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Article

A Key Role of Leptin in the Control of Regulatory T Cell Proliferation

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SUMMARY

We report here that leptin can act as a negative signal for the proliferation of human naturally occurring Foxp3$^+$CD4$^+$CD25$^+$ regulatory T (T_{reg}) cells. Freshly isolated T_{reg} cells produced leptin and expressed high amounts of leptin receptor (ObR). In vitro neutralization with leptin monoclonal antibody (mAb), during anti-CD3 and anti-CD28 stimulation, resulted in T_{reg} cell proliferation, which was interleukin-2 (IL-2) dependent. T_{reg} cells that proliferated in the presence of leptin mAb had increased expression of Foxp3 and remained suppressive. The phenomena appeared secondary to leptin signaling via ObR and, importantly, leptin neutralization reversed the anergic state of the T_{reg} cells, as indicated by downmodulation of the cyclin-dependent kinase inhibitor p27 (p27^{kip1}) and the phosphorylation of the extracellular-related kinases 1 (ERK1) and ERK2. Together with the finding of enhanced proliferation of T_{reg} cells observed in leptin- and ObR-deficient mice, these results suggest a potential for therapeutic interventions in immune and autoimmune diseases.

INTRODUCTION

Thymus-derived, naturally occurring, regulatory T (T_{reg}) cells are a subset of T lymphocytes that constitutes about 5%–10% of peripheral CD4$^+$ T cells. T_{reg} cells constitutively express the high-affinity interleukin-2 (IL-2) receptor α chain CD25 and can inhibit effector T cell responses in vitro and in vivo (Shevach, 2002; Sakaguchi, 2004; Suri-Payer et al., 1998; Thornton and Shevach, 1998; Ng et al., 2001). T_{reg} cells express the forkhead family transcription factor Foxp3, a key control gene for their development and function (Shevach, 2002; Sakaguchi, 2004). Given the importance of T_{reg} cells in the mechanisms of immune regulation and their protective role in several autoimmune conditions, there has been wide interest in finding strategies that expand T_{reg} cell numbers in the periphery (Kretschmer et al., 2005; Tang et al., 2004; Jiang et al., 2003). However, technical difficulties including hyporesponsiveness of freshly isolated T_{reg} cells to T cell receptor (TCR) stimulation in culture has hampered this process (Thornton and Shevach, 1998; La Cava et al., 2004). Nonetheless, murine and human T_{reg} cell numbers have been expanded in vitro up to 200-fold in the presence of high doses of IL-2 (Tang et al., 2004; Jiang et al., 2003). These proliferated T_{reg} cells continue to express cell-surface molecules and intracellular markers consistent with a regulatory phenotype and effectively suppress in vitro proliferative responses to either anti-CD3 or allogeneic stimuli, in a fashion similar to naturally occurring T_{reg} cells (Tang et al., 2004; Jiang et al., 2003).

Leptin is a cytokine-like hormone structurally similar to IL-6 and is involved in the control of food intake, metabolism, and T cell function (Friedman and Halaas, 1998; La Cava and Matarese, 2004). We report here that freshly isolated human T_{reg} cells constitutively expressed high amounts of both leptin and the leptin receptor (ObR) and that the leptin pathway can act as a negative signal for the proliferation of T_{reg} cells. These findings may partly explain why chronic leptin- and leptin-receptor deficiency associate with increased susceptibility to infection and resistance to autoimmunity (La Cava and Matarese, 2004; Ikejima et al., 2005; Farooqi et al., 2002; Matarese et al., 2002) and the increased risk of infection and reduced incidence of autoimmunity in individuals with low leptin (Matarese et al., 2002). These results may also suggest new possibilities for leptin-based manipulation of the T_{reg} cells.

RESULTS

Human T_{reg} Cells Express Higher Amounts of ObR than Do CD4$^+$CD25$^-$ Effector T Cells

Previous studies have shown that leptin receptor (ObR) is expressed on CD4$^+$ T cells and that it is able to switch immune responses toward a T helper 1 (Th1) phenotype (La Cava and Matarese, 2004). We sought to analyze the expression of ObR on T_{reg} cells and to correlate its

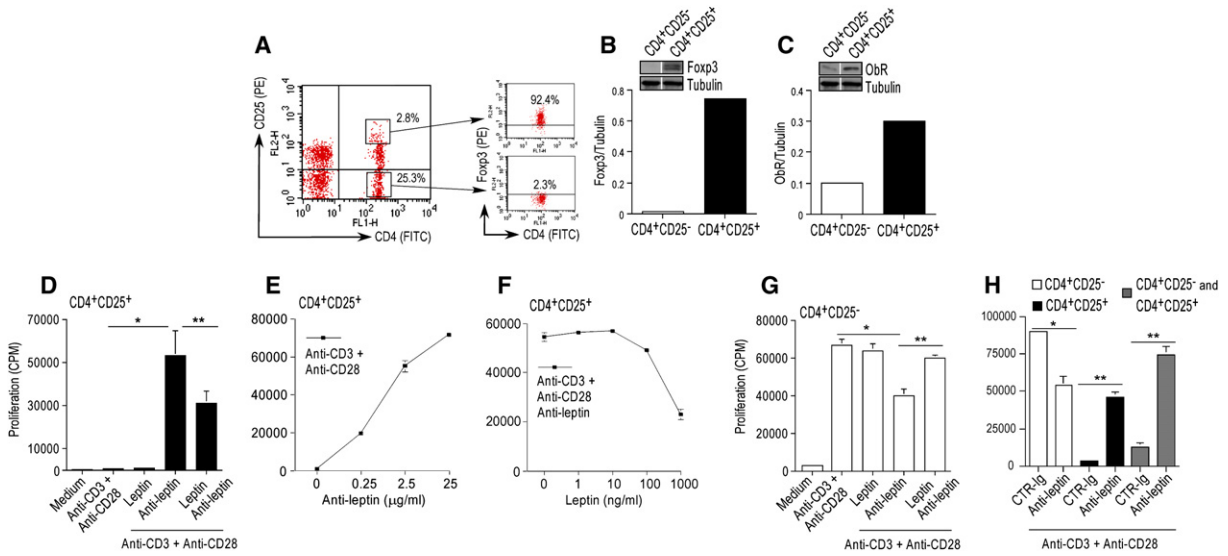

Figure 1. Human T_{reg} Cells Express High ObR, and Leptin Neutralization Reverses Their Hyporesponsiveness

(A) Representative flow cytometry plot of human T cells stained for CD4, CD25, and Foxp3.

(B and C) Immunoblot analysis of sorted $CD4^+$ T cells on the basis of their CD25 expression. Graphs show quantitation of Foxp3 and ObR with respect to tubulin. One representative out of five independent experiments is shown.

(D) Proliferation of $CD4^+CD25^+$ T_{reg} cells treated with recombinant human leptin (250 ng/ml) in the presence or absence of leptin mAb (10 μg/ml). The data are shown as mean ± SD (n = 5, *p < 0.0001; **p < 0.01).

(E) Dose dependency of T_{reg} cell proliferation induced by leptin mAb. Proliferation was measured after treatment with indicated doses of leptin mAb. The data are shown as mean ± SD (n = 5).

(F) Proliferation of T_{reg} cells induced by a fixed dose of leptin mAb in the presence of increasing concentration of recombinant leptin. The data are shown as mean ± SD (n = 5).

(G) Proliferation of $CD4^+CD25^-$ effector T cells treated with recombinant human leptin (250 ng/ml) in the presence or absence of leptin mAb (10 μg/ml). The data are shown as mean ± SD (n = 5, *p < 0.01; **p < 0.01).

(H) Proliferation of $CD4^+CD25^-$ effector T cells, T_{reg} cells, and of both cell types in coculture in the presence or absence of leptin mAb (10 μg/ml). The data are shown as mean ± SD (n = 5, *p < 0.01; **p < 0.0001).

expression with Foxp3. $CD4^+CD25^+$ T_{reg} cells and $CD4^+CD25^-$ effector T cells purified from human healthy donors (Figure 1A, left) were analyzed for Foxp3 expression by FACS analysis and immunoblot (Figure 1A, right, and Figure 1B, respectively). As expected, T_{reg} cells showed high amounts of Foxp3 protein whereas $CD4^+CD25^-$ did not show detectable amount of the protein in cell extracts (Figures 1A and 1B). Instead, ObR was expressed on both freshly isolated cellular subsets, although at significantly higher amounts ($p < 0.001$) in T_{reg} cells as compared to the $CD4^+CD25^-$ T cell effectors (Figure 1C).

Leptin Neutralization Results in the Proliferation of Human T_{reg} Cells Stimulated with Anti-CD3 and Anti-CD28

Human T_{reg} cells were hyporesponsive to anti-CD3 and anti-CD28 stimulation (Figure 1D), in agreement with previous findings (Ng et al., 2001). Addition of exogenous recombinant leptin to the cultures did not alter T_{reg} cells hyporesponsiveness (Figure 1D). However, addition of neutralizing leptin monoclonal antibody (mAb) reversed the hyporesponsiveness and promoted T_{reg} cell proliferation (Figure 1D) in a dose-dependent fashion (Figure 1E). Analogous results were observed with different leptin or ObR-blocking antibodies (see Figure S1 in the Supplemental Data available online). Confirming specificity, addition of exogenous recombinant leptin to anti-CD3- and anti-CD28-stimulated T_{reg} cells antagonized the proliferation induced by leptin mAb (Figure 1D). Moreover, dose-dependent increase of the proliferation of stimulated T_{reg} cells in the presence of leptin mAb (Figure 1E) was reversed by addition of increasing doses of recombinant leptin (Figure 1F). Interestingly, leptin mAb inhibited the proliferation of purified effector $CD4^+CD25^-$ T cells, a phenomenon that was reversed by addition of exogenous leptin (Figure 1G). Thus, neutralization of leptin had opposite effects on effector $CD4^+CD25^-$ T cells and T_{reg} cells—it inhibited proliferation on the former lymphocyte subset (Figure 1G), whereas it promoted expansion of the latter subpopulation (Figure 1D). This effect was also evident morphologically, as formation of cell clumps in the cultures of T_{reg} cells stimulated with anti-CD3 and anti-CD28 and leptin mAb, but not in the cultures of T_{reg} cells stimulated with anti-CD3 and anti-CD28 in the absence of leptin mAb (data not shown). Finally, in coculture experiments, T_{reg} cells efficiently suppressed the proliferation of $CD4^+CD25^-$ T cells, and leptin neutralization reversed the suppression by T_{reg} cells (Figure 1H).

