-deficient $db^{-/-}$ T cells had significantly reduced induction of Glut1 RNA following activation relative to $db^{+/+}$ controls (Fig. 4A). We next examined the expression of Glut1 protein in $db^{-/-}$ versus wt ($db^{+/+}$) control $CD4^+$ T cells. Like Glut1 mRNA, total Glut1 protein induction was reduced in LepR-deficient T cells following activation (Fig. 4B; note that Glut1 runs as a smear due to glycosylation; quantification of Glut1 protein levels normalized to actin from duplicate experiments is shown below).

To determine to what extent leptin-mediated regulation of Glut1 may account for the effect of leptin on T cell metabolism, we measured the effect of leptin on Glut1 Tg T cells (5). $CD4^+$ T cells were isolated from T cell–specific Glut1 Tg animals and age-matched wt controls and activated with Abs to CD3 and CD28 in serum-free media in the presence or absence of exogenous leptin. Leptin stimulation resulted in increased glucose uptake in activated wt T cells. As expected, glucose uptake was higher in activated Glut1 Tg T cells than in wt T cells. Leptin, however, did not further augment glucose uptake (Fig. 4C).

The effects of leptin on activated T cell function and metabolism are hematopoietic cell intrinsic

Leptin can act on a variety of cell types, including thymic stroma, and therefore, the role of leptin in regulation of T cell number and function may be from indirect effects on T cell development and environment (30, 33). To examine whether the effects of leptin on activated peripheral T cell function and metabolism are hematopoietic cell intrinsic or extrinsic, we performed bone marrow transplants of LepR-deficient ($db^{-/-}$) and wt ($db^{+/+}$) hematopoietic cells into irradiated Rag1 knockout (LepR wt) recipients. Following immune reconstitution, activated $CD4^+$ T cells from mice that received $db^{-/-}$ or $db^{+/+}$ bone marrow were compared in vitro for production of inflammatory cytokine IFN-γ as well as glucose metabolism. T cells from recipient mice that received LepR-deficient $db^{-/-}$ bone marrow had defects in both IFN-γ production (Fig. 5A) and glucose uptake (Fig. 5B) following activation compared with T cells from recipient mice that received bone marrow from $db^{+/+}$ controls. Similarly, recipient mice that

