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PH.D. THESIS

PBX-REGULATING-PROTEIN 1 (PREP1) AS A NOVEL TRANSCRIPTION FACTOR LINKING IMMUNE SYSTEM FUNCTION AND METABOLISM

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Summary

1. INTRODUCTION

1.1 Immunometabolism

1.1.1 Immuno-metabolism, a new branch linking metabolism and immunity

Metabolism is the phenomenon whereby our body monitors the production, maintenance and destruction of biomolecules in order to convey the energy necessary to maintain physiological functions. Therefore, biomolecular concentrations, within the body, continually vary and undergo specific regulation. Changes in food supply and energy expenditure closely regulate body fuel stores. Of course, adipose tissue is the most important deposit of body reserves. In Western and industrialized countries, sedentary and the excessive caloric intake has led to a marked increase in the number of obese subjects; obesity indeed, is reaching epidemic proportions in the last few years (*Ng et al., 2014*). Moreover it has been associated with a state of low grade chronic inflammation, which plays a role in a series of disorders including cancer, cardiovascular disease and type 2 diabetes and in the pathogenesis of insulin resistance and endothelial dysfunction. Recently, it emerged that the control of orexigenic and anorexigenic circuits not only influences body weight regulation but also significantly influences other important physiological and dominant functions, including immune homeostasis. In particular, many cytokines, hormones, neuropeptides and transcription factors play important roles not only at the metabolic level, but also at the immunological level (*Bruun et al., 2002*). The excess of adipose tissue, particularly in the visceral compartment, is associated with the dysregulation of adipocytokine production, resulting in adipocytic hypertrophy, hypoxia and an increase in immune cell infiltration, such as macrophages and lymphocytes. The link between obesity and inflammation has therefore raised the important issue that obesity-induced inflammation plays a pathogenic role in the development and progression of disorders such as insulin resistance and type 2 diabetes mellitus (*Xu et al., 2003*). In this *scenario* there is a new study topic which is attracting the attention of scientific community: “immunometabolism”. Immunometabolism is the field of research that links immunology and metabolism, originally considered two

separate and distinct disciplines (*Mathis and Shoelson, 2011*). However, recent scientific evidence and growing interest in this new branch has made it clear that metabolic alterations have an impact on immune system functions. Adipose tissue releases a number of molecules and factors that mediate, suppress or mitigate, the immune response.

Understanding the relationship between the endocrine and immune functions of the adipose tissue and defining the main factors working at the interface between metabolism and immunity, may have important implications for the development of new therapeutic strategies for obesity and associated diseases.

Immune cells are highly dynamic in terms of their growth, proliferation, and effector functions as they respond to immunological challenges. Different immune cells can adopt distinct metabolic assets that allow the cell to balance its requirements for energy, molecular biosynthesis, growth and longevity. In this context, it is now becoming clear that also intra-cellular metabolism has direct roles in regulating immune cell function (*Procaccini et al., 2016*). The coordination of metabolic programs with immune cell fate is a fundamental event in the control of immunity. During the course of an immune response, immune cells can traverse multiple tissues containing diverse conditions of nutrient and oxygen availability. Typically, their activation is accompanied by increased expression of effector molecules, an event which is also accompanied by extensive and rapid cellular proliferation. Recent studies have revealed the fundamental importance of metabolic reprogramming, including dynamic regulation of aerobic glycolysis (the Warburg effect), lipid synthesis and degradation, and mitochondrial activity, in immune cell activation and differentiation and the outcome of immune responses. This process depends upon the interplay between the metabolic machinery, immune signaling and transcriptional pathways. Malnutrition or hunger directly affect the function of T cells by altering the level and type of cellular metabolism. It is also well known that energy-demanding processes related to T cell differentiation directly link cellular function with energy-generating mode: the critical link between metabolism and immunity is indicated by findings that aberrant immune cell metabolism not only affects inflammatory responses and adaptive immunity, but also contributes to metabolic disorders (*Chandra, 1999; Hotamisligil et al., 2006*).