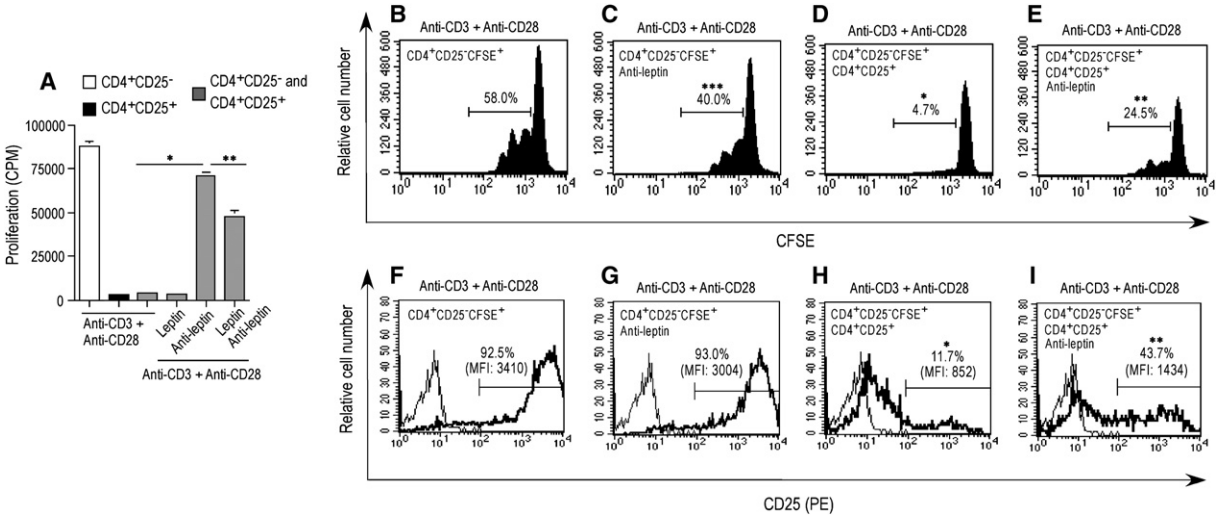

Figure 2. Human T_{reg} Cells Exhibit Partial Suppressive Capacity upon Leptin-mAb-Induced Proliferation

(A) Proliferation of $CD4^+CD25^-$ effector T cells, T_{reg} cells, and of both cell types in coculture treated with recombinant human leptin (250 ng/ml) in the presence or absence of leptin mAb (10 μg/ml). The data are shown as mean ± SD (n = 3, *p < 0.0001; **p < 0.01).

(B–I) Proliferative response (B–E) and CD25 expression analysis (F–I) of CFSE-labeled-$CD4^+CD25^-$ effector T cells alone or in coculture with untreated or leptin-mAb-treated unlabeled T_{reg} cells. The thin line represents the isotype-matched negative control and the thick line represents the CD25 staining. One representative out of three independent experiments is shown (*p < 0.0001; **p < 0.01; ***p < 0.05, as compared with $CD4^+CD25^-CFSE^+$).

Proliferated T_{reg} Cells Have Partial Suppressive Capacity that Attains Full Activity when They Enter the Resting Phase

The suppressive capability of T_{reg} cells in the presence of leptin mAb was tested in coculture experiments of T_{reg} cells together with $CD4^+CD25^-$ T cells. Addition of exogenous leptin did not affect suppression of T_{reg} cells on $CD4^+CD25^-$ T cells (Figure 2A). In contrast, suppression of proliferation was apparently abrogated in the presence of leptin mAb (Figure 2A, gray columns). Concomitant addition of leptin together with the leptin mAb partly reduced the anti-leptin-induced reversal of suppression, confirming specificity for leptin neutralization in the coculture experiments (Figure 2A, gray columns).

To understand whether T_{reg} cells during anti-leptin-induced proliferation could exert suppressive capacity, we performed coculture experiments with CFSE-labeled $CD4^+CD25^-$ T cells in the presence of unlabeled T_{reg} cells (Figures 2B–2E). As expected, T_{reg} cells suppressed the expansion of $CD4^+CD25^-$ effectors (Figure 2D), and suppression was partially maintained in the presence of leptin mAb (Figure 2E)—albeit at a lower degree when compared with untreated T_{reg} cells (Figure 2D). This suggested that T_{reg} cells during anti-leptin-induced proliferation are partially functional in terms of suppressive capability. Moreover, the coculture experiments of T_{reg} cells with $CD4^+CD25^-$ T cells, in the presence of leptin mAb, indicated that the reversal of suppression (Figure 2A, gray columns) was apparent only because $CD4^+CD25^-$ effectors were inhibited in part in their proliferation, as indicated by the CFSE dilution (Figure 2E). Thus, the high amount of [^{3}H]thymidine incorporation in coculture of T_{reg} cells with $CD4^+CD25^-$ T cells in the presence of leptin mAb (Figures 1H and 2A) has to be ascribed to both T_{reg} cell proliferation and partly to $CD4^+CD25^-$ T cells. These phenomena were confirmed in terms of expression of the activation marker CD25 on CFSE-labeled $CD4^+CD25^-$ effector T cells. The anti-CD3 and anti-CD28 activation induced a substantial upregulation of CD25 on the cell surface (Figure 2F) and was significantly inhibited in the presence of T_{reg} cells (Figure 2H). A significant reduction of CD25 expression on effector T cells was observed in the presence of anti-leptin-expanding T_{reg} cells (even if at lower extent when compared with untreated T_{reg} cells) (Figure 2I). In any case, the suppressive capacity of anti-leptin-expanding T_{reg} cells was diminished by about 40%, and a certain number of $CD4^+CD25^-$ T cells was still able to proliferate and divide (Figure 2E).

Next, we performed two-step experiments to address whether anti-leptin-proliferated T_{reg} cells in the resting phase could retain their suppressive capacity over time. 8 days after the first expansion, stimulated T_{reg} cells were still suppressive for $CD4^+CD25^-$ effector T cells in in vitro coculture experiments (Figures S2A–S2C). Although effector $CD4^+CD25^-$ T cells cultured in the presence of leptin mAb proliferated upon restimulation and did not suppress allogeneic $CD4^+CD25^-$ T cells expansion in coculture experiments (Figure S2B), the anti-leptin-expanded T_{reg} cells maintained their hyporesponsiveness after restimulation and were capable of suppressing proliferation of allogeneic $CD4^+CD25^-$ effector T cells (Figure S2C). These data suggested that the anti-leptin-proliferated T_{reg} cells, once in the resting phase, maintain their suppressive capacity over time.

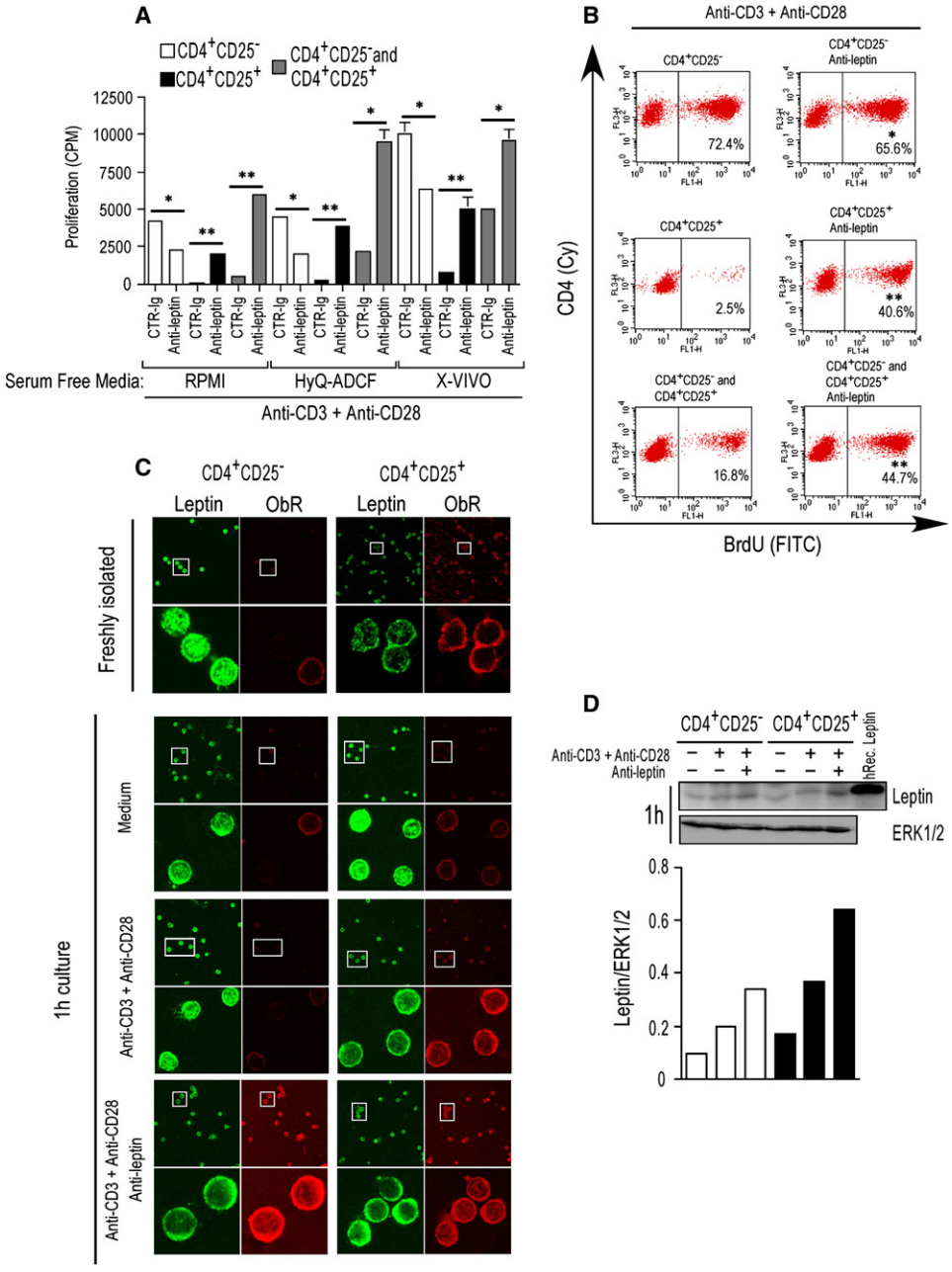

Figure 3. T_{reg} Cells Produce Leptin and Express High Amounts of ObR

(A) Proliferation of $CD4^+CD25^-$ effector T cells, T_{reg} cells, and of both cell types in coculture in three different serum- and leptin-free media in the presence or absence of leptin mAb. The data are shown as mean ± SD (n = 6, *p < 0.01; **p < 0.001).

