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Supporting Information

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Leptin deficiency impairs maturation of dendritic cells and enhances induction of regulatory T and Th17 cells

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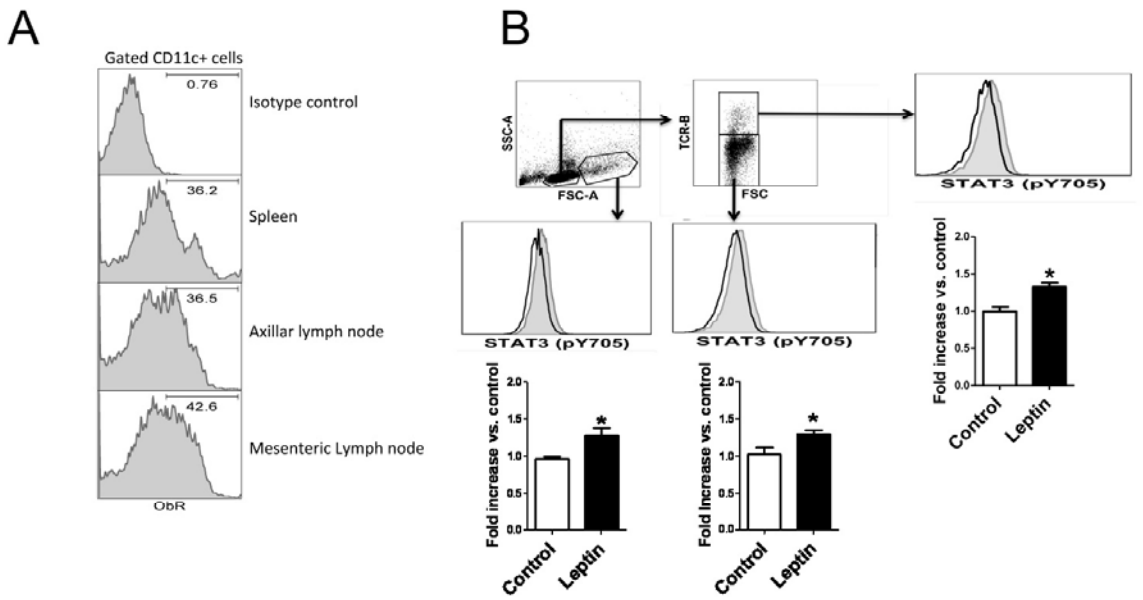
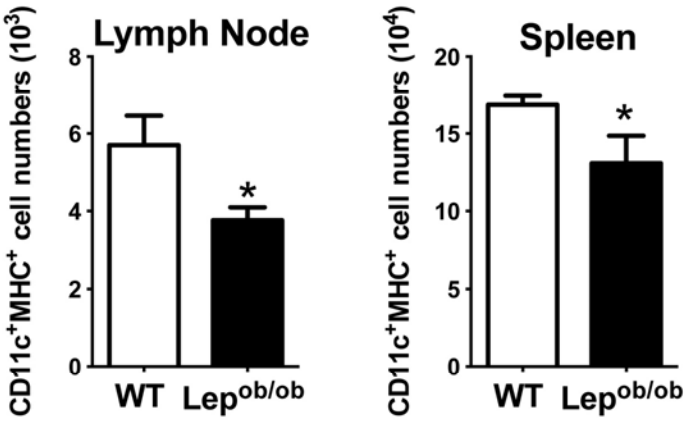
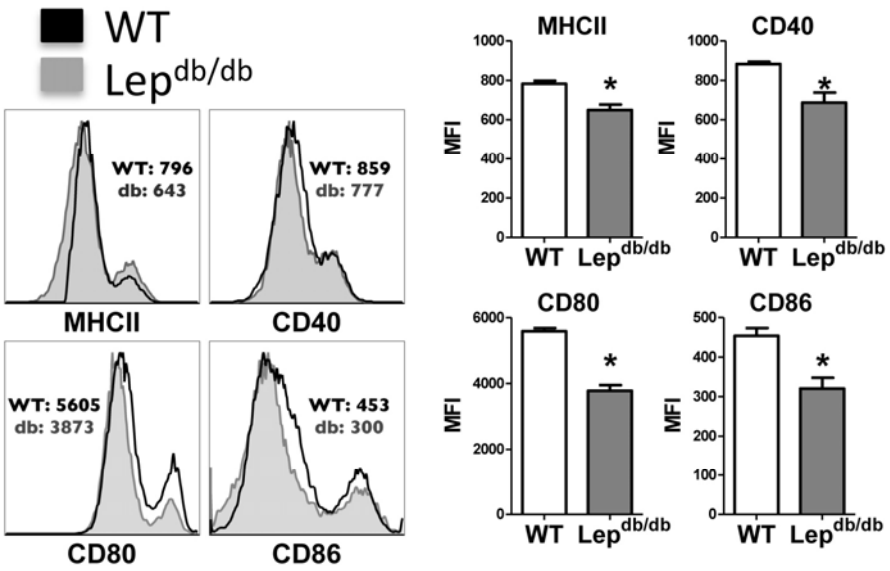
Supporting Information