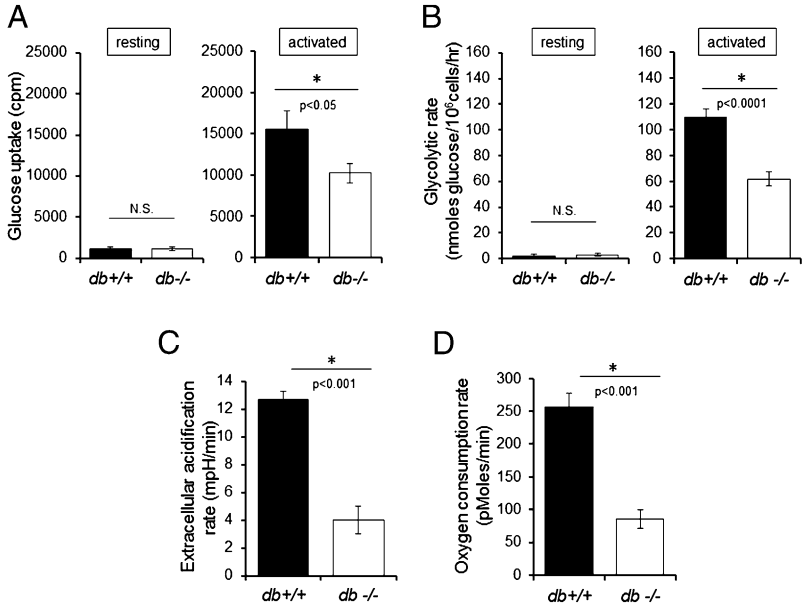

FIGURE 3. Leptin signaling is required for normal rates of glucose uptake, glycolysis, and mitochondrial respiration in activated T cells. (**A** and **B**) $CD4^+$ T cells were isolated from $db^{+/+}$ (black bars) or $db^{-/-}$ (white bars) mice. Glucose uptake was assessed by ^{3}[H]2-deoxyglucose uptake (A), and glycolytic flux was determined by the generation of tritiated water by enolase (B) in both resting cells and cells activated with anti-CD3 and anti-CD28 Abs for 48 h, as indicated. Representative data are shown. Extracellular acidification rates (**C**) and basal oxygen consumption rates (**D**) were measured using a Seahorse Extracellular Flux Analyzer in activated T cells isolated from wt ($db^{+/+}$, black bar) or LepR-deficient ($db^{-/-}$, white bar) mice. Data are representative of two independent experiments.

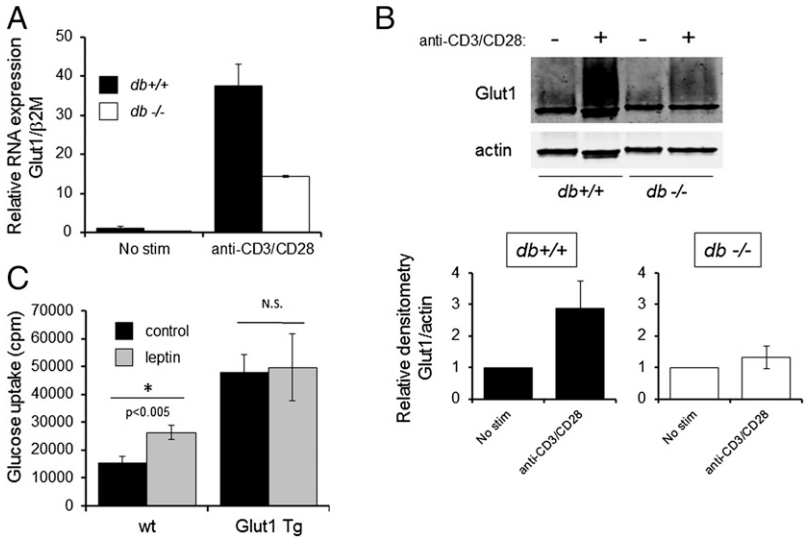

FIGURE 4. Leptin regulates glucose metabolism in part by upregulation of Glut1. (**A**) $CD4^+$ T cells were isolated from wt ($db^{+/+}$, black bars) or LepR-deficient ($db^{-/-}$, white bars) mice and activated with or without (No stim) anti-CD3 and anti-CD28 for 48 h. Glut1 mRNA expression was determined by quantitative PCR. Data are expressed relative to β_2-microglobulin (β2M) RNA levels and normalized to mRNA levels in unstimulated wt T cells. Data are representative of two independent experiments. (**B**) $CD4^+$ T cells were isolated from wt ($db^{+/+}$, black bars) or LepR-deficient ($db^{-/-}$, white bars) mice and activated with or without (No stim) anti-CD3 and anti-CD28 Abs, as indicated, for 48 h. Lysates were resolved by SDS-PAGE and immunoblotted using Abs to Glut1 and actin. Glut1 is heavily glycosylated and appears as a large smear on Western blot. Bar graphs depict densitometry of the Glut1 band, normalized to actin, in wt ($db^{+/+}$) versus LepR-deficient ($db^{-/-}$) animals, as averaged from two additional independent experiments. (**C**) $CD4^+$ T cells from wt or T cell–specific Glut1 Tg were isolated and stimulated with anti-CD3 and anti-CD28 in serum-free media for 24 h. Cells were then treated with (gray bars) or without (black bars) 250 ng/ml leptin for an additional 24 h, after which glucose uptake was measured. Data are representative of two independent experiments.

received LepR-deficient $db^{-/-}$ bone marrow had defects in glycolysis following T cell activation (Fig. 5C) compared with recipient mice that received bone marrow from $db^{+/+}$ controls. Importantly, resting T cells had equivalent glycolysis (Fig. 5C).