(B) Flow cytometry plot of BrdU incorporation of $CD4^+CD25^-$ effector T cells, T_{reg} cells, and of both cell types in coculture in serum-free medium (X-VIVO), in the presence or absence of leptin mAb during anti-CD3 and anti-CD28 stimulation. One representative out of three independent experiments is shown (*p < 0.05; **p < 0.001).

(C) Confocal microscopy of freshly isolated and 1 hr-stimulated T_{reg} cells and $CD4^+CD25^-$ effectors stained for leptin (in green) and leptin receptor (ObR) (in red). One representative out of three independent experiments is shown.

(D) Immunoblot for leptin on cell lysates from T_{reg} cells and $CD4^+CD25^-$ effectors. The graph shows quantitation of leptin with respect to total ERK1/2. One representative out of three independent experiments is shown.

Leptin Production from Human T_{reg} Cells

Leptin is present in media supplemented with human serum, such as the medium used in our experiments (see Experimental Procedures). To test whether human T_{reg} cells could expand in the absence of leptin, we stimulated T_{reg} cells with anti-CD3 and anti-CD28 in three different types of serum- and leptin-free media (Figure 3A). Under these conditions, T_{reg} cells maintained hyporesponsiveness

even in the absence of exogenous leptin. Surprisingly, addition of leptin mAb to the cultures resulted in T_{reg} cell proliferation (Figure 3A). This finding was also confirmed by BrdU incorporation in serum-free medium cultures (Figure 3B). This finding suggests the possibility that leptin may be produced by T_{reg} cells in a fashion similar to the production of leptin by $CD4^+$ T cells and monocytes in multiple sclerosis (Sanna et al., 2003; Matarese et al., 2005). To test this possibility, we examined leptin and ObR expression on T_{reg} cells and $CD4^+CD25^-$ T cells. Both freshly isolated T_{reg} cells and $CD4^+CD25^-$ T cells stained positive for leptin and ObR, with different intensity patterns (Figure 3C). Moreover, after 1 hr culture, T_{reg} cells showed higher leptin production than did $CD4^+CD25^-$ T cells, and this tendency was maintained during anti-CD3 and anti-CD28 stimulation, both in the presence and in the absence of leptin mAb (Figure 3C). Parallel quantitation by immunoblotting analysis for leptin on cell lysates confirmed the difference (Figure 3D). Indeed, the presence of a basal production of leptin increased significantly after anti-CD3 and anti-CD28 stimulation (Figure 3D), and T_{reg} cells always produced more leptin than did $CD4^+CD25^-$ T cells (Figure 3D). Interestingly, leptin neutralization induced a compensatory leptin production and ObR upregulation in both T_{reg} cells and $CD4^+CD25^-$ T cells, and again, to a higher amount in the T_{reg} cells (Figures 3C and 3D). These results were confirmed at 12 hr by confocal microscopy and immunoblotting studies (data not shown). Additionally, to address the capacity of T_{reg} cells and $CD4^+CD25^-$ T cells to secrete leptin, we performed a human leptin-specific ELISA (see Experimental Procedures) and confocal microscope analysis at different time points (12 hr and 36 hr) on culture supernatants (Figure S3). At 12 hr, the secretion of leptin was similar in both T_{reg} cells and $CD4^+CD25^-$ T cells, whereas at 36 hr the amount of leptin secreted was significantly higher in the T_{reg} cells, either unstimulated or treated with anti-CD3 and anti-CD28 plus leptin mAb (Figure S3). The finding was confirmed by the observation of a reduced content of leptin in T_{reg} cells at 36 hr (Figure S3) in confocal microscopy and immunoblotting studies on cell lysates (data not shown). Finally, these results on leptin secretion were also confirmed by immunoblotting for leptin on culture supernatants (data not shown). Thus, these results suggest the presence of an autocrine loop of leptin secretion by T_{reg} cells controlling their hyporesponsiveness.

Leptin-mAb-Induced Proliferation of T_{reg} Cells Is IL-2 Dependent, and IL-2-Supported Expansion of T_{reg} Cells Is Not Affected by Leptin

To test whether the leptin-mAb-induced proliferation of T_{reg} cells was IL-2 dependent, we evaluated the effects of IL-2 neutralization on the T_{reg} cell proliferative responses and IL-2 production. Addition of human IL-2-neutralizing mAb enhanced the inhibitory effects of leptin mAb treatment on the $CD4^+CD25^-$ T cell proliferation and IL-2 secretion (Figures 4A and 4B, white bars). IL-2 mAb reduced both T_{reg} cell proliferation and IL-2 production induced by the neutralization of leptin (Figures 4A and 4B, black bars). Moreover, the apparent reversal of suppression in coculture experiments resulting from anti-leptin-induced T_{reg} cells proliferation was inhibited by anti-IL-2 (Figures 4A and 4B, gray bars). IL-2 secretion was evaluated via CTLL-2 proliferation (Figure 4B). These results were also confirmed by intracellular staining for IL-2 in FACS analysis and ELISA (Figure S4).

Next we compared IL-2-induced proliferation versus anti-leptin-induced proliferation on T_{reg} cells. Addition of exogenous IL-2 reversed T_{reg} cells unresponsiveness upon anti-CD3 and anti-CD28 stimulation (Figure 4C, left), as expected. Of interest, IL-2-induced proliferation was less than that observed upon leptin blockade (Figure 4C, left). To also address whether anti-leptin treatment affected the ability of T_{reg} cells to proliferate in the presence of IL-2 but in the absence of TCR stimulation, we measured the proliferation of unstimulated T_{reg} cells cultured with leptin mAb or IL-2 and anti-leptin plus IL-2. No proliferation was observed in the absence of TCR engagement in all the above conditions (Figure 4C, left). Finally, we tested whether leptin could inhibit IL-2-induced expansion of T_{reg} cells. The anti-proliferative effect of increasing doses of recombinant leptin on T_{reg} cell proliferation induced by IL-2 was not significant (Figure 4C, right), suggesting that IL-2 signaling may be independent and dominant of the leptin-mediated inhibitory effects on the cell proliferation.

T_{reg} Cells Expanded with Leptin mAb Have Increased Expression of Foxp3

We next addressed whether leptin neutralization could affect the amount of Foxp3 in T_{reg} cells. Foxp3 expression was evaluated after leptin neutralization at 1 hr and 12 hr during anti-CD3 and anti-CD28 stimulation. As shown in Figure 4D, Foxp3 expression was increased at early time points after anti-CD3 and anti-CD28 stimulation, and leptin mAb treatment did not alter Foxp3 protein amounts. Conversely, at 12 hr, T_{reg} cells that proliferated after leptin mAb showed increased Foxp3 amounts when compared to those activated with anti-CD3 and anti-CD28 alone (Figure 4E). As expected, Foxp3 was undetectable in $CD4^+CD25^-$ T effectors after 1 hr of stimulation in all the experimental conditions (Figure 4D), whereas at 12 hr, there was a little expression of Foxp3 after anti-CD3 and anti-CD28 stimulation either in the presence or in the absence of leptin mAb that did not reach statistical significance (Figure 4E). All these data were confirmed by real-time PCR (data not shown).

The Effect of Leptin Neutralization on ObR and STAT3 Activation Pathway

The molecular effects of leptin mAb were studied at early (1 hr) or late (12 hr) time points on highly purified human T_{reg} cells or effector $CD4^+CD25^-$ T cells, activated or not with anti-CD3 anti-CD28.

We first analyzed the leptin-ObR-STAT3 biochemical pathway: 1 hr and 12 hr cultures confirmed that in unstimulated cells, the ObR was expressed at higher amounts in T_{reg} cells than in $CD4^+CD25^-$ T cells (Figures 3C, 5A,

