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*J Immunol* 2012; 189:2941-2953; Prepublished online 17 August 2012; doi: 10.4049/jimmunol.1200935

http://www.jimmunol.org/content/189/6/2941

Supplementary Material [http://www.jimmunol.org/content/suppl/2012/08/17/jimmunol.1200935.DC1](http://www.jimmunol.org/content/suppl/2012/08/17/jimmunol.1200935.DC1)

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Leptin-Induced mTOR Activation Defines a Specific Molecular and Transcriptional Signature Controlling CD4+ Effector T Cell Responses

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The sensing by T cells of metabolic and energetic changes in the microenvironment can determine the differentiation, maturation, and activation of these cells. Although it is known that mammalian target of rapamycin (mTOR) gauges nutritional and energetic signals in the extracellular milieu, it is not known how mTOR and metabolism influence CD4+CD25−FOXP3− effector T cell (Teff) responses. In this article, we show that leptin-induced activation of mTOR, which, in turn, controls leptin production and signaling, causes a defined cellular, biochemical, and transcriptional signature that determine the outcome of Teff responses, both in vitro and in vivo. The blockade of leptin/leptin receptor signaling, induced by genetic means or by starvation, leads to impaired mTOR activity that inhibits the proliferation of Teffs in vivo. Notably, the transcriptional signature of Teffs in the presence of leptin blockade appears similar to that observed in rapamycin-treated Teffs. These results identify a novel link between nutritional status and Teff responses through the leptin–mTOR axis and define a potential target for Teff modulation in normal and pathologic conditions.

Activated CD4+ T cells respond to Ags, cytokines, and costimulatory molecules to undergo proliferation, clonal expansion, and differentiation toward effector and memory functions. The integration of multiple extracellular signals affects transcriptional programs and signaling pathways that ultimately determine, in the CD4+ T cells, multiple events that include modulation of energy metabolism, glycolysis, and proliferation and the production of cytokines (1). A key player in this cellular control is mammalian target of rapamycin (mTOR), an evolutionarily conserved 289-kDa serine/threonine protein kinase inhibited by rapamycin (2–4) that directly influences T cell differentiation and proliferation by integrating environmental cues, including nutrients, energy stores, and growth factors (5–7). The central role of mTOR in T cell metabolism and function is reinforced by recent findings that it influences the generation of regulatory T cells (Tregs) (8–10), the generation of CD8+ memory cells (11), and the choice between T cell activation and anergy (12, 13).

The intracellular energy status is affected by the microenvironment. It was hypothesized that the hormone cytokine leptin can be a crucial “sensing” factor linking the availability of nutrients in the environment to intracellular metabolism and immune responses (14, 15). Circulating levels of leptin are proportional to the fat mass and associate positively with obesity (16, 17) and, importantly, to increased susceptibility to autoimmunity and chronic inflammation (18). Indeed, obese leptin-deficient mice (ob/ob) and leptin receptor (LepR)-deficient mice (db/db) display resistance to autoimmune diseases, including experimental autoimmune encephalomyelitis (19, 20), experimental colitis (21), Ag-induced arthritis (22), and type 1 diabetes (23). The ability of leptin to promote proinflammatory Th1 immune responses (24, 25) and suppress Treg activity (26) may explain, in part, this association.

Leptin signaling and mTOR activity influence each other in the hypothalamus and in the peripheral immune system. Cota and colleagues (27, 28) showed that mTOR activation in the hypothalamus is necessary for the leptin-induced effects on the hypothalamic axis modulation of food intake. mTOR is activated in response to leptin, and rapamycin inhibits the anorexigenic effects of leptin. At the immune level, mTOR activity controls leptin-induced activation of macrophages and the responsiveness of Tregs (9) and autoreactive T cell survival (29). Yet, the signaling events that lead to mTOR activation in effector CD4+CD25−
FOXP3+ T cells (Teffs) are only partly understood (3); the same holds true for the ability of leptin to influence Teff responses. In this study, we show that leptin has a key role in the control of mTOR activity in vitro and in vivo in Teffs, in which it determines defined biochemical and transcriptional activities that are directly linked to the intracellular energy status, metabolism, and immune response.

Materials and Methods

Teff purification, cultures, and proliferation assays

Human CD4+CD25- T cells were purified from PBMCs by buffy coats of human healthy donors by high-performance cell sorting (MoFlo; Dako) after staining with FITC-anti-human CD4 (BD Pharmingen; clone RPA-T4), PE-anti-human CD25 (BD Pharmingen; clone M-A251), or allophycocyanin anti-human CD127 (R&D Systems; clone 40131) or by magnetic cell separation with the Dynabeads Regulatory T Cell Kit (Invitrogen). Ab bead-free cells were 95–99% pure by FACS analysis. For proliferation assays, purified cells were cultured (5 × 10^4 cells/well) as previously described (26). Mouse effector T cells were isolated with the Regulatory T Cell Isolation Kit (Miltenyi Biotec, Gladbach, Germany) and by autoMACS Pro Separator (Miltenyi Biotec) or by high-performance cell sorting (MoFlo; Dako). Cells were then stimulated with Dynabeads mouse anti-CD3/CD28 (0.5 bead/cell; 5 × 10^4 cells/well). Teffs (98% pure by FACS analysis) were cultured, as previously described (26).