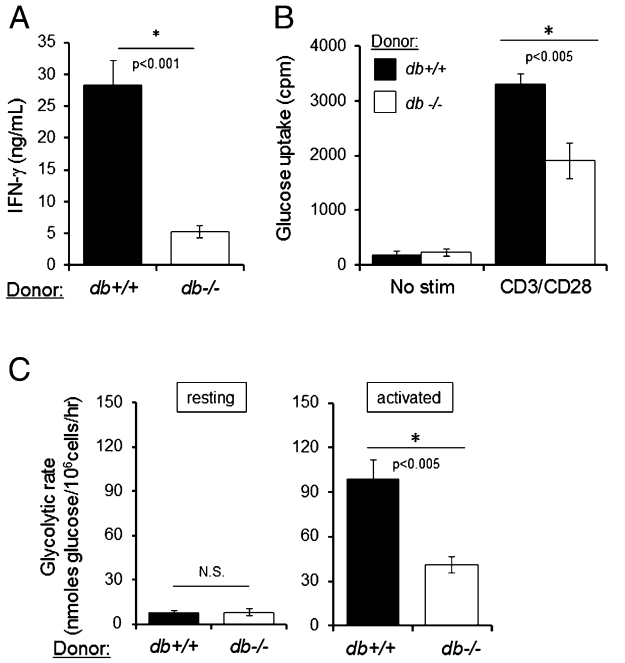

FIGURE 5. The effects of leptin on activated T cell function and metabolism are hematopoietic cell intrinsic. Irradiated Rag recipient mice were transplanted with bone marrow isolated from wt ($db^{+/+}$, black bars) or LepR-deficient ($db^{-/-}$, white bars) donors. Following full immune reconstitution at 18 wk, the following experiments were performed. (**A**) $CD4^+$ T cells were isolated and activated with anti-CD3 and anti-CD28 for 48 h, followed by measurement of IFN-γ production by ELISA. (**B** and **C**) Resting and activated $CD4^+$ T cells were evaluated for glucose uptake. Glucose uptake data are representative of five independent experiments (B) and glycolytic flux data are representative of three independent experiments (C).

Leptin directly regulates T cell metabolism and function

The intrinsic role of leptin in T cell inflammatory cytokine production and metabolism was confirmed in genetic studies using mice with a conditional LepR deletion. We generated conditional T cell–specific LepR knockout mice by crossing *LepR-fl/fl* animals with mice expressing a Cre recombinase transgene under the control of the proximal Lck promoter (*Lck-Cre*), and confirmed significantly lower LepR expression in the conditional knockout following activation using RT-PCR (Fig. 6A). Consistent with prior studies that showed induction of LepR expression after T cell activation, LepR expression was not detectable in resting T cells (22). Activated $CD4^+$ T cells isolated from T cell–specific LepR knockout mice secreted less IFN-γ than T cells isolated from heterozygous controls (Fig. 6B). Moreover, T cell–specific LepR deficiency reduced activated T cell glucose uptake relative to heterozygous *LepR-fl/*$^+$ controls (Fig. 6C). These studies confirm a direct effect of leptin on T cell metabolism and cytokine production.

Leptin has a known role in modulating $CD4^+$ T cell subset differentiation. As a proinflammatory cytokine, leptin upregulates Th1 and Th17 cells while inhibiting Treg proliferation (46–49). To examine the direct effect of leptin on $CD4^+$ T cell subset metabolism, we generated conditional $CD4^+$ T cell–specific LepR knockout mice (*LepR-fl/fl* animals crossed with *CD4-Cre*). $CD4^+$ T cells were isolated from LepR knockout and control mice and induced in vitro to differentiate into Th17 cells and Treg, as described previously (6). Following 3 d of in vitro cell differentiation, the cells were split and cultured for an additional 2 d in the presence of IL-2, after which glycolytic flux was determined. $CD4^+$ T cell–specific LepR knockout Th17 cells had significantly decreased glycolysis as compared with LepR-expressing controls, whereas Treg generated from $CD4^+$ T cell–specific LepR knockout had equal glycolytic rate as LepR-expressing controls (Fig.