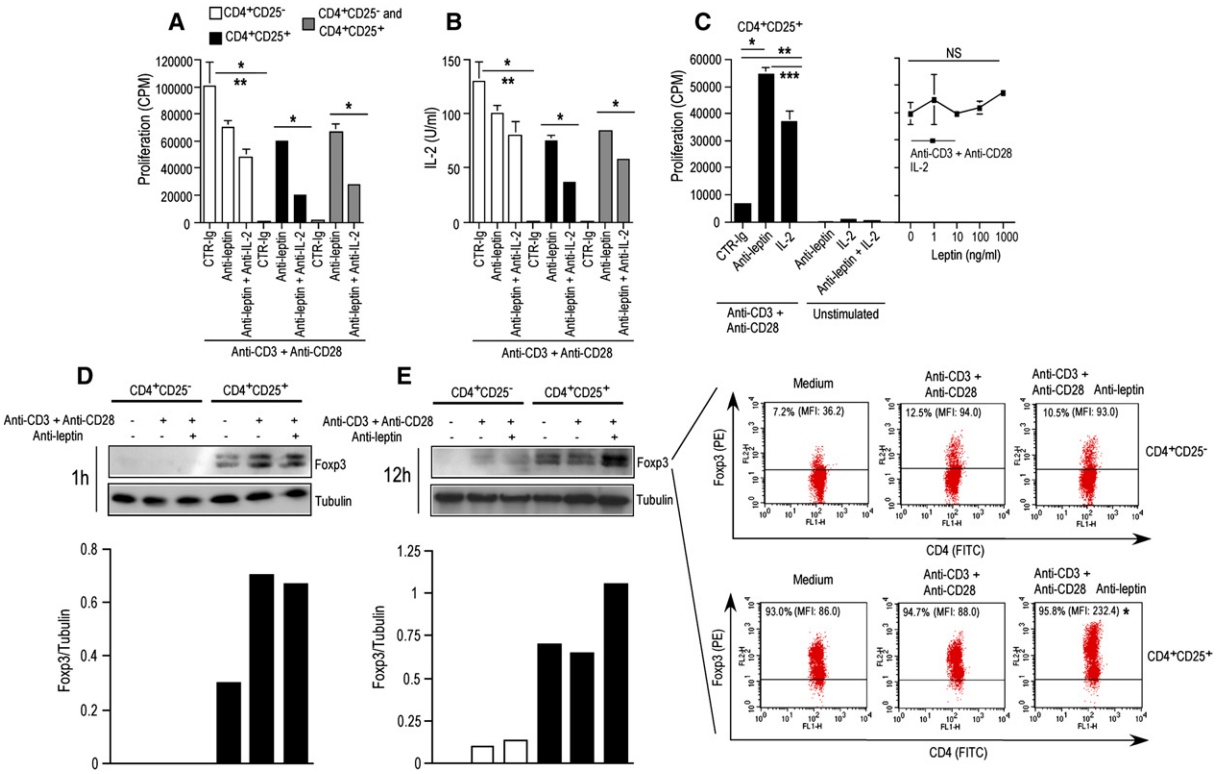

Figure 4. Leptin-mAb-Induced Proliferation of Human T_{reg} Cells Is IL-2 Dependent, and Foxp3 Expression Is Increased during Proliferation

(A) Proliferation of $CD4^+CD25^-$ effector T cells, T_{reg} cells, and of both cell types in coculture in the presence or absence of leptin mAb and IL-2-neutralizing mAb. The data are shown as mean ± SD (n = 5, *p < 0.01, **p < 0.05, white bars; *p < 0.001, black bars; *p < 0.01, gray bars).

(B) IL-2 secretion of $CD4^+CD25^-$ effector T cells, T_{reg} cells, and of both cell types in coculture in the presence or absence of leptin mAb and IL-2-neutralizing mAb. The data are shown as mean ± SD (n = 5, *p < 0.01 and **p < 0.05, white bars; *p < 0.001, black bars; *p < 0.01, gray columns).

(C) Proliferation of T_{reg} cells in the presence of either leptin mAb or recombinant IL-2 (left), stimulated or not with anti-CD3 and anti-CD28. The data are shown as mean ± SD (n = 5, *p < 0.0001; **p < 0.01; ***p < 0.05). Addition of scalar doses of recombinant leptin to proliferating T_{reg} cells (right) stimulated with anti-CD3 and anti-CD28 in the presence of recombinant IL-2. The data are shown as mean ± SD (n = 5, NS, not significant).

(D) Immunoblot analysis of $CD4^+CD25^-$ effector T cells and T_{reg} cells in the presence or absence of leptin mAb, at 1 hr stimulation with anti-CD3 and anti-CD28. The graph shows quantitation of Foxp3 with respect to tubulin. One representative out of five independent experiments is shown.

(E) Immunoblot analysis and flow cytometry plot of $CD4^+CD25^-$ effector T cells and T_{reg} cells in the presence or absence of leptin mAb, at 12 hr stimulation with anti-CD3 and anti-CD28. The graph shows quantitation of Foxp3 with respect to tubulin. One representative out of five independent experiments is shown (*p < 0.01, as compared with anti-CD3 and anti-CD28).

and 5B). In vitro stimulation with anti-CD3 and anti-CD28 induced an upregulation of the ObR expression on both cell types and was more evident at 1 hr than at 12 hr (Figures 3C, 5A, and 5B). Leptin neutralization on both T_{reg} cells and $CD4^+CD25^-$ T cells further upregulated the expression of ObR, particularly at 1 hr, suggesting that leptin blockade might induce a compensatory upregulation of its receptor.

As readout of ObR activity, we measured phospho-STAT3 (P-STAT3) amounts, because STAT3 is known to participate in the intracellular signaling pathways of ObR (reviewed in La Cava and Matarese, 2004). Activation of $CD4^+CD25^-$ T cells induced strong STAT3 phosphorylation at 1 hr and 12 hr and anti-leptin treatment reduced the P-STAT3 amounts, particularly at 1 hr (Figures 5A and 5B). Conversely, stimulation of T_{reg} cells was not associated with a marked increase of P-STAT3, whereas anti-leptin induced amounts of P-STAT3 at 1 hr and 12 hr comparable to those observed in proliferating $CD4^+CD25^-$ T cells (Figures 5A and 5B). Of note, at 1 hr, $CD4^+CD25^-$ T cells expressed both STAT3 spliced isoforms (Maritano et al., 2004)—STAT3α and STAT3β—whereas T_{reg} cells mainly expressed the STAT3α isoform. This phenomenon was not as evident after 12 hr when the two spliced isoforms were similar in both cell subsets, suggesting that the tissue-culture conditions could induce STAT3β in T_{reg} cells, independently of the different type of stimulation. Finally, in unstimulated or anti-CD3- and anti-CD28-stimulated T_{reg} cells, at both 1 hr and 12 hr, the amounts of P-STAT3 were higher than those observed in unstimulated $CD4^+CD25^-$ cells, suggesting an increased "basal" leptin-mediated signaling in T_{reg} cells. This result could be secondary to higher basal expression of ObR on T_{reg} cells (Figures 1C, 3C, 5A, and 5B). No induction of P-STAT3 was observed in T_{reg} cells treated with leptin mAb alone without TCR stimulation (Figure S5).

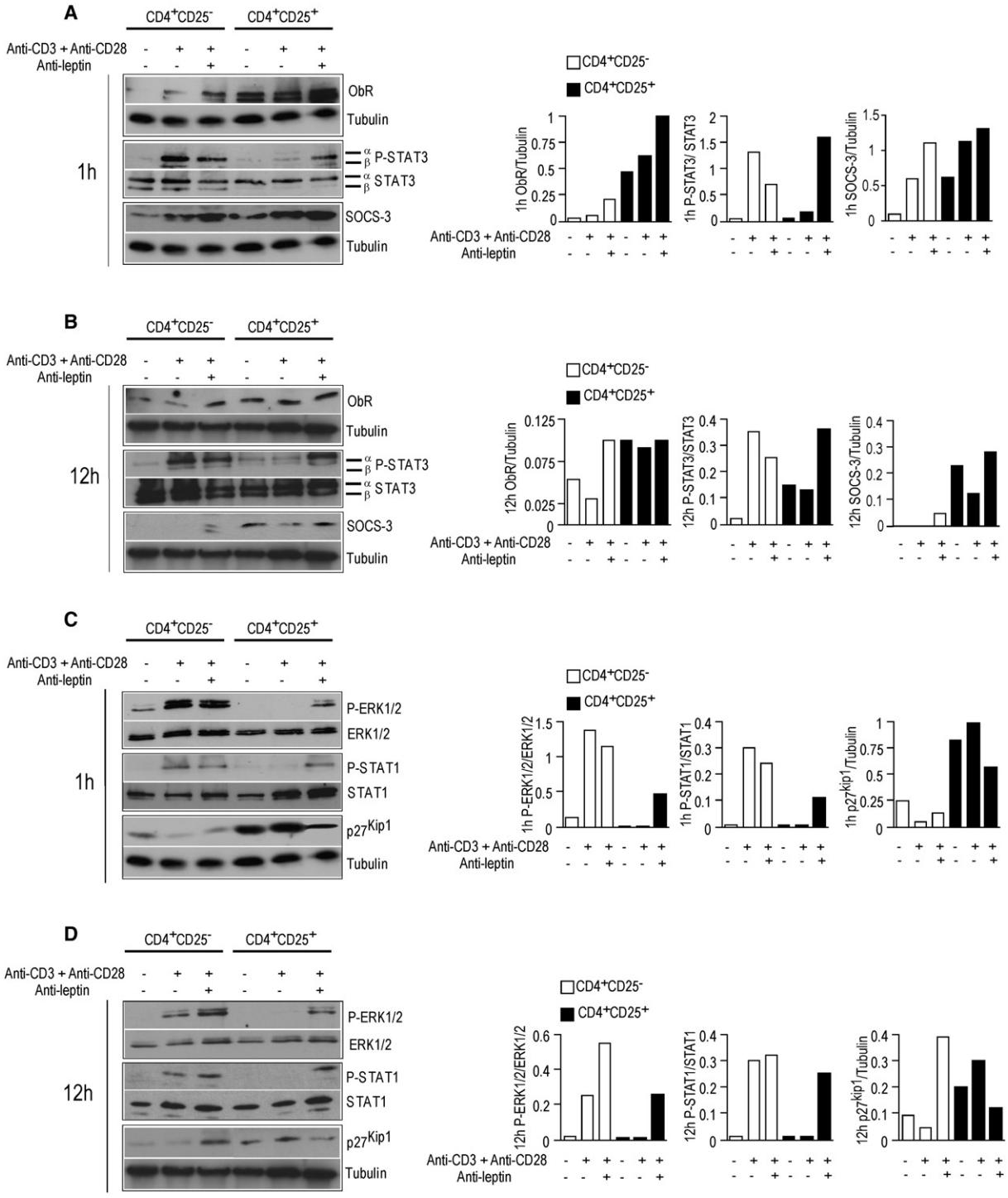

Figure 5. Molecular Effects of Leptin Neutralization on Human T_{reg} Cells

(A and B) Immunoblot for ObR, STAT3, and SOCS3 on $CD4^+CD25^-$ T cells and T_{reg} cells in the presence or absence of leptin mAb stimulated with anti-CD3 and anti-CD28 at 1 hr and 12 hr, respectively. Graphs show quantitation of each specific protein. One representative out of five independent experiments is shown.

(C and D) Immunoblot for ERK1/2, STAT1, and $p27^{kip1}$. Graphs show quantitation of each specific protein. One representative out of five independent experiments is shown.