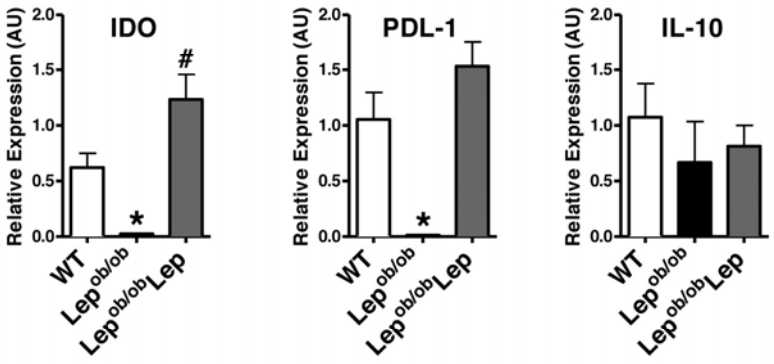
Supporting Information Figure 1**. ObR expression in dendritic cells and leptin-induced STAT3 phosphorylation in immune cells.** (A) Cells from LN and spleen were stained with anti-Leptin receptor (ObR) and anti-CD11c antibodies. The percentage of the cells expressing the ObR was evaluated in gated $CD11c^+$ cells in the spleen, axillar and mesenteric LN. The data are representative of one of two independent experiments each with three tested mice/group. (B) The lymph nodes of C57BL6/J wild type mice were obtained and the cells were isolated. The mononuclear cells were incubated for 5 hours in culture and then stimulated for 15 minutes with 10 µg/mL of recombinant mouse leptin. The phosphorylation of STAT3 in tyrosine 705 (pY705) was evaluated by flow cytometry in the region of the lymphocytes TCR^+ and TCR^- and in the region of potential monocytes. The data are representative of one of two independent experiments each with three tested mice/group. The results are presented as the means ± SEM of one of two independent experiments each with three tested mice/group. * $p<0.05$. Non-parametric Mann-Whitney test.



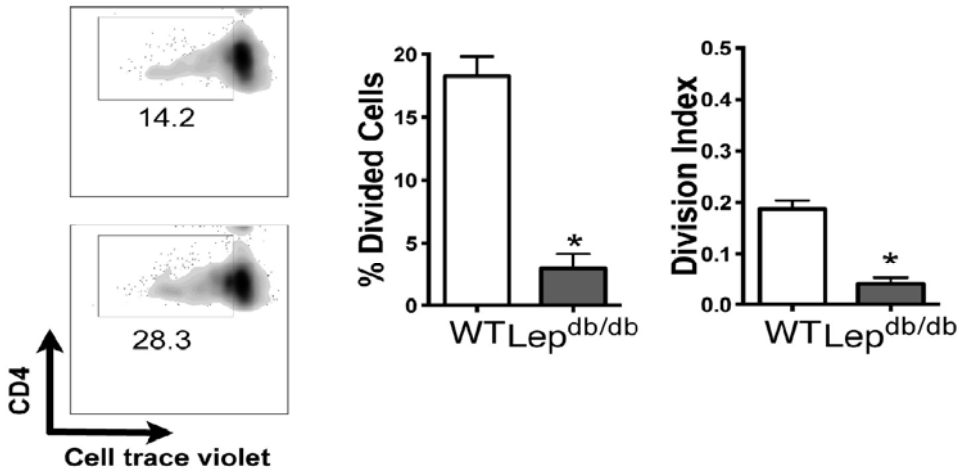
Supporting Information Figure **2. Total number of lymph node and spleen CD11c^{+}MHC-II^{+} DCs.** The frequency of CD11c^{+}MHC-II^{+} cells in un-manipulated C57BL6/J Lep$^{ob/ob}$ and WT control mice was assessed. Cells from spleen and lymph node were evaluated by Flow Cytometry, and the total number was calculated. The results are presented as the means ± SEM of one of two independent experiments each with five tested mice/group. * $p<0.05$. Non-parametric Mann-Whitney test.