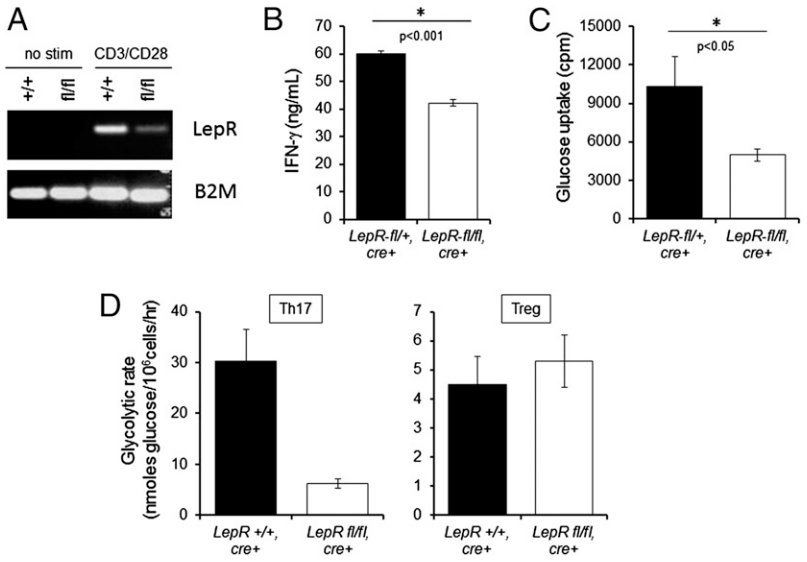

FIGURE 6. T cell–intrinsic LepR signaling is required for activated T cell inflammatory cytokine production and glucose metabolism. (**A**) T cell–specific LepR conditional knockout was generated using cre-lox recombination. LepR expression was determined by RT-PCR using primers to LepR and β_2-microglobulin (B2M, control). $CD4^+$ T cells were isolated from conditional LepR knockout (white bars) versus heterozygous control mice (black bars) and activated with anti-CD3 and anti-CD28 Abs for 48 h, after which IFN-γ concentration was measured in culture supernatants by ELISA (**B**), and glucose uptake was assessed (**C**). Data are representative of two independent experiments. (**D**) $CD4^+$ T cells were isolated from $CD4^+$ T cell–specific conditional LepR knockout (white bars) and homozygous control mice (black bars) and induced to differentiate into Th17 and Treg populations in vitro, after which glycolytic rate was determined. Data are representative of two independent single-mouse experiments. No stim, no stimulation.

6D). These data indicate that LepR signaling is critical for glucose metabolism in effector Th17 cells, but not Treg. This is consistent with previous reports that have found Th17 to be highly glycolytic and Treg to depend on an oxidative metabolism, rather than glucose uptake for energy metabolism (6, 7).

Persistent functional and metabolic defects of T cells from fasted animals can be rescued by either leptin or increased glucose uptake