We also investigated in our system the expression of suppressor of cytokine signaling 3 (SOCS3), a key negative regulator of cytokine signaling, including leptin (Kinjyo et al., 2006; La Cava and Matarese, 2004). SOCS3 in anti-CD3- and anti-CD28-stimulated $CD4^+CD25^-$ T cells increased markedly and was further increased by leptin neutralization at 1 hr (Figure 5A). This finding inversely correlated with the corresponding P-STAT3 expression. At a later time point (12 hr), SOCS3 was undetectable and could be found at low amounts upon leptin neutralization (Figure 5B). At 1 hr, SOCS3 in T_{reg} cells was markedly higher than in $CD4^+CD25^-$ cells in all the experimental conditions. Specifically, SOCS3 expression was high in unstimulated T_{reg} cells and increased after anti-CD3 and anti-CD28 stimulation despite little STAT3 activation. Treatment with anti-leptin, which induced P-STAT3, did not alter the SOCS3 amounts (Figure 5A). At 12 hr, basal SOCS3 expression was higher in the T_{reg} cells than in $CD4^+CD25^-$ cells and was markedly reduced after anti-CD3 and anti-CD28 stimulation. In conclusion, leptin mAb treatment—which associates with STAT3 activation in T_{reg} cells—also induced SOCS3 expression (Figure 5B).

The Effect of Leptin Neutralization on Biochemical Pathways Involved in T Cell Activation and Anergy

To evaluate whether leptin neutralization could affect T cell activation and anergy, we studied tyrosine-phosphorylation of the extracellular signal-regulated kinases 1 and ERK2 (ERK1/2). Leptin neutralization in the presence of anti-CD3 and anti-CD28 in $CD4^+CD25^-$ T cells increased ERK1/2 phosphorylation as compared to anti-CD3 and anti-CD28 stimulation alone, particularly at 12 hr (Figures 5C and 5D). Treatment of T_{reg} cells with anti-CD3 and anti-CD28 did not induce phospho-ERK1/2 (P-ERK1/2), confirming their anergic state. However, leptin mAb induced high amounts of ERK1/2 tyrosine-phosphorylation at both 1 hr and 12 hr, concomitantly with the induction of T_{reg} cell proliferation and a reversal of their hyporesponsiveness (Figures 5C and 5D).

Moreover, we investigated whether leptin neutralization could alter STAT1, another transcription factor whose activity is required for the development and function of T_{reg} cells (Nishibori et al., 2004). We found that leptin neutralization did not affect STAT1 phosphorylation (P-STAT1) in $CD4^+CD25^-$ effectors at both time points. In contrast, P-STAT1 was markedly induced in the leptin-neutralized T_{reg} cells (Figures 5C and 5D).

Last, we studied the modulation of the cyclin-dependent kinase inhibitor p27 ($p27^{kip1}$), a molecule involved in the control of cell cycle, T cell anergy and known to block the cell-cycle progression in T_{reg} cells (Li et al., 2005; Wells et al., 2001). $p27^{kip1}$ was downmodulated by anti-CD3 and anti-CD28 stimulation in $CD4^+CD25^-$ effectors whereas anti-leptin did not downmodulate $p27^{kip1}$ expression at 1 hr and markedly increased its expression at 12 hr (Figures 5C and 5D), explaining, at least in part, the inhibition of $CD4^+CD25^-$ T cells proliferation induced by leptin neutralization. In contrast, T_{reg} cells showed elevated amounts of $p27^{kip1}$ before and after anti-CD3 and anti-CD28 stimulation, confirming their anergic state associated with cell-cycle arrest. Leptin mAb treatment induced degradation of this molecule at both 1 hr and 12 hr, which could explain reversal of their anergic state and subsequent proliferation (Figures 5C and 5D).

Leptin Deficiency Promotes Proliferation of T_{reg} Cells in Mice

T_{reg} cells, despite their in vitro hyporesponsiveness, can expand in vivo in normal, nonlymphopenic hosts (Trenado et al., 2003; Gavin et al., 2002). We used nonirradiated, nonlymphopenic recipient mice to avoid a homeostatic expansion of the T_{reg} cells that would occur in lymphopenic hosts (Trenado et al., 2003; Gavin et al., 2002). The in vivo proliferative capacity of T_{reg} cells in anti-leptin-treated wild-type (WT) mice versus control-Ig-treated mice was tested with CFSE-labeled-$CD4^+$ T cells from normal WT mice and by measuring the CFSE dilution in the $CD4^+Foxp3^+$ cells (Figure 6A). Mouse T_{reg} cells expanded in vivo more robustly and earlier (day 4 and day 7 after transfer) when adoptively transferred into leptin-neutralized WT mice (Figure 6A). This result was confirmed by adoptive transfer of CFSE-labeled-$CD4^+$ T cells from normal WT mice into leptin-deficient *ob/ob* mice, in which the T_{reg} cells expanded more robustly and earlier (day 4 and day 7 after transfer) when compared with cells transferred into normal WT mice (Figure 6B). We also confirmed our data in a transgenic antigen-specific system utilizing donor mice carrying the (TcrAND)53Hed transgene (AND-TCR Tg mice) expressing the clonotypic $V\alpha11.1/V\beta3$ TCR chains, specific for the carboxy-terminal fragment of pigeon cytochrome c (PCC) (Kaye et al., 1989). Assessment 4 and 7 days after adoptive transfer revealed that transferred CFSE-labeled clonotypic AND-TCR T_{reg} cells proliferated more vigorously in *ob/ob* mice as compared with normal WT controls (Figure 6C). This phenomenon was reversed by recombinant leptin administration to leptin-deficient *ob/ob* mice (Figure 6C). To avoid interference of $CD4^+$ T cell lymphopenia on the T_{reg} cell expansion in vivo, in the adoptive transfer experiments in *ob/ob* mice, we utilized 6-week-old mice (in which the leptin deficiency has not yet determined significant reduction of the $CD4^+$ T cell number). The in vivo results were also confirmed by CFSE dilution experiments gating on the $CD4^+CD25^+$ T_{reg} cells in both leptin-neutralized WT and leptin-deficient *ob/ob* mice, respectively (Figures S6A and S6C, respectively). Foxp3 expression was increased in the T_{reg} cells of the leptin-neutralized WT mice (Figure S6B). Finally, to confirm our data, we tested the ability of T_{reg} cells to expand in the presence of an anti-leptin receptor (anti-ObR)-blocking antibody. The data showed a marked increase in the percentage of $CD4^+Foxp3^+$ T_{reg} cells in treated WT mice (data not shown).

Proliferative Potential and Functional Capacity of T_{reg} Cells from Leptin Receptor-Deficient Mice

We studied the in vitro proliferation and suppressive capacity of $CD4^+CD25^-$ effectors and T_{reg} cells from congenitally leptin-receptor-deficient *db/db* mice and normal

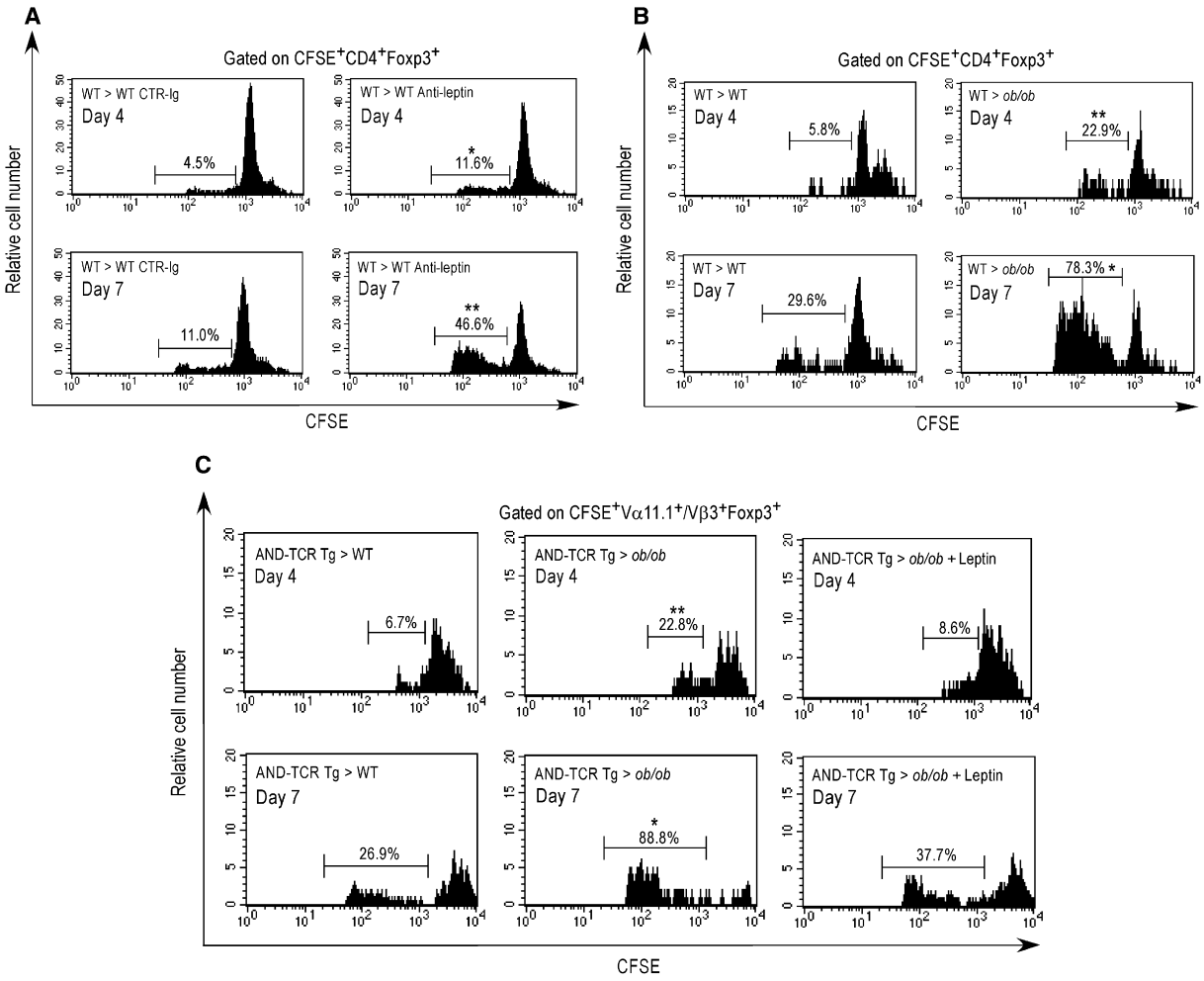

Figure 6. In Vivo Leptin Neutralization or Congenital Leptin Deficiency Associate with Proliferation of T_{reg} Cells

(A) Proliferation measured as CFSE dilution of CFSE-labeled T_{reg} cells obtained from WT mice and transferred into control (CTR)-Ig or mouse leptin neutralizing Ab-treated WT mice. The histogram shows the percent of $CFSE^+$ T_{reg} cells (gated on $CD4^+Foxp3^+$ cells) that had divided 4 and 7 days after transfer. One representative out of three independent experiments with 3 mice per group is shown (*$p < 0.01$; **$p < 0.001$).