1.1.2 Adipose tissue and inflammation

Adipose tissue is a well-vascularized and innervated tissue that contains a connective tissue matrix and numerous immune cells, including macrophages. The white adipose tissue (WAT) is the type of fat in which triglycerides are stored and from which lipids are mobilized for systemic use when other tissues need energy. WAT is often subdivided into subcutaneous and abdominal deposits, whose physiologies can be distinct and whose functions in pathological conditions can also be distinct. This is contrasted with brown adipose tissue, whose main function is thought to be non-shivering thermogenesis, a process of heat production through the uncoupling of oxidative phosphorylation. Originally considered as a passive warehouse for energy storage, WAT is now known to secrete a variety of substances that help to regulate immune homeostasis (*Tilg and Moschen, 2006*). Adipose tissue is not only a reservoir for energy, but also an immune organ (*Grant and Dixit, 2015*). In the context of obesity, the development of insulin resistance is now recognized to be initiated by inflammation of the adipose tissue. The first discovery of inflammation in obese tissues in the mouse revealed increased levels of the cytokine TNF-α in adipose tissue (and in adipocytes themselves) of obese mice compared with lean controls. This report was soon followed by a wealth of studies describing the inflammatory differences between obese and lean animals as well as humans. It is now appreciated that not only TNF-α but an array of inflammatory cytokines are increased in obese tissues, including interleukin (IL)-6, IL-1β, CCL2, and others (*Cawthorn and Sethi, 2007*).

Immune cells in adipose tissue, especially macrophages, have always attracted the attention of the scientific community. Both in humans and mice, the number of bone marrow macrophages is closely related to obesity and inflammatory states. Adipose tissue in obese subjects is characterized by macrophage infiltration, which has been shown to be stimulated from the adipocyte production of MCP-1 (monocytic chemoattractant), able to attract monocytes into the adipose tissue, where they become activated. The recruitment of macrophages into adipose tissue is the initial event in obesity-induced inflammation and insulin resistance (*Kanda et al., 2006*).

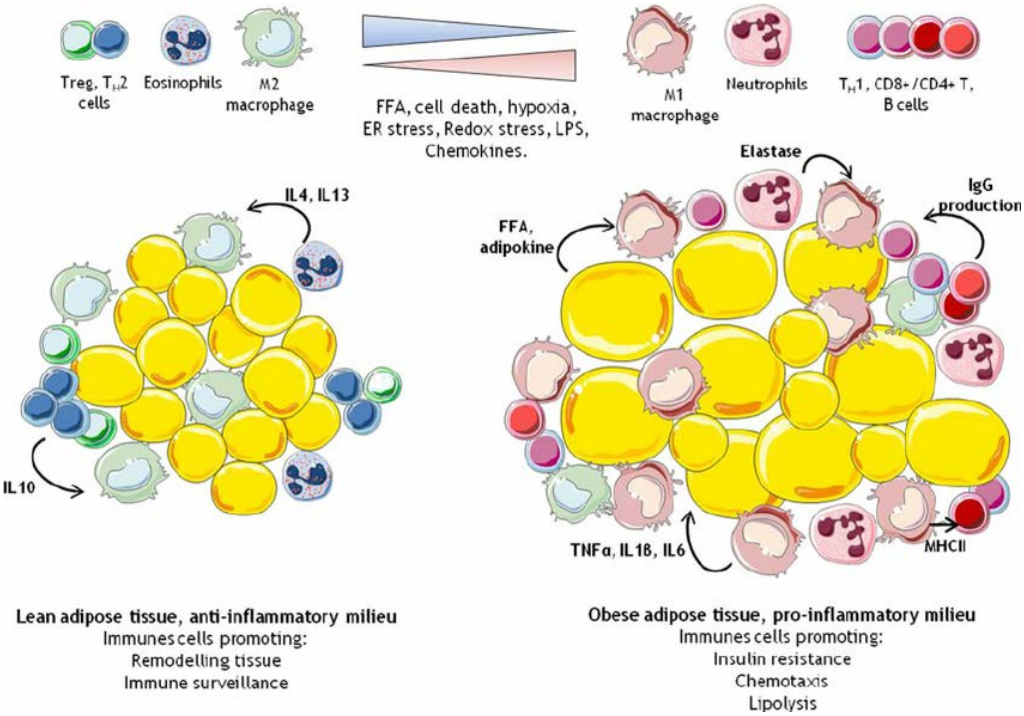


Figure 1. Immune cells in adipose tissue during obesity. In the lean adipose tissue, eosinophils and Th2 cells and Treg cells promote an anti-inflammatory milieu through the secretion of IL4, IL10 and IL13 inducing macrophage M2 polarization. In the obese adipose tissue, over nutrition leads to bigger adipocyte, leading to the recruitment of different immune cells and the development of a pro-inflammatory environment. In this context, macrophages are recruited and induced to become pro-inflammatory M1 macrophages, leading to enhanced immune cells activation and increased secretion of pro-inflammatory cytokines.
(image taken from H.L. Kammoun et al, 2014)