In addition to reduced leptin, fasting leads to a variety of systemic hormonal and nutritional effects that could impact T cell metabolism and function (37). To examine the contribution of reduced leptin alone in fasting-induced peripheral metabolic T cell defects, T cells were isolated from control fed and fasted animals and activated in vitro in control media or with addition of exogenous leptin. Despite the presence of leptin in serum, a low concentration was required (1%) to maintain cell viability in T cells from fasted animals. Importantly, even with a low level of serum-derived leptin, provision of additional leptin was sufficient to restore glucose uptake in activated T cells from fasted mice to levels equivalent to that of control T cells isolated from animals fed ad libitum (Fig. 7A). Similarly, leptin addition restored IL-2 and IFN-γ production of T cells from fasted animals to levels at or above those of T cells isolated from fed controls (Fig. 7B, 7C). In these low-serum conditions, additional leptin did not, however, augment IL-2 production beyond what was seen in T cells isolated from the fed controls and incubated in media with 1% serum. Exogenous leptin did indeed increase IFN-γ secretion above that seen in T cells from the fed controls. These results suggest that, whereas leptin is sufficient to reverse the persistent defects in T cell function and metabolism induced by fasting, the effect of leptin on IL-2 and IFN-γ production is likely concentration dependent.

Rescue of persistent metabolic and functional defects in T cells from fasted and hypoleptinemic animals by in vitro provision of

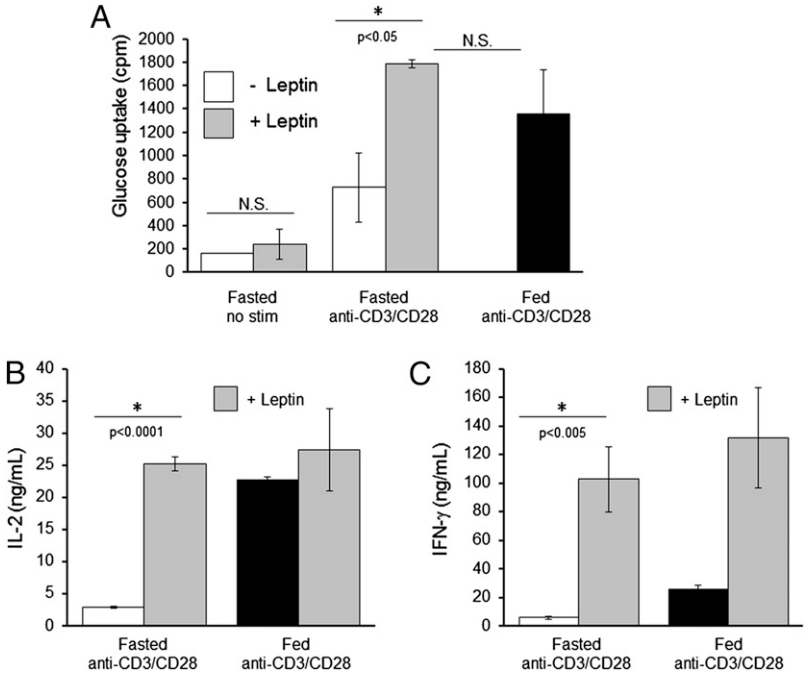

FIGURE 7. Defective glucose metabolism and inflammatory cytokine production in activated peripheral T cells from fasted mice are rescued by leptin in vitro. T cells were isolated from control or fasted C57BL/6 mice, activated with anti-CD3 and anti-CD28 for 24 h in low-serum (1%) conditions, and then treated with or without 250 ng/ml additional leptin for an additional 24 h, after which glucose uptake was measured (**A**), and cytokine production (**B**, **C**) was measured by ELISA. Data are representative of two independent experiments, three mice per experimental group.

leptin suggested that leptin provides a signal essential for T cells to upregulate glucose metabolism upon activation. The contribution of leptin to peripheral T cell function and metabolism in fasted animals was, therefore, studied in vivo. Fasted mice received either twice-daily i.p. injections of PBS or recombinant leptin in PBS. Fed control mice received PBS injections. Thymocytes and peripheral T cells were isolated after 48 h of treatment. In vivo leptin administration during fasting partially rescued the cell-number abnormalities acquired from fasting (37) and reduced the loss of thymocytes and peripheral T cell numbers (Supplemental Fig. 2A, 2B). To test if leptin altered the functional defects imparted by fasting, peripheral T cells were isolated and activated with Abs to CD3 and CD28 in full nutrient media for an additional 48 h in the absence of any additional leptin. Importantly, IL-2 and IFN-γ production of in vitro–stimulated T cells from fasted mice was fully restored to control levels by in vivo leptin treatment (Fig. 8A, 8B). In addition, in vivo leptin treatment prevented the T cell metabolic defects and maintained glucose uptake of in vitro–stimulated T cells from fasted mice to a level similar to that of T cells from normally fed animals (Fig. 8C).