The leptin-sensitive BA/F3 1442-CI4 cells, stably transfected with the long form of human LepR, were kindly provided by Prof. Arieh Gertler (Hebrew University, Rehovot, Israel). Human LepR<sup>B</sup>BA/F3 cell proliferation is leptin dependent. Human LepR<sup>B</sup>BA/F3 cells were cultured in flat-bottom 96-well microtiter plates (Becton-Dickinson Falcon, Franklin Lakes, NJ) at a density of 5 × 10^3 cells/well in a total volume of 100 μl RPMI 1640 medium supplemented with 2% FCS (HyClone-Pierce; Thermo Fisher Scientific, Rockford, IL), 2 mmol/l L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (Life Technologies, Carlsbad, CA). Cells were cultured at 37˚C in 100% humidity and 5% CO<sub>2</sub> in the presence of increasing doses of leptin (0.01–10 ng/ml).

Treatment in vitro with rapamycin, lepin, and anti-leptin, and LY294002

For transient mTOR inhibition, Teffs were pretreated in vitro for 1 h with rapamycin (Sigma-Aldrich) at a final concentration of 100 nM. Cells were washed extensively with serum-free culture medium and cultured, as described previously (9). For in vitro leptin neutralization, human leptin (R&D Systems; Minneapolis, MN) was used at a final concentration of 20 μg/ml. Human recombinant leptin was purchased from R&D Systems, and it was used at a final concentration of 100 ng/ml. For transient PI3K inhibition, BA/F3 cell lines were pretreated in vitro for 1 h with LY294002 (Calbiochem) at a final concentration of 20 μM. Cells were then washed extensively with serum-free culture medium and stimulated or not with recombinant leptin for an additional 30 or 60 min.

Cytokine measurement

The FlowCytomix Human Basic Kit (Bender MedSystems) was used according to the manufacturer’s instructions to measure IL-2, lepin, IFN-γ, IL-17A, MCP-1, and soluble ICAM-1 (sICAM-1) production. Both mouse and human serum leptin were also measured by ELISA kits from mouse and human serum leptin were also measured by ELISA kits from R&D Systems. Human IL-2–neutralizing mAb was used as previously described (26).

Flow cytometry

FITC–anti-human CD4 (clone RPA-T4), PE–anti-human CD25 (clone M-A251), and FITC–anti-human phospho-S6 (clone N4-41) were from BD Pharmingen; FITC–anti-human CCR7 (clone 150503) was from R&D Systems; the anti-human FOXP3 staining kit was from BioLegend (clone PCH101; San Diego, CA); PE–anti-human CD152 (clone BN13), FITC–anti-human CD62L (clone FMC46), FITC–anti-human CD71 (clone DF1513), and PE–anti-human CD39 (clone A1) were from BD Serotec; and PE–anti-human GITR (clone DT3D5) was from Miltenyi Biotec. Incubation of BrdU and FACS analysis were performed with the BrdU Flow Kit (BD Pharmingen), in accordance with the manufacturer’s instructions. Flow cytometry experiments were performed with a FACSCanto (Becton-Dickinson, San Diego, CA) and a Dako CyAn (Dako cytometry) and analyzed by FlowJo software (Tree Star, Ashland, OR).

Western blots and biochemical analyses

Total cell lysates and Western blot analysis were performed as previously described (26). The following Abs were used: anti-p21<sup>WAF1/CIP1</sup>, anti-phospho-mTOR, anti-mTOR, anti–phospho-p7056 kinase (p70<sup>S6K</sup>), anti-p70<sup>S6K</sup>, anti–phospho-S6 ribosomal protein (S6), anti–S6, anti-AMPK, anti–phospho-Lck (Ty305), anti-phospho-ZAP70, anti–phospho-AKT (Ser473), anti-AKT, anti–phospho-STAT5 (Tyr694), anti-STAT5, anti–phospho-STAT3 (Tyr705), anti-STAT3, and anti–NF-kB (all from Cell Signaling Technology, Beverly, MA); anti–ERK1/2 and anti–phospho-ERK1/2 (both from Santa Cruz Biotechnology, Santa Cruz, CA); and anti–FOXO3 (eBioscience). The filters were also probed with a tubulin Ab (Sigma-Aldrich) to normalize for the amount of loaded protein. All filters were quantified as previously described (26).

Confocal microscopy

Confocal microscopy was performed on freshly isolated or cultured Teffs. Cells were washed in PBS, and 10<sup>4</sup> cells were seeded on a multistest slide (ICN Biomedicals, Aurora, OH), air-dried, fixed in 4% paraformaldehyde in PBS, washed in NH<sub>4</sub>Cl 50 mM in PBS, permeabilized for 5 min in PBS containing 0.2% Triton X-100, and incubated in blocking solution (PBS 1% BSA) for 1 h. Cells were washed three times in PBS and incubated overnight at 4˚C with primary Abs (tobacco polyclonal) and rabbit polyclonal anti–leptin and mouse anti-LepR mAbs (both from Santa Cruz Biotechnology), diluted 1:50 in blocking solution. Cells were washed extensively in PBS and incubated for 1 h at room temperature with the secondary Ab (goat anti-rabbit 488 and goat anti-mouse 543; both from Molecular Probes) diluted 1:200 in PBS 0.5% BSA, washed in PBS, and mounted in 1:1 PBS/glycerol. Quantitative analysis of multicolor confocal immunofluorescence microscopy images was performed using LSM software (Carl Zeiss).