1.1.3 Inflammation and insulin-resistance

Obesity-induced chronic inflammation is one of the most important mechanisms that can lead to the development of insulin resistance and metabolic syndrome. Therefore, obesity, inflammation and insulin resistance are not disconnected but they are pathological conditions, which are closely related, each other (*Asghar and Sheikh, 2017*). Although a connection between inflammation and diabetes was suggested more than a century ago, the evidence that inflammation is an important player in the development of insulin resistance came approximately 20 years ago, when Feingold and Grunfeld observed that administration of the proinflammatory cytokine tumor necrosis factor-α (TNF-α) led to increased serum glucose concentrations, thus suggesting that hyperglycemia may be exacerbated by cytokine overproduction (*Grunfeld and Feingold, 1992*).

However, the first studies that established the concept of obesity-induced adipose tissue inflammation were performed by Hotamisligil et al., who showed that TNF-α was elevated in obese rodents and that neutralization of TNF-α was able to ameliorate insulin resistance (*Hotamisligil, 1999*). Additionally, mice deficient in TNF-α showed improved insulin sensitivity during diet-induced obesity. A mechanistic link between inflammatory processes and insulin resistance was further established by showing that the signaling pathways leading to activation of inhibitor of κB kinase-β (IKK-β) and nuclear factor-κB (NF-κB) are stimulated in obesity and insulin resistance (*Solinas and Karin, 2010; Cai et al., 2005*).

Several scientific evidence now supports the idea that pro-inflammatory cytokines can cause insulin resistance in adipose tissue, skeletal muscle and liver, reducing the signal transduction of insulin. Cytokine sources in insulin-resistant states are mainly fat and liver, but to a greater extent activated tissue macrophages. Chronic inflammation in these tissues could cause localized insulin resistance via autocrine/paracrine cytokine signaling and systemic insulin resistance via endocrine cytokine signaling all of which contribute to the abnormal metabolic state (*Pirola and Ferraz, 2017*). The proof that an anti-inflammatory strategy may be successful in the treatment of insulin resistance has come from studies using salicylates. Several years ago, it has been shown that high doses of salicylates administered to obese mice inhibit NF-κB activity, leading to improved insulin sensitivity and glucose metabolism and were able to lower glucose levels in diabetic patients, improving glycemic control. The anti-hyperglycemic and anti-inflammatory effects of salicylates are important indications for the link of inflammation to type 2 diabetes (T2D). Another therapeutic approach to modulate the immune system involves TZDs (thioazolinediones). TZDs are PPAR-γ ligands with well-known insulin-sensitizing effects in the clinical setting (*Hammarstedt et al., 2005*). These compounds also have potent anti-inflammatory actions that contribute to the overall improvement in insulin resistance with type 2 diabetes treatment. In addition to TNF-alpha, several clinical studies have been conducted using different strategies to inhibit IL-1 signaling. Studies in rodents and humans have shown that blockade of IL-1 action results in a modest improvement in glycemic control attributable to enhanced B cell function, but insulin sensitivity was not increased (*McLaughlin et al., 2017*).

These new findings help to reinforce the idea that obesity increases the risk of developing T2D and metabolic syndrome, by modulating immune system functions. This finding showing that insulin resistance might result from immunological alteration represents a new scientific focus for setting up anti-inflammatory strategies to modulate the consequences of metabolic alteration, such as obesity (*Huh et al., 2014*).

1.2 Immune cells and metabolic alterations

As previously mentioned, one of the feature of the inflammatory state of obesity is the increased infiltration of immune cells into the metabolic tissues. For example, the macrophage population is increased in the adipose tissue of obese mice or mice fed a high-fat diet (HFD) compared with lean mice fed normal chow, and these cells also contribute to the increased tissue cytokine expression (*Xu et al., 2003; Weisberg et al., 2003*). Although the factors attracting and/or activating immune cells in obese tissues are not yet fully understood, what is clear is the consensus that metabolic processes, especially obesity and obesity related complications, activate both the innate and adaptive arms of the immune system. Obesity associated inflammation appears to be caused by infiltration of inflammatory immune cells and a parallel loss, or functional reprogramming, of immunoregulatory cells. Together, these changes lead to a variety of positive feedback pathways that not only sustain chronic inflammation, but also contribute to the development of insulin resistance.