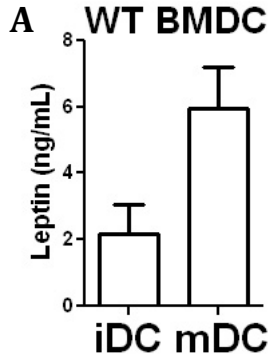
Supporting Information Figure **3. Leptin receptor deficiency affects the phenotype of BMDCs.** BMDCs were generated from $Lep^{db/db}$ and WT BALB/c mice. Non-adherent cells from the bone marrow (BM) cultures of $Lep^{db/db}$ and WT mice were analyzed by Flow Cytometry. The iDCs and mDCs from the $Lep^{db/db}$ (gray line) and the WT (black line) mice were evaluated for the expression of MHC-II, and the co-stimulatory molecules CD40, CD80 and CD86 by Flow Cytometry. The BMDCs were gated on $CD11c^{+}$ cells. The results are presented as the means ± SEM of one of three independent experiments each with three tested mice/group.. * $p<0.05$. Non-parametric Mann-Whitney test. The results are presented as the means ± SEM.



Supporting Information Figure **4. DCs from Lep$^{ob/ob}$ mice cultivated with autologous leptin-free serum, have reduced expression of IDO and PDL-1 and no effect in IL-10.** DCs were generated from the bone marrow of WT and Lep$^{ob/ob}$ mice. To guarantee the complete absence of leptin during the DC generation process, the culture medium was prepared using autologous Lep$^{ob/ob}$ serum. To ensure the action of leptin on the DCs, recombinant leptin was added to the Lep$^{ob/ob}$ DC cultures as a positive control. Immature dendritic cells (iDCs) were obtained by harvesting non-adherent cells from the Lep$^{ob/ob}$ and control bone marrow (BM) cultures on day seven of culture, and the mRNA was extracted. The total mRNA was isolated and converted to cDNA. *Ido, Pdl-1* and *Il-10* gene expression in Lep$^{ob/ob}$ DCs generated in the presence of leptin were compared to those expressed by WT control DCs. Data is representative of a single experiment with three tested mice/group. Each sample was run in duplicate and the relative expression levels were determined using the $2^{-\Delta\Delta Ct}$ method with the normalization of the target gene expression levels to GAPDH. The results are presented as the means ± SEM of three individual mice/group. Non-parametric Kruskal-Wallis test.



Supporting Information Figure **5. Leptin receptor deficiency affects the function of BMDCs.** BMDCs were generated from leptin receptor deficient ($Lep^{db/db}$) and WT BALB/c mice. The proliferation of syngeneic responder $CD4^+$ T cells was used to determine the immunostimulatory capacity of the BMDCs derived from leptin receptor knockout mice ($Lep^{db/db}$). mDCs generated from $Lep^{db/db}$ and WT mice were co-cultured with syngeneic Cell Trace Violet labeled $CD4^+$ T cells plus an anti-CD3 antibody for 4 days. Proliferation was determined by measurement of Cell Trace dilution by flow cytometry. The percentage of divided cells and the division index were calculated with FlowJo 8.7 software. The results are presented as the means ± SEM of one of three independent experiments each with three tested mice/group. * $p<0.05$. Non-parametric Mann-Whitney test.