Real-time PCR

Total RNA was extracted using EUROzol reagent (Euroclone), according to the manufacturer’s instructions. Total RNA (100 ng) from each sample was transcribed into cDNA using the RT-PCR Superscript III kit (Invitrogen), according to the manufacturer’s instructions. A total of 2.5 μl (10% of reverse-transcription reaction) each cDNA preparation was subsequently used as template for 25 μl PCRs containing 1 μmol/l in-house-designed primer pairs (forward: 5'-TAGGATCTGCAGCCTCAGC-3'; reverse: 5'-TGGTCTTGATGAGGTTTGGTGC-3') and 7.875 μl SYBR Green PCR master mix (Applied Biosystems). Real-time PCR was performed using an ABI Prism 7500 Sequence Detection System (Applied Biosystems) under the following conditions: 50˚C for 2 min, 95˚C for 5 min, 40 cycles of 95˚C for 45 s, and 55˚C for 1 min.

ATP measurement

Intracellular levels of ATP were evaluated in freshly isolated and cultured T cells by a luminescence assay with ATP lite 1 Step kit (Perkin Elmer), according to the manufacturer’s instructions.

Mice and in vivo experiments

Eight- to ten-week-old female leptin-deficient C57BL/6J-ob/db-ob/db (ob/ob), C57BL/6J lean control wild type (WT), leptin-receptor deficient C57BL/Ks-J db/db, and C57BL/Ks-db/db+ lean control (db/+). These mice were purchased from Harlan Italy (Corezzana, Italy). B6.Cg-Foxp3<sup>tm2tch</sup> (eGFP-Foxp3) mice were purchased from The Jackson Laboratory; these mice coexpress enhanced GFP and the Treg-specific transcription factor FOXP3 under the control of the endogenous Foxp3 promotor (30). Experiments were conducted in accordance with animal welfare guidelines under an approved protocol of the Istituto Superiore di Sanita (Rome, Italy). Mice were age matched for individual experiments and housed with a 12 h light/dark cycle in the animal facility at the Università di Napoli “Federico II” (Napoli, Italy).

ob/db and WT mice were injected i.p. once either with mTOR recombinant leptin (R&D Systems) dissolved in 200 μl PBS at a dose of 100 μg/mouse or with rapamycin at a dose of 100 μg/mouse. Normal mice were treated daily with BrdU (1 μg/mouse) in basal conditions and upon Ag immunization with CFA (Difco, BD Diagnostics - Diagnostic Systems) and were treated with a single dose of rapamycin (100 μg/mouse) or vehicle, 12 h before CFA priming, to follow proliferation of Teffs over time. Samples were obtained from tail veins at 5 d or from draining lymph nodes at days 8 and 12. For fasting experiments, eGFP-Foxp3 mice, treated daily with BrdU (1 μg/mouse), were fasted for 48 h in the presence or absence of exogenous mouse recombinant leptin (R&D Systems) dissolved in 200 μl PBS at a dose of 1 μg/g initial body weight twice daily at 9 am and
6 ng; they were compared with mice fed ad libitum (24). Draining lymph nodes from each group of mice were harvested after 48 h of starvation and stained for BrdU.

**Microarray analysis**

The microarray data used in this study are available at Gene Expression Omnibus under accession number GSE29606 (for human Teffs) and GSE29607 (for mouse Teffs) (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE29608). Human and mouse Teffs were purified, as previously described. The cells were preincubated for 1 h with rapamycin and then stimulated for an additional hour with anti-CD3/CD28 beads. Total RNA was isolated from human and mouse Teffs using the RNAqueous-4PCR Kit (Ambion, Austin, TX), following the manufacturer’s instruction. RNA was quantified by NanoDrop, and the amount and its quality were confirmed in an Agilent 2100 Bioanalyzer (Agilent Technologies, Rockville, MD). For each experimental group, three independent pools of five RNAs each were formed. The samples (500–1000 ng) were indirectly labeled using the T7 amplification method (Amino Allyl MessageAmp II amplification RNA [aRNA] Amplification Kit; Ambion), according to the manufacturer’s recommendations. aRNA (5 µg/sample) was labeled using monoreactive Cy3 and Cy5 dyes (GE Healthcare, Buckinghamshire, U.K.) or monoreactive Alexa Fluor 488 (Invitrogen, Gaithersburg, MD), followed by hybridization according to the manufacturer’s instructions. Labeled aRNAs (800 ng/sample) were hybridized onto Agilent Whole Human Genome 4x44K or Agilent Whole Mouse Genome 4x44K slides, according to the manufacturer’s recommendations. The slides were then washed and scanned by an Axon GenePix 4200 AL (Molecular Devices, Downington, PA) scanner. Microarray digital images were segmented, and the median intensity of each spot was estimated using GenePix Pro 6.0 software (Molecular Devices). The data were then imported into R software and preprocessed by the Bioconductor package Limma (31). After quality control of the data, the probes with undetectable signal throughout all the arrays of the dataset were removed; the remaining raw intensities were log-2 transformed and normalized by the quantile method across the arrays. The linear model, followed by the moderated t test, was used to find the differentially expressed genes (nominal p value < 0.01). The lists of significant genes were screened by DAVID 6.7 annotation tools (32, 33) to identify the functional categories. We then used the Kruskal–Wallis ANOVA test for unrelated two-group analyses, and a p value of 0.01 was considered significant.