(B) CFSE dilution profile of CFSE-labeled T_{reg} cells (gated on $CD4^+Foxp3^+$ cells) obtained from WT mice and transferred into WT or leptin-deficient *ob/ob* mice, 4 and 7 days after transfer. One representative out of three independent experiments with 3 mice per group is shown (*$p < 0.01$; **$p < 0.001$).

(C) CFSE dilution profile of PCC-specific CFSE-labeled AND-TCR T_{reg} cells (gated on $V\alpha11.1^+/V\beta3^+Foxp3^+$ cells) adoptively transferred into WT, *ob/ob*, and *ob/ob* treated with recombinant leptin, 4 and 7 days after transfer. One representative out of two independent experiments with 4 mice per group is shown (**$p < 0.001$; *$p < 0.01$).

db/+ heterozygous controls (Figures 7A–7C). Stimulation with anti-CD3 and anti-CD28 of $CD4^+CD25^-$ effector T cells from *db/db* mice was less effective in inducing proliferation than stimulation on $CD4^+CD25^-$ from *db*/+ mice (Figure 7A). Conversely, in vitro stimulation of T_{reg} cells from *db/db* mice induced significantly higher proliferation than that of T_{reg} cells from *db*/+ heterozygous controls (Figure 7B). These data suggested that the absence of the ObR impaired the expansion of $CD4^+CD25^-$ effectors and enhanced the proliferative potential of T_{reg} cells. The ObR deficiency seemed to affect the proliferative potential of the T_{reg} cells rather than their qualitative or functional activity, because T_{reg} cells from *db/db* mice suppressed the proliferation of $CD4^+CD25^-$ T cells in a fashion similar to that of T_{reg} cells from *db*/+ control mice (Figure 7C), confirming the finding that addition of exogenous leptin did not alter the suppressive capacity of T_{reg} cells in vitro (Figure 2A).

Subsequent studies were performed analyzing antigen-specific responses in an autoimmune disease model: the in vitro proliferative capacity of T_{reg} cells against the pancreatic autoantigen glutamic acid decarboxylase (GAD)65 was evaluated in autoimmune diabetes nonobese diabetic (NOD/LtJ) mice versus leptin-receptor mutant NOD-$Lepr^{db5J}$/LtJ mice (Figure 7D; Lee et al., 2005). These recently isolated ObR mutant mice display obesity, hyperphagia, and resistance to the development of pancreatic β-islets infiltration (Lee et al., 2005). We found that after 5 days of splenocyte cultures, T_{reg} cells from NOD-$Lepr^{db5J}$/LtJ proliferated more robustly against mouse

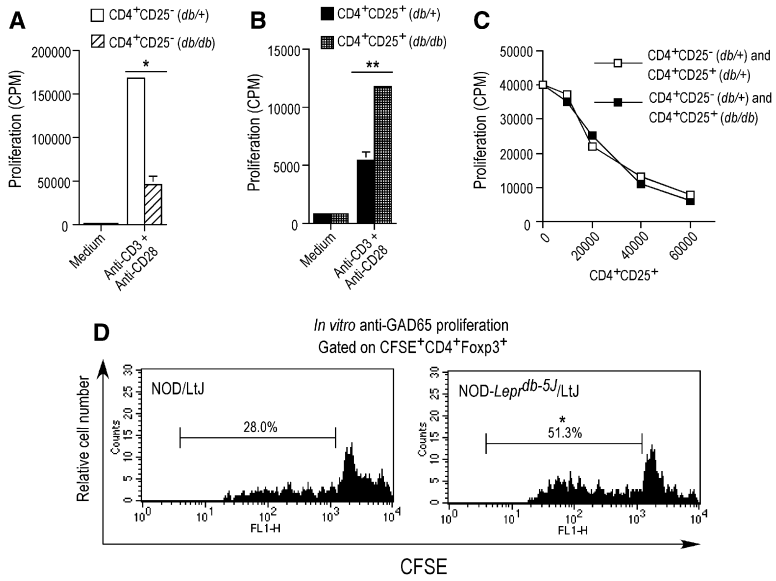

Figure 7. ObR Deficiency Increases T_{reg} Cells Proliferative Potential, Does Not Alter Their Suppressive Capacity, and Impairs $CD4^+CD25^-$ Proliferation

(A) Proliferation of $CD4^+CD25^-$ effector T cells from *db*/+ and leptin receptor-deficient *db*/*db* mice stimulated with anti-CD3 and anti-CD28. The data are shown as mean ± SD (n = 5, *p < 0.001).

(B) Proliferation of T_{reg} cells from *db*/+ and *db*/*db* mice stimulated with anti-CD3 and anti-CD28. The data are shown as mean ± SD (n= 5, **p < 0.01).

(C) Proliferation of $CD4^+CD25^-$ effector T cells from *db*/+ mice in the presence of increasing number of either *db*/+ or *db*/*db* T_{reg} cells, stimulated with anti-CD3 and anti-CD28. The data are shown as mean ± SD (n = 5).

(D) CFSE dilution of T_{reg} cells (gated on $CD4^+Foxp3^+$ cells) from leptin receptor mutant NOD-*Lepr*$^{db-5J}$/LtJ mice stimulated with mouse recombinant GAD65, after 5 days culture. One representative out of five independent experiments is shown (*p > 0.01).

GAD65 than T_{reg} cells from NOD/LtJ females, suggesting an increased autoantigen-specific proliferative potential of the T_{reg} cells from mice with impaired ObR signaling (Figure 7D). In contrast, $Foxp3^-$ T cells from NOD-*Lepr*^{db5J}/LtJ proliferated significantly less than that of NOD/LtJ controls (data not shown), suggesting that an absence of ObR impaired the expansion of $Foxp3^-$ effectors and enhanced the proliferative potential of T_{reg} cells.

Leptin-Deficient Mice Have Increased Numbers of T_{reg} Cells that Can Be Reduced by Administration of Leptin

Mice with genetic deficiency of leptin (*ob*/*ob*) or leptin-receptor (*db*/*db*) have reduced susceptibility to autoimmunity (Matarese et al., 2001; Sanna et al., 2003; Siegmund et al., 2004; Faggioni et al., 2000; Lee et al., 2005). T_{reg} cells play a central role in regulating autoimmunity (Shevach, 2002; Sakaguchi, 2005), so we tested whether genetic deficiency of leptin associated with effects on T_{reg} cells. A significant increase of the percentage of peripheral T_{reg} cells was observed in *ob*/*ob* as compared to WT (Figures S7A and S7B, respectively). Administration of leptin reduced the elevated number of T_{reg} cells in the *ob*/*ob* mice to a number comparable to that found in the WT mice (Figures S7A and S7B).

DISCUSSION

In this study we establish a unique link between T_{reg} cells and leptin by showing that leptin can modulate the hyporesponsiveness and proliferation of T_{reg} cells both in vitro and in vivo. Freshly isolated human T_{reg} cells express high amounts of ObR and produce substantial amounts of leptin that are responsible for an autocrine inhibitory loop that constrains the expansion of T_{reg} cells. Although leptin neutralization inhibits the proliferation of effector $CD4^+CD25^-$ T cells, this condition leads to an expansion of the T_{reg} cells, which maintain their suppressive phenotype in the resting phase. The opposite effects of leptin blockade on $CD4^+CD25^-$ T cells and T_{reg} cells associate with a differential expression of intracellular leptin and cell-surface ObR in the two cell subsets.

It must be noted that in our experimental system, the culture media supplemented with human serum contained low—albeit significant—concentrations of human leptin (0.5–1 ng/ml in the 5% human serum [HS]/95% RPMI). The contribution of HS-derived leptin versus T_{reg} cell-derived leptin was analyzed in cultures with three different serum-free media—all tested for the absence of leptin. The anti-leptin-induced expansion of the T_{reg} cells in serum-free conditions indicated that the T_{reg} cell-derived leptin was sufficient to act as negative signal for the expansion of the T_{reg} cells. It is possible to speculate that a leptin-mediated negative autocrine loop may operate on T_{reg} cells, because both freshly isolated and cultured T_{reg} cells stain positive for and secrete leptin. On the other hand, the leptin present in the culture medium and secreted by the $CD4^+CD25^-$ T cells could act as a positive signal for T cell proliferation. These differential effects induced by leptin could rely upon the different ObR levels and leptin secretory capacity, which would ultimately affect the intracellular signaling differentially.

Our T_{reg} cells were cultured in medium supplemented with 5% HS because fetal calf serum (FCS) is rich in bovine leptin (10–20 ng/ml in RPMI 10% FCS) that is not neutralized by human leptin mAb (data not shown). We also found that 10% FCS abrogated the effects induced by human leptin mAb on human T_{reg} cells (data not shown). Therefore, it was crucial, for efficient leptin neutralization, to avoid exposure to FCS for the ObR-expressing T_{reg} cells. The same issue has to be taken into account had the T_{reg} cells been isolated by positive selection and thus undergone repeated steps of FCS exposure compromising leptin neutralization. Moreover, we also noted that

T_{reg} cells isolated by positive selection with anti-CD25 mAb (either by FACS or magnetic bead cell sorting) had a reduced capacity to proliferate in vitro after leptin neutralization when the anti-CD25 mAb was not removed from the cell surface after isolation. The reasons for an interference of the anti-CD25 mAb with leptin neutralization process and T_{reg} cell expansion are currently being investigated and might include an interference with IL-2 signaling, a functional inactivation of the T_{reg} cells by the anti-CD25 mAb recently demonstrated (Kohm et al., 2006), and an influence on the kinetics and the degree of leptin secretion by the T_{reg} cells. In any case, detachment of the anti-CD25 mAb after cell isolation completely eliminates possible problems of proliferation for the anti-leptin-treated stimulated T_{reg} cells (see Experimental Procedures for details).