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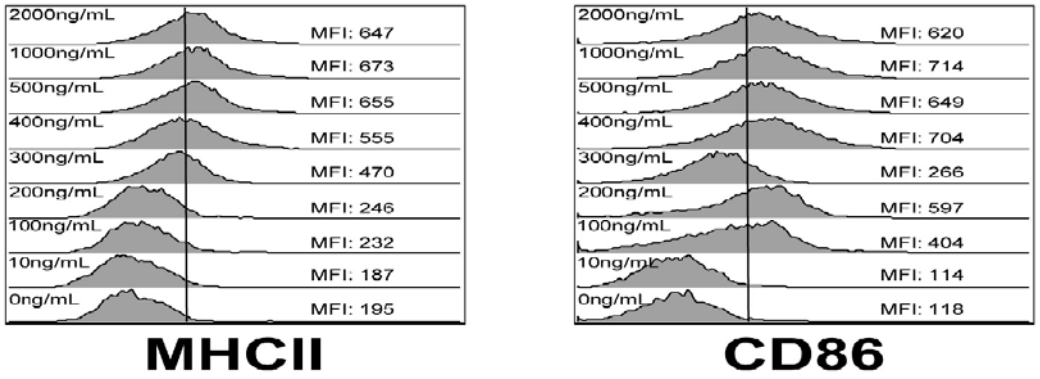
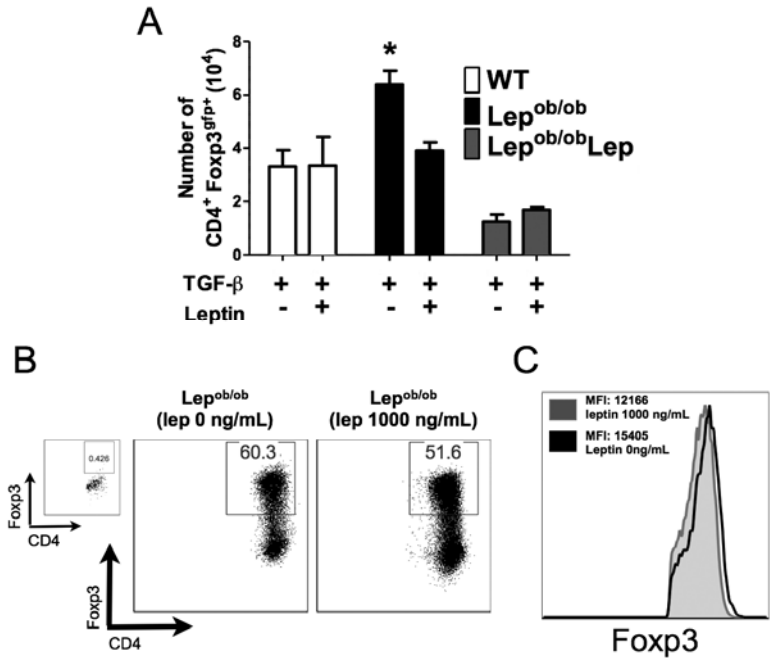
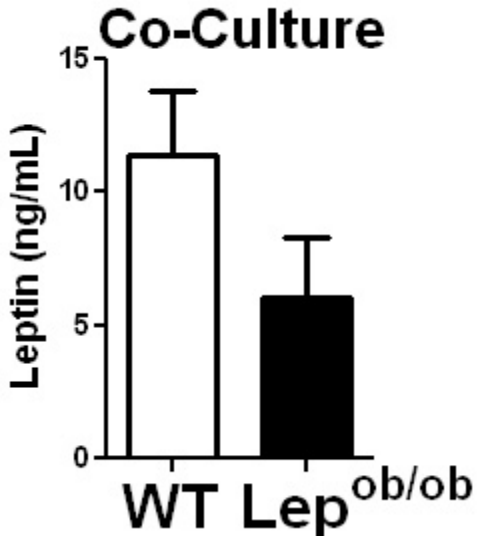


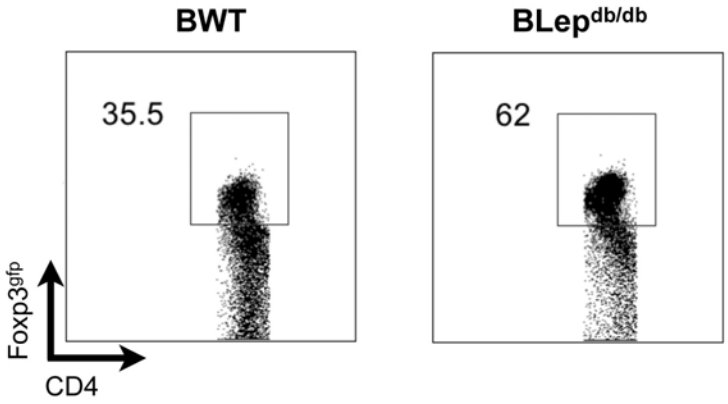
Supporting Information Figure **6. Lep$^{ob/ob}$ DCs respond to leptin in a dose dependent manner.** (A) DCs were generated from the Bone Marrow of wild-type C57BL/6 mice. iDC and LPS-induced mDC supernatant were harvested, and the level of leptin secretion was determined by ELISA. DMEM low glucose medium supplemented with 10% FBS was used as blank. The results are presented as the means ± SEM from a single experiment with five individual mice/group. (B) DCs were generated from Lep$^{ob/ob}$ mice supplemented from day 0 of differentiation with increasing doses of recombinant leptin (0-2000 ng/mL). CD11c^{+} iDCs were analyzed for the expression of MHC-II and CD86. The results are presented as the means ± SEM a single experiment with three individual mice/group.