**Results**

**Cellular and functional signature induced by acute inhibition of mTOR in human Teffs**

We transiently inhibited mTOR with rapamycin in Teffs 1 h prior to anti-CD3/CD28 stimulation. mTOR inhibition resulted in an impaired proliferation of Teffs, as indicated by a robust reduction in thymidine incorporation and reduced cell clustering observed at 60–72 h in culture (Fig. 1A, 1B).

The inhibition of Teff proliferation after pretreatment with rapamycin was associated with a significant decrease in IL-2 production (Fig. 1C). The addition of anti–IL-2–neutralizing Abs to cell cultures further inhibited proliferation (Fig. 1D), whereas, confirming specificity, the addition of exogenous IL-2 to cultures abolished this effect (Fig. 1D), indicating IL-2 dependency of the rapamycin-induced Teff inhibition of proliferation.

To study the effects of mTOR inhibition on the function of Teffs, we measured INF-γ and IL-17A production in culture media of Teffs pretreated or not with rapamycin (Fig. 1E, 1F). Consistent with the reduced proliferation of the Teffs, mTOR inhibition also was associated with a decrease in effector cytokine production, including MCP-1 and sICAM-1 (Fig. 1G, 1H), suggesting that mTOR inhibition impairs proinflammatory responses.

After 36–48 h of culture, rapamycin-pretreated Teffs showed a reduced expression of surface activation markers CD25, CD62L, cytotoxic T lymphocyte Ag-4 (CTLA-4), and CD71 (Fig. 1I); they also upregulated the expression of markers that are typically expressed by immune cells with regulatory/suppressive functions, such as CCR7, GITR, and CD39 (Fig. 1J). Altogether, these data suggest that mTOR inhibition is associated with a reduced activity of Teffs and a propensity toward the acquisition of regulatory immune cell phenotypes.

**Biochemical events induced by acute mTOR inhibition in human Teffs**

The early biochemical events induced by the inhibition of mTOR in human Teffs were studied in 1-h cultures (Fig. 2). The consistent amount of activated ERK1/2 and the low levels of the cell cycle inhibitor p27kip1 reflected the high proliferative state of Teffs stimulated by anti-CD3/CD28 (26). After TCR stimulation, the inhibition of mTOR reduced ERK1/2 phosphorylation and increased the levels of p27kip1, suggesting that rapamycin promoted cell cycle arrest in these cells (Fig. 2A, Supplementary Fig. 1A). When the mTOR pathway activity was evaluated in its entirety as phosphorylation of all of its downstream translational targets, such as p70S6K and S6, an increase was observed after TCR stimulation, and a decrease was observed of the phosphorylation of the whole pathway by rapamycin (Fig. 2B, Supplementary Fig. 1A). These data suggest that a reduced activity of mTOR is associated with a block in the cell cycle and the inhibition of Teff proliferation.

Next, we studied the effects of mTOR inhibition on TCR signaling. Rapamycin increased the phosphorylation of the inhibitory tyrosine kinase Lck盛5盛5 in TCR signaling, which resulted in a concomitant decrease in ZAP70 phosphorylation, as expected, considering the impairment of Teff proliferation (Fig. 2C, Supplementary Fig. 1A). When examining the PI3K/protein kinase B (or AKT) pathway downstream of TCR activation, we observed that the phosphorylation of AKT at Ser盛7盛1 was decreased by rapamycin upon TCR engagement (Fig. 2D, Supplementary Fig. 1A), suggesting that mTOR inhibition hampers the proper TCR signaling required for Teff proliferation.

Because we found that mTOR inhibition was associated with impaired IL-2 secretion (Fig. 1D), we analyzed the activity of STAT5 as a key element in the signal transduction of IL-2R. STAT5 phosphorylation increased upon TCR stimulation, as expected, whereas rapamycin reduced p-STAT5 levels (no induction upon TCR ligation) (Fig. 2D, Supplementary Fig. 1A); these phenomena were associated with impaired activation of NF-κB upon mTOR inhibition (Fig. 2D, Supplementary Fig. 1A). Like for STAT5, rapamycin inhibited phosphorylation of STAT3, another
downstream molecule involved in cytokine signaling, even upon TCR engagement (Fig. 2D, Supplemental Fig. 1A). Concomitantly with the suppression of mTOR activity, FOXP3 expression was partly increased in Teffs after 1 and 36 h of mTOR inhibition (Fig. 2E, 2F, Supplemental Fig. 1A). Because FOXP3 suppresses IL-2 gene transcription (34), rapamycin-induced upregulation of FOXP3 could account for the decreased IL-2 production and the inhibition of Teff proliferation, which is consistent with the reduced STAT5 phosphorylation. In addition, we found increased phosphorylation of AMP-activated protein kinase (Fig. 2G, Supplemental Fig. 1A), a master sensor of intracellular energy status that negatively regulates mTOR according to changes in the ATP/ADP ratio (3). In this context, the intracellular levels of ATP were also significantly reduced in Teffs upon mTOR inhibition at 36 h (Fig. 2H, Supplemental Fig. 1B), confirming that mTOR inhibition impairs the metabolic rate in Teffs.