Previous experimental evidence suggests that the growth, homeostasis, and function of the T_{reg} cells is dependent on IL-2 in vitro and in vivo (Setoguchi et al., 2005; Fontenot et al., 2005). Our data also indicated that leptin neutralization could induce IL-2 secretion by T_{reg} cells and that leptin mAb-induced-proliferation was IL-2 dependent. When comparing the efficiency of leptin neutralization and IL-2 to expand T_{reg} cells in vitro, we observed that leptin mAb had a better efficiency than recombinant IL-2 (as indicated by T_{reg} cell proliferation and number after 8 days culture). Conversely, addition of increasing doses of recombinant leptin to the cell cultures did not affect the IL-2-mediated proliferation of the T_{reg} cells, suggesting that the control of leptin on the expansion of the T_{reg} cells was independent and not overcome by IL-2 signaling. McHugh et al. (2002) demonstrated that cultures of T_{reg} cells with neutralizing glucocorticoid-induced TNF receptor (GITR) mAb allowed the T_{reg} cells to respond to exogenous IL-2 in the absence of TCR stimulation. We also tried to induce T_{reg} cell proliferation in the absence of TCR stimulation by adding high concentration of IL-2 and anti-leptin, but we found lack of proliferation, indicating that anti-leptin-induced proliferation of the T_{reg} cells needs concomitant TCR engagement. This evidence was also confirmed at biochemical level; anti-leptin alone in the absence of TCR stimulation did not induce significant P-STAT3 levels in T_{reg} cells.

Foxp3—the master gene for T_{reg} cell development and function (Sakaguchi, 2005)—was induced in leptin-neutralized T_{reg} cells at late time points (12 hr), supporting the evidence that removal of leptin from culture medium not only expanded the T_{reg} cells but also allowed the maintenance of their phenotype. The phenomenon might possibly be ascribed to the increased IL-2 secretion induced by leptin neutralization, because others have shown that IL-2 is capable of upregulating Foxp3 expression in human T_{reg} cells (Zorn et al., 2006).

At the molecular level, T_{reg} cells expressed high levels of ObR and of $p27^{kip1}$ but no phosphorylation of ERK1/2 or STAT1 and little phosphorylation of STAT3. In contrast, leptin neutralization upon anti-CD3 and anti-CD28 stimulation and T_{reg} cell expansion was associated with a rapid degradation of $p27^{kip1}$ as well as a marked phosphorylation of ERK1/2, STAT1, and STAT3 (phosphorylation of STAT3 in T_{reg} cells could be partly explained by both the induction of the ERK1/2, which is known to phosphorylate STAT3 independently of ObR [Quadros et al., 2004; Barboza et al., 2004], as well as by the secretion of STAT3-activating cytokines, i.e., IL-2 and IL-6 [Doganci et al., 2005]). Recently, Zorn and collegues (Zorn et al., 2006) showed that IL-2 upregulates Foxp3 expression in human T_{reg} cells through a STAT3-dependent mechanism, confirming that induction of Foxp3 (in our case, during leptin neutralization) may occur via a STAT3-dependent mechanism. Intriguingly, $CD4^+CD25^-$ T cells, after leptin neutralization, showed an increase of $p27^{kip1}$ associated with sustained ERK1/2 phosphorilation—a phenotype often observed in anergic T cells (Wells et al., 2001; Chen et al., 1999; Waiczies et al., 2005) and in effector T cells from mice treated with anti-leptin antibodies during autoimmune encephalomyelitis (De Rosa et al., 2006). The fact that leptin neutralization reduced the levels of STAT3 phosphorylation and had little effect on STAT1 needs an explanation. This partial effect on the ObR-STAT3-mediated signaling could be ascribed to the fact that also other cytokines activate STAT3 (Doganci et al., 2005). As such, removal of leptin could only inhibit in part STAT3 phosphorylation. In this context, it is interesting to note that SOCS3, a key negative regulator of STAT3-activating cytokines (Kinjyo et al., 2006), also was differentially expressed in the T_{reg} cells and in the $CD4^+CD25^-$ T cells. The regulation of STAT3 signaling by SOCS3 in $CD4^+CD25^-$ effectors was biphasic, with a first peak at 1 hr and a rapid degradation at 12 hr, as previously described (Wormald et al., 2006). Leptin neutralization increased SOCS3 amounts concomitantly with a reduced phosphorylation of STAT3. This is also consistent with the SOCS3 inhibition of IL-2 production and T cell proliferation that we observed in our work (Matsumoto et al., 2003). In T_{reg} cells, SOCS3 was highly expressed in basal conditions and markedly induced during acute stimulation, in agreement with the anergic state of the T_{reg} cells and their reduced IL-2 secretion.

Our results indicate that leptin neutralization can "unlock" the hyporesponsiveness of T_{regs} via a rapid degradation of the cell-cycle inhibitor $p27^{kip1}$, associated with the phosphorylation of ERK1/2. These biochemical events would allow the T_{reg} cells to enter the G1/S phase of the cell cycle, induce IL-2 gene transcription, and, consequently, reverse their anergic state (Li et al., 2005). Our data also indicate that these changes associate with the activation of STAT1 and STAT3, two transcription factors related with cytokine signaling and proliferation of the T_{reg} cells (Doganci et al., 2005; Nishibori et al., 2004).

Incidentally, this study also describes a novel strategy to expand human peripheral T_{reg} cells, via leptin neutralization, although this approach needs to be further investigated. Current strategies to expand T_{reg} cells employ ex vivo addition of cytokines to cultured cells during TCR stimulation (Tang et al., 2004; Jiang et al., 2003). Because neutralization of the leptin present in human serum of tissue cultures and of leptin produced by T_{reg} cells may be sufficient to determine an expansion of the T_{reg} cells, it

might be possible that leptin neutralization, in addition with exogenous IL-2, may improve the protocols of expansion for T_{reg} cells. We are currently investigating this possibility.

In vivo studies in leptin-deficient *ob/ob* mice show higher percentage and absolute number of circulating T_{reg} cells. They can expand up to 90% in vivo in normal nonlymphopenic hosts by day 28 after transfer (Trenado et al., 2003). Adoptive transfer experiments of CFSE-labeled T_{reg} cells from WT mice into leptin-deficient *ob/ob* mice showed a significant in vivo expansion of T_{reg} cells. It is also noteworthy that chronic leptin deficiency allows higher expansion of T_{reg} cells when compared with acute leptin neutralization induced by leptin Ab (the percentage of proliferating T_{reg} cells in *ob/ob* mice was always higher than that observed in leptin-neutralized WT mice). Importantly, in vivo leptin neutralization also determined an increased Foxp3 expression in the T_{reg} cells, suggesting maintenance of their suppressive phenotype after expansion in vivo. Taken together, our data suggest that the presence of ObR may be crucial in the control of the expansion of T_{reg} cells because stimulated T_{reg} cells from *db/db* mice proliferated better than T_{reg} cells from controls in vitro, whereas the suppressive capability of T_{reg} cells from *db/db* mice was similar to that of control mice. Confirmation of the crucial function of ObR came in an antigen-specific system of autoimmune diabetes in the NOD/LtJ mouse with the finding of increased anti-GAD65 proliferative response of T_{reg} cells from ObR mutants NOD-*Lepr*^{db5J}/LtJ mice.

The fact that leptin can act as a negative signal for the proliferation of T_{reg} cells envisions new possibilities of anti-leptin-based approaches for the immunotherapy of conditions characterized by low numbers of T_{reg} cells. Leptin might act as an endogenous "sensing" factor linking the environment (availability of nutrients) to circulating T_{reg} cell number. Because nutritional deprivation increases susceptibility to infection and associates with amelioration of clinical manifestations of autoimmunity (Kuchroo and Nicholson, 2003; Payne, 2001), it will be important to address how this relates to the influence of leptin on T_{reg} cells and whether anti-leptin-based intervention can be applied to tune cognate T cell responses in immune regulation.

EXPERIMENTAL PROCEDURES

Purification, Cultures, and Assays with T Cells

Human $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells were purified from human healthy donors PBL either by magnetic cell separation with the Dynal $CD4^+CD25^+$ T_{reg} Kit (Dynal-Biotech, Oslo, Norway) or by flow cytometry cell sorting (MoFlo high-performance cell sorter, Dako, Glostrup, Denmark) and were rapidly cleaned with the Detach reagent (Dynal-Biotech) from surface-bound CD25 mAb. Both magnetic beads-based and flow cytometry-based purification techniques yielded a highly expressing $CD25^+$ population (95%–98% pure by FACS analysis), 90% of which expressed Foxp3. The T_{reg} cells:effector ratio in the suppression experiments was 1:1. Cells were cultured (5×10^4 cells/well) in round-bottom 96-well plates (Becton-Dikinson Falcon, Franklin Lakes, NJ) with RPMI medium supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin (all from Life Technologies Inc., Gaithersburg, MD) and 5% AB human serum (Sigma-Aldrich, St. Louis, MO). Cells were stimulated for 3 days in the presence of anti-CD3/CD28 Dynabeads (0.1 bead/cell) (Dynal-Biotech). On the last day, [^{3}H]thymidine (0.5 μCi/well) (Amersham-Pharmacia Biotech, Cologno Monzese, Italy) was added to the cultures and cells harvested after 12 hr. Radioactivity was measured with a β-cell-plate scintillation counter (Wallac, Gaithersburg, MD).