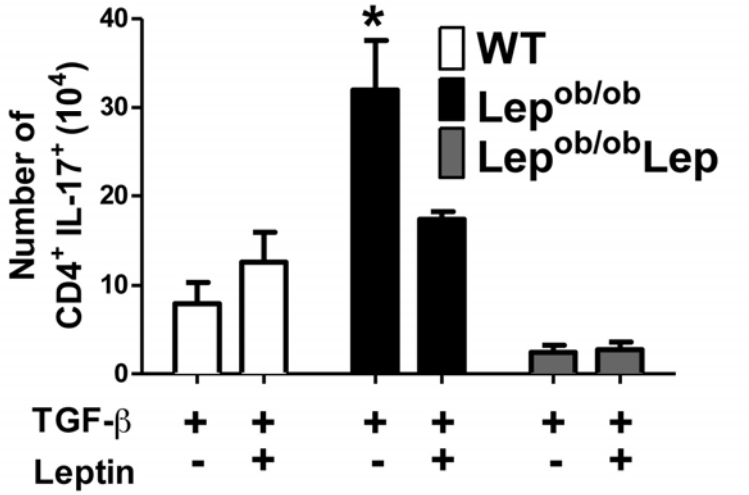
Supporting Information Figure **7. Leptin deficiency promotes the BMDCs-mediated generation and proliferation of CD4^{+}Foxp3^{gfp+} and high dose of leptin inhibits Foxp3 expression and Treg generation.** (A) iDCs generated from the bone marrow of Lep$^{ob/ob}$ mice cultured with (Lep$^{ob/ob}$Lep) or without (Lep$^{ob/ob}$) recombinant leptin (rLep), and wild-type (WT) C57BL/6 DCs were used to assess the potential to polarize naïve CD4 T cell precursors to CD4^{+}Foxp3^{gfp+} T cells. The naïve CD4^{+} T cells from WT syngeneic C57BL/6J Foxp3^{GFP} knock-in mice were co-cultured with DCs in the presence of TGF-β plus an anti-CD3 antibody, and the total number of differentiated Tregs was obtained. The results are presented as the means ± SEM of one of three independent experiments each with three tested mice/group. *p<0.05: versus all other groups. Non-parametric Kruskal-Wallis tests. (B) DCs were generated from Lep$^{ob/ob}$ mice with autologous Lep$^{ob/ob}$ serum alone or supplemented with 1000 ng/mL of rLep, and were co-cultured with naïve CD4 T cells from WT Foxp3^{gfp} knock-in mice plus TGF-β and an anti-CD3 antibody for 5 days. (C) The expression of Foxp3 was evaluated in the gated CD4^{+}Foxp3^{gfp+} T cells. The results are presented as the means ± SEM from a single experiment with three tested mice/group).