**Transcriptional signature induced by acute mTOR inhibition**

Given the difference in functional activity and proliferative response between Teffs pretreated or not with rapamycin, we used gene-expression profiling to characterize the effects of mTOR inhibition at the molecular level. We calculated the fold change ratio of gene expression in Teffs pretreated or not with rapamycin. A total of 413 genes was differentially expressed (nominal $p < 0.01$) between anti-CD3/CD28–stimulated Teffs pretreated or not with rapamycin; 273 genes were upregulated, and 140 genes were downregulated. The downregulated genes (Fig. 3A, 3B) included those encoding for proteins involved in the induction of transcription (46 genes), inhibition of apoptosis (9 genes), DNA metabolism (7 genes), organization of vesicles (21 genes), protein transport (11 genes), and immune function (7 genes). The genes that were upregulated by mTOR inhibition included genes that inhibit transcription (26 genes), ribosomal proteins (20 genes), genes that induce cell death (6 genes) and catabolic and proteolytic processes (22 genes), as well as immune regulatory functions (10 genes) (Fig. 3A, 3C). Taken together, these data suggest that rapamycin influences Teff functions by inhibiting the expression of genes directly involved in the assembly of cell-transcription machinery, in organizing vesicles and proteins/ion transport, in controlling apoptosis and DNA damage as well as in proinflammatory mediator synthesis. Conversely, mTOR inhibition upregulated the expression of genes that repress transcription and increase catabolic and proteolytic processes.

**Inhibition of mTOR impairs the leptin axis in Teffs**

We previously showed that leptin can be produced by T cells, and it can favor their proliferation as a result of an autocrine loop of leptin secretion that sustains their proliferation. We also showed that
neutralization of leptin during T cell activation inhibits Teff proliferation (26). Because these phenomena resemble the effects induced by the inhibition of mTOR with rapamycin (Fig. 4A), we investigated, at the transcriptional level, whether leptin neutralization could determine a genetic profile similar to that observed in mTOR-inhibited Teffs. Interestingly, both conditions upregulated or downregulated genes belonging to similar functional classes (Supplemental Fig. 1C, 1D) and sharing several genes (Supplemental Fig. 1E), revealing an impairment in the processes controlling cell cycle progression, proliferation, RNA maturation, transcriptional activity, and mitochondrial functions (Supplemental Fig. 1C, 1D); this suggested overlap of leptin signaling with the mTOR pathway. We also evaluated the effects of leptin on rapamycin-induced Teff impairment. Hypothesizing that leptin antagonized the effect of mTOR inhibition, we found that leptin neutralization further inhibited Teff proliferation (Fig. 4A) and that the addition of leptin to rapamycin-pretreated Teffs partially reversed the inhibition of their proliferation (Fig. 4B). We next addressed whether rapamycin-induced inhibition of the proliferation of Teffs could be ascribed to a downmodulation of leptin

FIGURE 2. Molecular events induced by mTOR inhibition on human Teffs. Immunoblot for p-ERK and p27kip1 (A), p-mTOR, p-p70S6K, and p-S6 (B), p-Lck and p-ZAP70 (C), p-AKT, p-STAT5, p-NF-κB, and p-STAT3 (D), and FOXP3 (E) on human Teffs pretreated or not with rapamycin and then stimulated with anti-CD3/CD28 for 1 h. One representative of five independent experiments. (F) FOXP3 expression in Teffs pretreated or not with rapamycin after a 36-h stimulation with anti-CD3/CD28. Representative of six independent experiments. (G) Immunoblot for p—AMP-activated protein kinase on human Teffs pretreated or not with rapamycin and then stimulated with anti-CD3/CD28 for 1 h. One representative of five independent experiments. (H) ATP production in human Teffs pretreated or not with rapamycin and stimulated or not with anti-CD3/CD28 mAb for 36 h. Data are mean ± SD (n = 3). *p < 0.01.
and/or LepR expression. TCR stimulation induced both leptin and LepR expression (Fig. 4C), whereas pretreatment with rapamycin reduced both intracellular leptin levels and the cell surface expression of LepR (Fig. 4C), also at transcriptional level, as confirmed by real-time PCR analysis (Fig. 4D). To translate these findings to in vivo systems and at the systemic level, we measured serum leptin in the blood of patients with acquired cystic kidney disease who had normal kidney function and were treated with rapamycin. Serum leptin levels were significantly inhibited after both 6 and 12 mo of mTOR-inhibition treatment by rapamycin (Fig. 4E). To exclude that fluctuations in serum leptin levels in these patients could be ascribed to changes in body fat (measured as body mass index [BMI]), we measured BMI over time and found no differences, suggesting that mTOR controls leptin secretion and that these effects are also systemic, similar to what was observed in normal C57BL/6 mice that were treated or not with rapamycin (Fig. 4F).