As leptin signal promotes Glut1 expression, and fasting is associated with both lower leptin levels and decreased glucose uptake and metabolism, we tested if independent support of glucose uptake by Glut1 Tg was sufficient to rescue T cells from persistent metabolic and functional defects in fasting. T cells from wt and Glut1 Tg–fed control and fasted mice were isolated and cell counts obtained. The Glut1 transgene did not augment T cell count in either the fed control (5) or fasted condition (Supplemental Fig. 2C), demonstrating that glucose uptake alone is not sufficient to prevent T cell loss in fasting. Next, T cells from wt and Glut1 Tg–fed control mice were compared with cells from fasted mice that received either twice-daily i.p. injections of PBS or recombinant leptin in PBS. T cells were isolated and stimulated in vitro with anti-CD3 and anti-CD28 for 48 h, and glucose uptake and cytokine production were measured. Transgenic expression of Glut1 increased and maintained T cell glucose uptake even in fasting (Fig. 8D). Although leptin rescued defects in T cell glucose metabolism and cytokine production in fasted wt mice, T cells from Glut1 Tg mice, which already resist fasting-induced defects in metabolism and function, did not further upregulate glucose uptake or IFN-γ production in response to leptin. Importantly, Glut1 Tg T cells also resisted fasting-induced dysregulation of T cell cytokine production and maintained the ability to secrete IFN-γ (Fig. 8E). Thus, persistent fasting-induced T cell defects appear due, in part, to an inability of T cells to increase glucose uptake and metabolism even when activated in the presence of abundant nutrients. We conclude that leptin acts directly on T cells to license metabolic reprogramming in activation, linking systemic nutrient status with T cell metabolism and function.

Discussion

Nutritional status is a critical regulator of overall immune function. Malnutrition reduces immunity and markedly increases the risks and mortality from severe infections (2). In contrast, obesity predisposes to systemic inflammation that promotes the development of certain forms of autoimmunity, asthma, and insulin resistance, leading to type 2 diabetes (3, 50–53). There is growing evidence that adipocytes and immune cells coordinate nutritional status and immunity. One mechanism for this interaction is through secretion of adipokines, such as leptin, that can directly convey the state of nutrient availability to peripheral lymphocytes and other immune cells (10, 50). This communication of nutritional status to immune cells is critical to maintain energy balance in immunological defense, as activated effector lymphocytes have a very high metabolic demand essential for growth, proliferation, and the production of proteins required for a successful immune response (54–56). Leptin, in particular, has been shown to link adipose stores to immunity, as leptin deficiency has been associated with decreased immune reactivity in both mouse and human studies (14, 16, 19). Both neutralization of leptin in wt mice and genetic leptin deficiency lead to decreased susceptibility to autoimmunity in animal models of experimental autoimmune encephalitis, glomerulonephritis, colitis, and hepatitis and increased susceptibility to intracellular infections (11). Additionally, calorie restriction has been shown to attenuate autoimmune diseases such as experimental autoimmune encephalitis in mice (57), and fasting-induced hypoleptinemia was seen to expand Treg in a lupus-prone mouse model (58). Our study now shows leptin acts directly on activated effector T cells to allow glucose uptake and metabolic reprogramming that link systemic nutrient availability to metabolic licensing of T cells essential for activation.