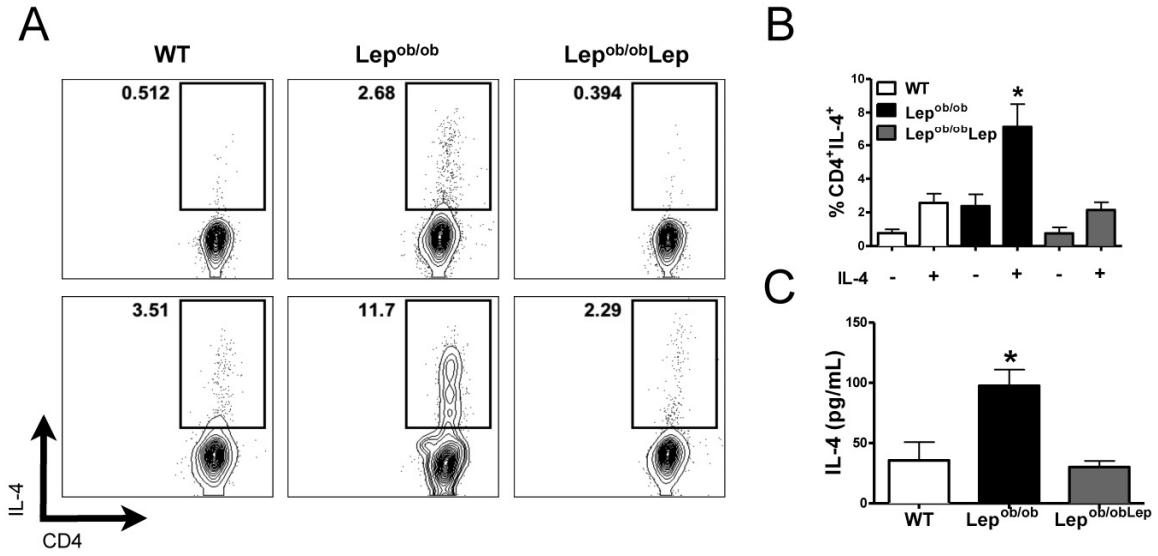
Supporting Information Figure 8**. Leptin secretion by CD4 T cells.** iDCs were derived from $Lep^{ob/ob}$ mice and cultured with $Lep^{ob/ob}$ autologous serum. iDCs were incubated with syngeneic splenic CD4 T cells from WT C57BL/6 mice plus an anti-CD3 antibody for 5 days. The supernatants were harvested and leptin secretion in the co-culture was evaluated by ELISA. Since DCs were generated from $Lep^{ob/ob}$ mice with autologous serum, it assures us to assume that the leptin present in the supernatant is from the WT CD4 T cells. The results are presented as the means ± SEM from a single experiment with 3 tested mice/group.



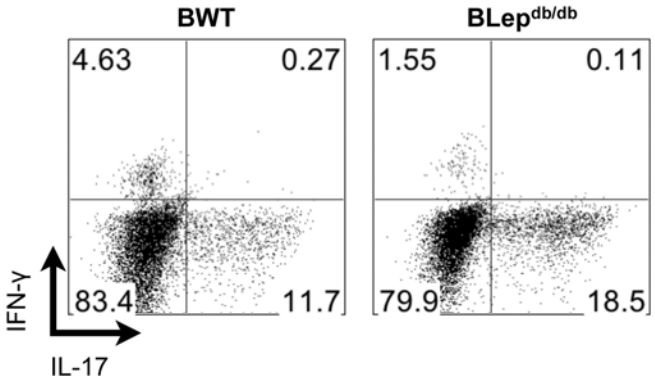
Supporting Information Figure 9**. Leptin receptor deficiency in BMDCs promotes increased induction of Tregs under optimal polarizing conditions**. iDCs generated from bone marrow of Lep$^{db/db}$ and WT BALB/c mice were used for Tregs polarization, from naïve precursors. CD4^{+}CD62L^{+}CD44^{-}Foxp3^{gfp-} naïve T cells were sorted from WT syngeneic BALB/c Foxp3^{GFP} knock in mice, and were co-cultured with BMDCs in Tregs polarizing conditions. The differentiation of Tregs was induced by addition of TGF-β (5 ng/mL). The data are representative of one of two independent experiments each performed with 3 pooled mice/group with similar results.