**Leptin modulates Teff responses in vitro and in vivo via mTOR activation**

The effects of leptin on mTOR activation were studied in Baf/3 cells stably transfected with the long form of human leptin receptor (their growth is dependent on leptin). The absence of leptin decreased STAT3 phosphorylation, whereas the addition of leptin for 30 or 60 min increased it, confirming LepR activation (Fig. 5A, left panel, Supplemental Fig. 2A). Leptin induced both AKT and S6 phosphorylation, suggesting that mTOR activation occurs through an AKT-dependent mechanism (Fig. 5A, left panel, Supplemental Fig. 2A). Confirming specificity, pretreatment with the inhibitor of PI3K (LY294002) decreased STAT3, AKT, and S6 phosphorylation, whereas exogenous leptin induced their activation (Fig. 5A, right panel, Supplemental Fig. 2A). Finally, the addition of leptin to rapamycin-treated Baf/3 cells induced STAT3 and S6 phosphorylation, confirming leptin’s involvement in mTOR activity (Fig. 5A, right panel, Supplemental Fig. 2A).

The contribution of leptin to mTOR activation in human Teffs was studied by treating unstimulated cells with leptin for 1 h. As shown in Fig. 5A, leptin treatment had little effect on mTOR phosphorylation, but it induced a significant increase in p70S6K and S6 phosphorylation, concomitant with a consistent increase in AKT phosphorylation. Phosphorylation of STAT3 was evaluated as an indicator of leptin-related signaling activation (Fig. 5B, Supplemental Fig. 2B). The induction of mTOR, as well as AKT phosphorylation induced by TCR engagement, was significantly reduced by leptin blockade (Fig. 5C, Supplemental Fig. 2C), and this inhibition was partially reversed by the addition of recombinant leptin to cultures (Fig. 5C, Supplemental Fig. 2C). Taken together, these results suggest a link between autocrine secretion of leptin and mTOR activation in Teffs through an AKT-dependent mechanism.

To depict, in vivo, the role of leptin in mTOR activation in Teffs, we analyzed S6 phosphorylation in Teffs from LepR-deficient mice.
rapamycin (100 μg/mouse) or a single dose of either leptin or rapamycin/leptin (Fig. 6A). Nonimmunized mice served as controls. We found that the percentage and absolute number of Teffs were decreased by rapamycin to a greater extent after immunization compared with nonimmunized mice (Fig. 6B, 6C, Supplemental Fig. 3E, 3F). Conversely, leptin coadministered with rapamycin reversed the inhibition of Teffs induced by rapamycin alone (Fig. 6B, 6C). The reduction in Teffs induced by rapamycin was associated with an impaired proliferative capacity, as suggested by the reduced BrdU incorporation both in the peripheral blood (data not shown) and in the draining lymph nodes (Fig. 6D). Teff proliferation was restored by leptin replacement, which induced mTOR activity (Fig. 6E), thus confirming the role of leptin in the control of Teff responsiveness via mTOR modulation. Similar results were observed with regard to Ag-specific Teff proliferation in all groups of mice (Supplemental Fig. 3G, 3H). Teffs from rapamycin-treated mice 8 d (Supplemental Fig. 3G) or 12 d (Supplemental Fig. 3H) postimmunization had impaired proliferative capability upon in vitro stimulation with tuberculin-purified protein derivative or with the polyclonal stimulator anti-CD3ε mAb (2C11). Again, leptin replacement restored Teff proliferation.

LepR signaling effects on the functional and transcriptional signature of Teffs

To analyze whether the impairment of LepR signaling could affect Teff functions, we evaluated the proliferation of Teffs from db/db mice and their littermate controls (db/+). As shown in Fig. 7A, LepR deficiency inhibited Teff proliferation upon TCR-mediated stimulation (Fig. 7A). At the biochemical level, Teffs from db/+ mice had a significant induction of ERK1/2 activation and S6 phosphorylation after a 1-h culture in vitro; in contrast, Teffs from db/db mice had an impairment in both ERK1/2 and S6 activation,

(db/db) compared with their WT counterparts (db/+). Fig. 5D shows that the impaired leptin signaling in db/db mice was associated with reduced S6 phosphorylation. In leptin-deficient ob/ob mice, leptin treatment induced mTOR hyperactivation, as indicated by the increased phosphorylation of S6 (Fig. 5E), whereas it had little effect on WT mice in which leptin was present (Supplemental Fig. 2D). To avoid the interference of obesity and metabolic dysfunctions associated with congenital leptin deficiency, we also inhibited leptin signaling through acute starvation for 48 h, which dramatically reduces circulating leptin levels (24). To exclude Tregs from the analyses, we used eGFP-Foxp3 reporter mice to study only GFP2 Teff (Teff) cells (30). Mice were starved for 48 h, given leptin or not, and compared with mice fed ad libitum. Starvation induced a reduction in body weight (Supplemental Fig. 3A) and a decrease in serum leptin levels (Supplemental Fig. 3B) that was associated with a marked reduction in the numbers of splenocytes and CD4+ cells (Supplemental Fig. 3C, 3D); these events were reversed by leptin administration, as expected. The animals were treated with BrdU daily, and in vivo proliferation of Teffs in relation to leptin-mediated mTOR activity was evaluated. Starved mice displayed a strong inhibition of the proliferative Teff response, in terms of BrdU incorporation, compared with mice fed ad libitum and starved mice treated with leptin (Fig. 5F). These events were associated with a reduction in S6 phosphorylation in starved mice that was partially reversed by leptin replacement (Fig. 5G), suggesting a direct link in vivo between leptin and mTOR activity in Teffs.