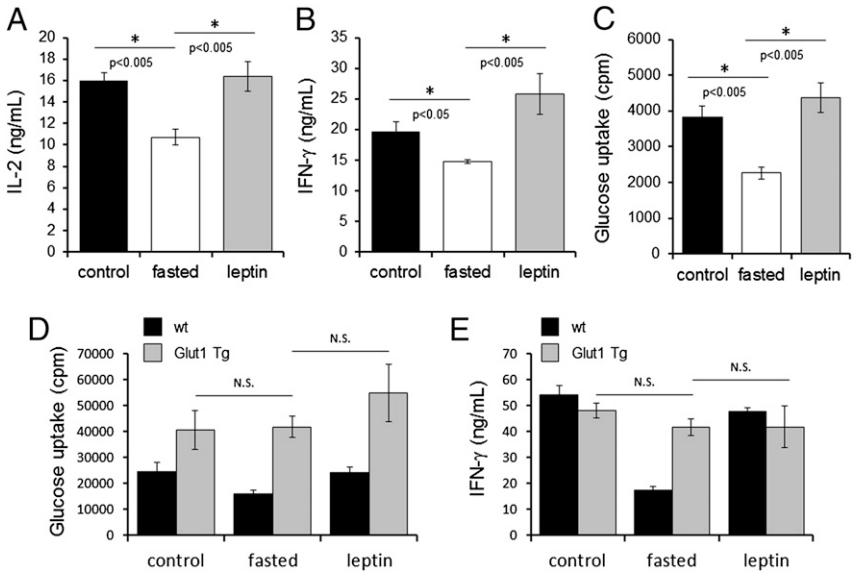

FIGURE 8. Both in vivo leptin administration and Glut1 overexpression can rescue peripheral T cell function and metabolism in fasted mice. $CD4^+$ T cells were isolated from control C57BL/6J mice (black bars), fasted mice (white bars), or fasted mice receiving leptin injections (gray bars) and activated with anti-CD3 and anti-CD28 for 48 h, after which cytokine production was measured by ELISA (**A**, **B**) and glucose metabolism was analyzed by comparing relative glucose uptake (**C**). Data are representative of two independent experiments, three mice per experimental group. (**D** and **E**) wt C57BL/6J mice (black bars) or T cell–specific Glut1 Tg (gray bars) were fed ad libitum (control), fasted, or fasted while receiving leptin injections (leptin), as indicated, for 48 h. Peripheral $CD4^+$ T cells were isolated and activated with anti-CD3 and anti-CD28 for an additional 48 h, after which glucose metabolism was analyzed by measuring glucose uptake (D), and IFN-γ production was measured in culture supernatants by ELISA (E). Data are representative of two independent experiments, three mice per group.

Given the dual roles of leptin in regulating both overall body metabolism and T cell function, we hypothesized that leptin signaling may control T cell activation in part through direct regulation of lymphocyte metabolism. This role for leptin has been established in metabolic cells such as muscle and adipose (40, 41). In support of this role for leptin in T cells, recent publications have highlighted the ability of leptin to activate mTOR, which is a signaling molecule well known to affect T cell metabolism and function (59). Indeed, we found LepR signal was required for increased Glut1 expression in peripheral T cells and upregulation of glucose uptake and glucose metabolism following T cell activation. This leptin-mediated increase in glucose metabolism was observed in effector Th17 cells but not Treg, which is consistent with findings that leptin promotes Th17 proliferation while inhibiting proliferation of Treg (47, 60). Therefore, a key role for leptin in T cell activation may be to allow effector T cells to increase Glut1 expression to fuel the demands of T cell growth, cytokine production, and proliferation. To test if directly augmenting T cell glucose metabolism bypassed the leptin requirement, we used T cell–specific Glut1-overexpressing mice. These studies showed that Glut1 overexpression was sufficient to maintain normal glucose uptake and rescue inflammatory cytokine production in T cells from fasted animals. The ability of Glut1 and glucose uptake to overcome persistent fasting-induced T cell dysfunction demonstrates that the ability of leptin to upregulate glucose metabolism is a key mechanism by which leptin directly enhances or supports T cell activation.