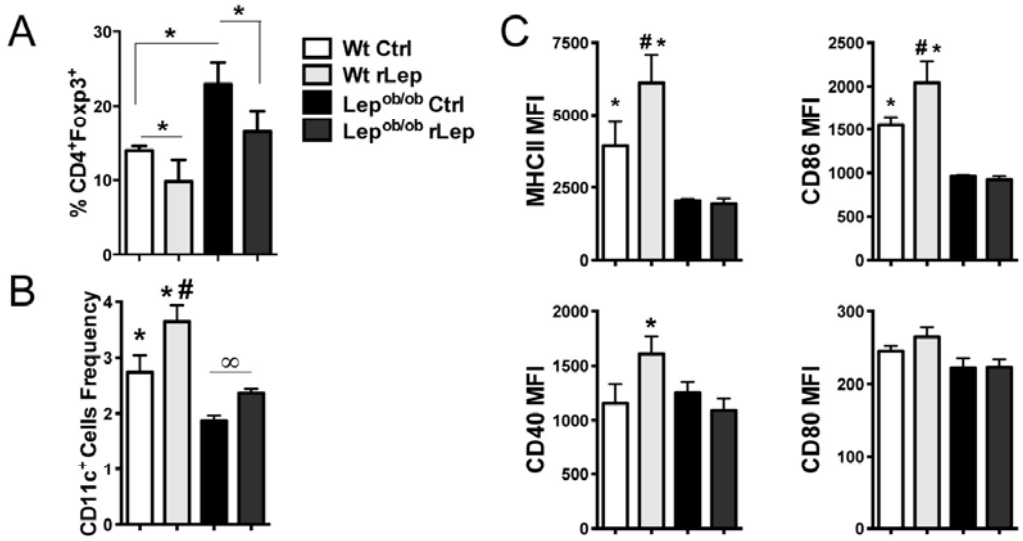
Supporting Information Figure 10**. Leptin deficiency promotes the BMDC-mediated generation of Th17 T cells.** iDCs generated from the bone marrow of $Lep^{ob/ob}$ mice, cultured with ($Lep^{ob/ob}$Lep) or without (autologous $Lep^{ob/ob}$ serum) recombinant leptin, and from WT C57BL/6 mice were used to assess the ability of DCs to generate $CD4^+IL\text{-}17^+$ T cells from naïve precursors. The $CD4^+CD62L^+CD44^-Foxp3^{gfp-}$ T cells were sorted from WT syngeneic C57BL/6J $Foxp3^{GFP}$ knock-in mice and co-cultured with the DCs in the presence of TGF-β (1 ng/mL), IL-6 (50 ng/mL) and an anti-CD3 (1 μg/mL) antibody and the total number of differentiated Th17 T cells obtained. The results are presented as the means ± SEM of one of three independent experiments each with three tested mice/group. *$p<0.05$: versus all other groups. Non-parametric Kruskal-Wallis test.



Supporting Information Figure 11. **Leptin deficiency promotes the BMDC-mediated generation Th2 T cells.** iDCs generated from the bone marrow of $Lep^{ob/ob}$ mice, cultured with ($Lep^{ob/ob}$Lep) or without (autologous $Lep^{ob/ob}$ serum) recombinant leptin, and from the WT C57BL/6 mice were used to assess the ability to generate $CD4^{+}IL\text{-}4^{+}$ T cells from naïve precursors. The $CD4^{+}CD62L^{+}CD44^{-}Foxp3^{gfp-}$ T cells were sorted from WT syngeneic C57BL/6J $Foxp3^{GFP}$ knock-in mice and were co-cultured with DCs in the presence of IL-4 (20 ng/mL) plus an anti-CD3 (1 µg/mL) antibody. (A) A dot plot representation of Th2 cell differentiation. (B) Graphical representation of the percentage of differentiated Th2 cells. (C) The measurement of IL-4 in the supernatant of Th2 cell differentiation. The results are presented as the means ± SEM of one of three independent experiments each with three tested mice/group. *$p<0.05$: versus all other groups. Non-parametric Kruskal-Wallis test.



Supporting Information Figure 12**. Leptin receptor deficiency in BMDCs promotes increased induction of Th17 cells under optimal polarizing conditions**. iDCs generated from bone marrow of $Lep^{db/db}$ and WT Balb mice were used for the differentiation of Th17 cells from naïve precursors. The $CD4^{+}CD62L^{+}CD44^{-}Foxp3^{gfp-}$ T cells were sorted from WT syngeneic Balb Foxp3 knock in mice and were co-cultured with BMDCs in Th17 polarizing conditions with an anti-CD3 antibody. The differentiation of Th17 cells was induced by TGF-β (5 ng/mL) and IL-6 (50 ng/mL). The data are representative of one of two independent experiments each performed with with 3 pooled mice/group.



Supporting Information Figure 13**. Leptin affects both DCs CD4⁺Foxp3⁺ T cells *in vivo*.** WT and $Lep^{ob/ob}$ mice were immunized with the MOG_{35-55} peptide with or without recombinant leptin (200 μg/mouse), and the draining lymph node (dLN) mononuclear cells were analyzed 7 days after the immunization. (A) Percentage of $CD4^+Foxp3^+$ T cells in the dLN. (B) Percentage of $CD11c^+$ cells in the dLN. (C) Expression of MHC-II, CD40, CD80 and CD86 by $CD11c^+$ cells in the dLN. (n=5 mice/group). The results are presented as the means ± SEM of one single experiment with three tested mice/group. * $p<0.05$ versus all other groups or as indicated. $^{\#}p<0.05$ versus WT control. $^{\infty}p<0.05$ as indicated. Non-parametric Kruskal-Wallis test.