To assess Teffs in vivo after Ag immunization (with CFA) and to assess the role of mTOR in the control of their proliferation, 12 h before CFA priming we injected mice with a single dose of rapamycin (100 μg/mouse) or a single dose of either leptin or
Protein kinase mTOR plays an essential role in maintaining homeostasis and in the regulation of protein synthesis in response to changing nutrient conditions (3). We report in this article that, in addition to its capacity to act as a nutrient sensor-signaling pathway, mTOR participates in the control of T eff responses. We found that mTOR inhibition with rapamycin impaired T eff proliferation via early downmodulation of ERK1/2 phosphorylation and increased accumulation of the cell cycle inhibitor p27kip1, and it inhibited IL-2 secretion (with reduced phosphorylation of STAT5, a central molecule in IL-2R signaling, and NF-κB). mTOR inhibition also decreased proinflammatory cytokine production in T effs, downmodulated the expression of activation markers, and inhibited TCR signaling, as revealed by low ZAP70 phosphorylation, elevation of the inhibitory LckTyr505, and decreased Akt signaling after TCR stimulation (Fig. 8). Of note, mTOR inhibition over time induced the expression of FOXP3, the master gene of Tregs. We can speculate that the increased expression of this molecule would switch T effs toward a regulatory phenotype and inhibit T effs (e.g., by downmodulating IL-2 secretion) (34).

Gene-expression profiling showed that mTOR inhibition led to a downmodulation of genes related to the control of transcription and apoptosis, contributing to a molecular explanation for the observed inhibition of T eff proliferation. In particular, mTOR inhibition downregulated the expression of several zinc fingers (ZF207, ZF689, ZF845), as well as genes encoding heat shock protein DnaJ (Hsp40), p53 binding protein, and superoxide dismutase, which are involved in transcription, mitotic phase assembly, response to DNA damage, and production of radicals, respectively. mTOR inhibition with rapamycin also inhibited genes involved in vesicle assembly, such as syntaxin, Na+/K+ ATPase, and potassium channel and amino acid transporters, further sup-

significant difference was detected between ad libitum fed mice and 48-h fasted + leptin mice. Representative of two independent experiments (n = 3 mice/group). *p < 0.001 versus ad libitum fed mice, **p < 0.05 versus ad libitum fed mice.
porting the idea that mTOR inhibition affects secretory function and cytokine release by Teffs. In contrast, mTOR inhibition upregulated the expression of genes related to the inhibition of transcription, such as histone deacetylase and methyl transferase, and the induction of catabolic processes, such as proteases, hydrolases, deaminases, and proteasome activators, suggesting the block of the transcription machinery. Altogether, these data suggest that mTOR inhibition blocks the transcriptional machinery in Teffs. Also, together with a decreased production of IFN-γ and IL-17 induced by mTOR inhibition in Teffs, rapamycin induced the expression of IL-27, which suppresses proinflammatory cytokine production and the Th17 switch (35).

We next characterized the role of leptin, a master regulator of intracellular metabolism, in the modulation of the intracellular metabolic pathway and confirmed that activation of the mTOR pathway in Teffs is clearly dependent on this adipocytokine.
Microarray data from leptin-neutralized Teffs revealed a transcriptional signature comparable to that observed in rapamycin-treated Teffs. Indeed, we detected a downmodulation of genes associated with cell cycle progression and proliferation, such as Septin7, anaphase-promoting complex, and tubulin, or connected to activation of transcription (zinc finger, eukaryotic translation initiation factor 3 and 4) and of immune system (MHC1, T cell activation RhoGTPase activating protein, hematopoietic cell-specific Lyn substrate), suggesting an impairment of Teff functions induced by leptin neutralization. These data were confirmed by either genetic or induced ablation of LepR signaling, which impairs mTOR pathway activation. It needs to be investigated further whether these results came from a direct Teff leptin/LepR reduction or inhibition of systemic adipocyte-derived leptin secretion. However, the evidence that rapamycin treatment was able to inhibit leptin production suggests that mTOR kinase may be involved in the control of leptin production at the adipocyte level. Because it is not known how adipocyte mass/cell size...
controls transcription of the leptin gene, we can speculate that this mechanism is mediated by mTOR kinase-mediated control of leptin secretion.

Of note, the model chosen in our investigations considered that most studies on mTOR have been performed in animals deficient in mTOR-related molecules (i.e., Rheb, Tsc1/Tsc2, mLST8, raptor, rictor) (36–38). Although these models have proven very valuable for the comprehension of key mechanisms of molecular action of related factors, we elected to study, in a relatively more physiological system, the leptin–mTOR axis, because the ablation of selected genes might cause compensatory activities and, more importantly, could not address the dynamic and oscillatory changes that we wanted to investigate in Teffs. Therefore, we used transient pharmacologic inhibition of mTOR in normal human and mouse Teffs and in vivo, rather than genetic means and chronic inhibition of the leptin–mTOR pathway.