This ability of leptin to upregulate T cell Glut1 expression and glucose metabolism was observed only in lymphocytes activated through TCR together with costimulation. Resting T cells or T cells activated with anti-CD3 alone did not increase glucose metabolism following leptin therapy in vitro or in vivo. This finding correlates with the upregulation of LepR expression on T cells following full activation (22). This is also consistent with previous reports that CD28 costimulation is required for increased glycolysis in activation (61). Further, these data suggest that leptin, which is secreted in proportion to fat mass and is therefore a surrogate for nutritional sufficiency, is not required for resting T cell metabolism but is selective to license T cells to increase metabolism to support activation. Therefore, in the absence of adequate nutritional stores, T cells are unable to upregulate glucose metabolism and cannot become fully activated.

Mice fasted for 48 h were expected to have a rapid and significant drop in both thymocyte and T cell numbers (29, 37). Fasting has a number of important systemic effects, including increasing circulating levels of glucocorticoids (62, 63), which have potent effects on lymphocyte survival and function. Rising glucocorticoids in fasting, therefore, may contribute significantly to decreased overall thymocyte and T cell numbers (64). Injection of leptin alone during fasting only partially rescued thymocyte and T cell numbers. Moreover, Glut1 overexpression did not rescue T cell numbers in fasting, suggesting that the ability of leptin to partially rescue T cells numbers in fasting is via a mechanism independent of increased glucose uptake. Leptin may rescue cell number by opposing the apoptotic actions of glucocorticoids on both thymocytes and peripheral lymphocytes, as there is evidence in the literature for such as role (34), whereas in vivo injection of leptin alone during fasting fully rescued the persistent T cell defects in cytokine production and glucose metabolism following activation. Moreover, addition of leptin to cultured peripheral T cells from fasted animals in vitro rescued cytokine production and glucose metabolism to levels at or above the fed control. These data suggest a critical role of leptin in directly regulating activated T cell function and metabolism during states of malnutrition. However, the effects of leptin on activated peripheral T cell metabolism and function likely differ from other indirect mechanisms by which leptin affects resting T cell number and development in the thymus.

The relationship between leptin deficiency and immunodeficiency is complex. Although multiple studies have shown direct effects of leptin on T cell function (29, 31, 32), others have demonstrated that leptin may influence immune cell number and function indirectly through regulation of stromal cells or other hormones (30, 65, 66). More recent reports suggest leptin may also mediate its effects on immune cell populations via central signaling; in one study, intracerebroventricular injections of leptin were sufficient to rescue B cell numbers in fasted mice (67). However, the results from our studies, using both bone marrow transplant of LepR-deficient $db^{-/-}$ donors into Rag1 knockout recipients and the T cell–specific conditional LepR knockout mouse, support a direct and cell-intrinsic role for leptin in modulating peripheral T cell metabolism during activation. Leptin, therefore, links nutritional status to multiple levels of the immune system.

Collectively, our studies indicate that leptin serves as a nutritional rheostat that can communicate energy stores to peripheral lymphocytes undergoing activation. As lymphocyte activation is energetically costly, it is important to limit immunity in times of nutritional deficiency. Decreased leptin that occurs in malnutrition thus limits the capacity of T cells to consume the limiting availability of nutrients. Upregulation of LepR following peripheral T cell activation allows leptin signal to metabolically license T cells for activation when nutritional stores are at sufficient levels, thus providing a key link between systemic and cellular metabolism in the immune system. Evolutionarily, this response may be critical to balance the energy costs of immunity with more immediately critical systems, such as neurologic, cardiovascular, respiratory, and musculoskeletal.

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Disclosures

The authors have no financial conflicts of interest.

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