For suppression experiments in the mouse, T_{reg} cells were isolated with the Regulatory T Cell Isolation Kit (Miltenyi Biotec, Gladbach, Germany) and stimulated with Dynabeads mouse anti-CD3/CD28 (0.5 bead/cell; 5×10^4 cells/well). The T_{reg} cells:effector ratio in the suppression experiments was 1:2. Purified cells (98% pure by FACS analysis) were cultured in round-bottom 96-well plates (Becton-Dikinson Falcon) with RPMI medium supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin (Life Technologies) and 5% FCS or 5% mouse serum (Hyclone-Pierce, Rockford, IL). Cells were stimulated for 3 days, labeled with [^{3}H]thymidine (0.5 μCi/well) for the last 16 hr of culture, and harvested similarly to what done for human T cell cultures.

Incorporation of Bromodeoxyuridine (BrdU) and FACS analysis were performed in T cell cultures in serum-free medium (X-VIVO) with the BrdU Flow Kit from BD-Pharmingen in accordance to the manufacturer's instructions.

IL-2 Measurement

IL-2 measurement was done with the CTLL-2 cell line (kindly provided by N. Davey, Imperial College London, UK) according to a previously described method (Ng et al., 2001). Cells were maintained in culture in supplemented RPMI medium with 10% FCS (Hyclone-Pierce) and human recombinant (rh)-IL-2 (10 U/ml, Boehringer Mannheim, Mannheim, Germany). They were subcultured every 2 to 3 days. Cells were rested in medium without IL-2 overnight prior to use in the assays.

Reagents, Leptin-Neutralizing Antibodies, and Leptin Measurement

For in vitro blocking experiments, human leptin-neutralizing mAb (R&D Systems, Minneapolis, MN) was used at a final concentration of 0.25 to 25 μg/ml; controls were irrelevant isotype-matched antibodies (Biovendor Laboratory Medicine Inc., Brno, Czech Republic). Human recombinant leptin was purchased from R&D Systems. Human IL-2-neutralizing mAb (MQ1-17H12) was from BD Pharmingen and was utilized at final concentration of 5–10 μg/ml. FITC-anti-human-CD4 and PE-anti-human-CD25 were from BD Pharmingen; the anti-human Foxp3 staining set was from eBiosciences (San Diego, CA). Recombinant mouse GAD65 was purified from GAD65-producing cells that were kindly provided by R. Tisch (University of North Carolina, Chapel Hill, NC); recombinant GAD65 was tested for purity by SDS-PAGE and silver staining before the experimental use. The endotoxin content in the preparations was below the detection limit (about 10 pg of endotoxin/μg of protein) of the Limulus amebocyte lysate (LAL) method. Human leptin-specific ELISA was purchased from R&D Systems and measurements were performed according to the manufacturer's instructions (Matarese et al., 2005). Serum-free media were RPMI (Life Technologies), HyQ-ADCF (Animal Derived Component Free, from Hyclone-Pierce), and X-VIVO (BioWittaker).

Mice and In Vivo Experiments

6-week-old female leptin-deficient C57BL6/J-*ob/ob* (*ob/ob*), C57BL6/J, leptin-receptor deficient C57BL/Ks-*db/db* (*db/db*), and C57BL/Ks-*db*/+ lean controls (*db*/+) mice were purchased from Harlan Italy s.r.l. (Corezzana, Italy); B10.Cg.Tg(TcrAND)53Hed/J (AND-TCR Tg) PCC-specific transgenic mice were purchased from The Jackson Laboratory (Bar Harbor, ME); NOD/LtJ and NOD-*Lepr*^{db5J}/LtJ mutants were kindly provided by E. Leiter from The Jackson Laboratory. Experiments were conducted in accordance with the animal welfare guidelines under an approved protocol of the Istituto Superiore di Sanità, Roma, Italy. Mice were age-matched for individual experiments and housed with a 12 hr light/dark cycle in the animal facility at the

Università di Napoli "Federico II." *ob/ob* mice were injected intraperitoneally twice daily for 10 days with mouse recombinant leptin (R&D Systems) dissolved in 200 μl of PBS at a dose of 1 μg/g of body weight.

The mouse leptin-blocking antibody was produced in our laboratory after immunization of C57BL/6J mice with mouse recombinant leptin (R&D Systems) emulsified in complete Freund's adjuvant (CFA) CFA (Difco Laboratories, Detroit, MI); mouse leptin-specific antibodies (of the IgM class) were affinity purified with recombinant mouse leptin (R&D Systems) bound to AminoLink Plus Immobilization Gel (AminoLink Plus Immobilization Kit from Pierce, Rockford, IL) from serum and ascites of immunized mice. Affinity-purified IgM were used as control (BD Pharmingen). WT mice were treated for 3 days either with 100 μg of control mouse IgM or with mouse leptin Abs intraperitoneally in a total volume of 100 μl of PBS.

Adoptive transfer experiments were performed by labeling highly purified (98% pure by FACS analysis) $CD4^+$ T cells obtained from C57BL6/J WT mice (cells were purified with the mouse $CD4^+$ negative isolation kit from Dynal) with the fluorescent dye CFSE (5-, 6-carboxyfluorescein diacetate succinimidyl ester) from Molecular Probes (Eugene, OR) used at 1 μg/ml. In brief, 10^7 CFSE-labeled $CD4^+$ T cells were adoptively transferred into mice intravenously. 4 and 7 days later, spleen cells were harvested from mice and stained with PE-anti-Foxp3 (eBioscience) and Cy-anti-CD4 (BD PharMingen). In experiments of adoptive transfer performed with CFSE-labeled AND-TCR Tg $CD4^+$ T cells (98% pure), after 4 and 7 days, spleen cells were harvested from recipient mice and stained with anti-clonotypic PE-anti-Vα11.1 (RR8.1), biotynilated-anti-Vβ3 (KJ25) (both from BD PharMingen), and APC-anti-Foxp3 (eBioscience). Flow cytometric analysis of CFSE dilution was performed by gating on $CFSE^+CD4^+Foxp3^+$ cells (in non-Tg mice) (Figures 6A and 6B) and on $CFSE^+Vα11.1^+/Vβ3^+Foxp3^+$ cells (in AND-TCR Tg mice) (Figure 6C) with a FACScalibur (Becton-Dickinson, San Diego, CA) and analyzed by Cell Quest software (Becton-Dickinson).

Western Blots, Biochemical Analyses, and Confocal Microscopy

Total cell lysates were obtained in 50 mM HEPES (pH 7.5), 250 mM NaCl, 1 mM EDTA, 1.0% Triton X-100, 10 mM sodium fluoride, 1 mM sodium orthovanadate, and 2 μg/ml aprotinin, 2 μg/ml leupeptin, and 2 μg/ml pepstatin. 50 μg of total proteins were subjected to SDS-PAGE under reducing conditions. After electrophoresis, proteins were transferred onto a nitrocellulose filter membrane (Protan, Schleicher & Schuell) with a Trans-Blot Cell (Bio-Rad) and transfer buffer containing 25 mM Tris, 192 mM glycine, 20% methanol. Membranes were placed in 5% nonfat milk in phosphate-buffered saline, 0.5% Tween 20 (PBST) at 4°C for 2 hr to block the nonspecific binding sites. Filters were incubated with specific antibodies before being washed three times in PBST and then incubated with a peroxidase-conjugated secondary antibody (Amersham Biosciences). After washing with PBST, peroxidase activity was detected with the ECL system (Amersham Biosciences) or Femto (Pierce). The antibodies used were the following: anti-$p27^{Kip-1}$, anti-STAT3 and anti-phospho-STAT3 (Y705), anti-STAT1 and anti-phospho-STAT1 (Y701) (Cell Signaling Technology, Beverly, MA); anti-leptin, anti-ObR, anti-ERK 1/2, and anti-phospho-ERK 1/2 (all from Santa Cruz Biotechnology Inc., Santa Cruz, CA); anti-Foxp3 (eBioscience); anti-SOCS3 (Serotec Ltd, Kidlington, UK). The filters were also probed with a tubulin antibody (Sigma) to normalize the amount of loaded protein. Only for the human leptin western blots, performed on a 15% SDS-PAGE gel to better visualize 16 kDa leptin, normalization was performed against total ERK1/2. All filters were quantified by densitometric analysis of the bands utilizing the program ScionImage 1.63 for Mac (Scion Corporation, Frederick, MD).

Confocal microscopy was performed in parallel with proliferation and biochemistry at 1 hr and 36 hr on 98% pure T_{reg} cells and $CD4^+CD25^-$ cells. Cells were washed in PBS and 10^4 cells were seeded on multitest slide (ICN Biomedicals Inc., Aurora, OH), air-dried and fixed for 1 min in methanol, washed in PBS, permealized in PBS containing 0.2% Triton X-100 for 3 min, and incubated in blocking solution (PBS 1% BSA) for 1 hr. Cells were then washed three times in PBS and incubated overnight at 4°C with primary antibodies (rabbit polyclonal anti-leptin and mouse anti-ObR mAb, both from Santa Cruz Biotechnology Inc.) diluted 1:100 in blocking solution, washed in PBS, and incubated with the secondary antibody (goat anti-rabbit 488 and goat anti-mouse 543, both from Molecular Probes Inc.) for 1 hr at room temperature diluted 1:100 in blocking solution, washed in PBS, and finally mounted in PBS/Glycerol 1:1. Immunofluorescence analysis was performed with a confocal laser scanner microscope Zeiss LSM 510. The wavelength of the Argon ion laser was set at 488 nm; that of the HeNe laser was set at 543 nm. Fluorescence emission was revealed by BP 505–530 band pass filter for Alexa Fluor 488 and by BP 560-615 band pass filter for Alexa Fluor 543. Double-staining immunofluorescence images were acquired simultaneously in the green and red channels at a resolution of 1024 × 1024 pixels.

Statistical Analysis

Analyses were performed with the Mann-Whitney U-test for unrelated two-group analyses and the Kruskal-Wallis ANOVA test for three or more group analyses with the StatView software (Abacus Concepts Inc., Cary, NC). Results are expressed as mean ± SD. p values < 0.05 were considered statistically significant.

Supplemental Data

Seven Supplemental Figures can be found with this article online at http://www.immunity.com/cgi/content/full/26/2/241/DC1/.

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