Moreover, Teffs from LepR-deficient mice had reduced mTOR activity associated with a transcriptional signature of cell cycle arrest, impaired secretory functions, and downregulated expression of genes involved in the induction of transcription and protein translation, such as the initiation factor 3, the replication initiator factor, ribosomal proteins, and transcription factors, like TFIIA. Concomitantly, the impaired expression of genes connected to DNA repair function and cell cycle, such as cyclin-dependent kinase 2 and 8, suggests the presence of a state of hyporesponsiveness in Teffs from db/db mice. LepR deficiency also reduced vesicle formation and ion transport secretory capability and downmodulated NFAT, which plays a pivotal role in the induction of IL-2 (39). In the meantime, db/db mice upregulated the expression of genes related to cell death, such as caspase 8, annexin V, Gadd45g (which induces apoptosis and inhibits transcription), and proteases. Moreover, the induction of IL-24, which belongs to the Th2 cytokine family, of IL-18 binding protein, which binds to IL-18 inhibiting Th1 cytokine response (40), and of TGF-β–stimulated clone 22 (TSC22), which suppresses NF-κB DNA-binding activity and whose expression inversely correlates with T cell activation (41), support the hypothesis of impaired proliferation/activation process of Teffs in LepR-deficient mice, in line with the fact that mTOR inhibition decreases NF-κB activation (Fig. 2D). Importantly, mTOR inhibition and impaired leptin signaling had similar effects on Teff responses; the genetic profile observed in mTOR-inhibited Teffs had genes, which were upregulated or downregulated by rapamycin, of the same functional classes detected in the Teffs from LepR-deficient mice (Fig. 8, Supplemental Table I).

In this study, we also showed that leptin produced by Teffs contributes to the activation of the mTOR pathway in Teffs. In the cross-talk between leptin and mTOR in Teffs, rapamycin and subsequent mTOR inhibition significantly inhibit leptin secretion and LepR expression on this cellular subset. Taken together, these data suggest that leptin might be considered a pivotal element linking metabolic state to Teff function and response through mTOR pathway modulation. These results are in line with recent evidence showing that rapamycin inhibits adipogenesis in vitro and displays antiobesity effects in mice fed a high-fat diet. Indeed, mice treated with rapamycin had reduced body weight and epididymal fat pads/body weight, reduced daily food efficiency, and lower serum leptin and insulin levels compared with control mice fed a high-fat diet (42). Other studies showed that malnutrition impaired IFN-γ signaling by inhibiting mTOR kinase activity, thus linking again nutrient availability with immune cell response through modulation of the mTOR pathway (43). Moreover, recent evidence showed that adipose tissue, through leptin, has a key role in the survival of autoreactive CD4+ T cells directly through the activation of mTOR and the survival gene Bcl-2, as well as indirectly through the reduced secretion of cytokines important for autoreactive CD4+ T cell survival (IL-6, IL-15, IL-21, and GM-CSF) (29). In view of our recent findings that indicated hyperactivity of the leptin/mTOR pathway in Tregs...
(responsible for their hyposensitivity) (9), we think that the opposite effects of mTOR inhibition on Teffs and Tregs could be associated with a different expression of mTOR in the two cell subsets, together with differences in intracellular leptin and cell-surface LepR expression, which would affect their different responses to leptin.

Taken together, our report is in line with the epidemiological evidence that the frequency of autoimmunity is increasing in more affluent countries, and it correlates with increased body fat mass, higher leptin levels, and body weight at birth (44, 45). The studies published by Piccio et al. (46) showed that either nutritional deprivation or caloric restriction can profoundly modulate and reduce the magnitude and disease score of experimental autoimmune encephalomyelitis. The so-called “frugal phenotype,” in which survival of chronically food restricted mice is higher than mice fed ad libitum, might fit with the possibility of a reciprocal influence between nutritional/metabolic state and immune response in the control of self-tolerance. Similar results were obtained in mice in which chronic rapamycin treatment significantly increased their overall survival (47) by reducing the absorption of amino acids and glucose, as well as by dampening the level of a series of proinflammatory adipocytokines produced by adipocytes (including leptin) (48). The concept that T cells can sense the local milieu to respond accordingly in dependence on the environmental cues is expanded in this study by the finding that the environmental energy sensor leptin can directly modulate mTOR activity in Teffs to determine their responsiveness and activity (Fig. 8). These results can have relevance for the leptin-targeted approaches in the modulation of Teff activity in conditions where the effects of Teff can be either beneficial or detrimental to the host’s health status.

Acknowledgments

We thank Salvatore De Simone for technical help in the cell sorting MoFlo Core Facility at the Istituto di Endocrinologia e Oncologia Sperimentale, Consiglio Nazionale delle Ricerche, Napoli, Italy. This work is dedicated to the memory of Eugenia Papa and Serafino Zappacosta.

Disclosures

The authors have no financial conflicts of interest.

References