1.2.1 Macrophages

Different subsets of macrophages are involved in obesity-induced adipose tissue inflammation. Macrophages that accumulate in the adipose tissues of obese mice mainly express genes associated with an M1 or “classically activated” macrophage phenotype, whereas adipose tissue macrophages from lean mice express genes associated with an M2 or ‘alternatively activated’ macrophage phenotype (*Lumeng et al., 2007*). Both M1-like and M2-like populations express F4/80 and CD11b, and the M1-like macrophages also expresses CD11c (*Lumeng et al., 2007*). In the obese state, M1-like macrophages can accumulate

lipids, assuming a foamy appearance in the adipose tissue. Stimulation with T helper 1 (Th1-type) cytokines, including interferon-γ (IFN-γ), or with bacterial products leads to the generation of M1 macrophages(*Gordon, 2003*). Conversely, T helper 2 (Th2-type) cytokines such as IL-4 and IL-13 polarize macrophages to the M2 phenotype, which up-regulate IL-10 production and downregulate synthesis of pro-inflammatory cytokines. Thus, obesity-induced perturbations in the balance between Th1- and Th2-type signals may influence the recruitment and activation of macrophages in adipose tissues, thereby generating either a pathogenic and inflammatory environment or a non-inflammatory and protective environment (Figure 1). Tissue macrophages respond to changes in the local environment by changing their polarization status, and thus, the M1 and M2 classifications are oversimplifications of the more dynamic and varied polarization states of macrophages that can be observed in vivo. There are several scientific evidence that proinflammatory polarized macrophages can cause insulin resistance. In fact, they can secrete proinflammatory cytokines that directly reduce insulin sensitivity (*Brestoff and Artis, 2015*). The evidence of this action comes from experiments in which the elimination or inactivation of the main inflammatory pathways mediated by macrophages have been performed. For example, the elimination of IKK-β, JNK1, insulin receptor or fatty acid binding protein 4 (FABP4) in mice leads to protective action from insulin resistance induced by obesity (*Arkan et al., 2005; Mauer et al., 2010; Furuhashi et al., 2008*).

1.2.2 $CD4^+$ T cells

The total number of T cells increases in obesity in the visceral adipose tissue (VAT) and in parallel increases their proliferation and infiltration in response to specific adipose tissue factors (*Gerriets and MacIver NJ, 2014; Schipper et al., 2012*). In addition, it has been shown that one of the T-cell chemoattractors, the RANTES factor, is induced in adipocytes after activation by IFN-γ and/or TNF-α (*Kabir et al., 2014*). Therefore, obesity-induced factors would contribute both quantitatively and qualitatively to changes in T cell populations, leading to accumulation of pro-inflammatory molecules in adipose tissues of obese subjects. $CD4^+$ T lymphocytes recognize peptide antigens loaded on MHC Class II molecules of antigen-presenting cells. $CD4^+$ T naive cells can be divided in

differ subtypes of $CD4^+$ T cells, such as Th1, Th2, Th17, and T (Treg) regulatory cells. The principal actors of CD4 T cell differentiation are a variety of cytokines, including IFN-γ for Th1, IL-4 for Th2, IL-6, and TGF-β for Th17 and TGF-β for T-cell regulators. Th1 and Th17 cells mediate pro-inflammatory reactions, while Th2 and Treg cells contribute to anti-inflammatory responses. Th1 cells and Th17 cells are immune cell subsets that play critical roles in the onset of autoimmune diseases and tissue inflammatory responses. Th1 cells primarily secret IFN-γ which stimulates monocyte differentiation into M1 type macrophages. Consistent with the above observations, IFN-γ KO mice, display improved insulin sensitivity, accompanied by a decrease in HFD-induced adipose tissue inflammation, because of impaired Th1 differentiation (*O'Rourke et al., 2008*). In contrast, it has been proposed that a relative decrease in anti-inflammatory/regulatory cell types such as Th2 and Treg cells is associated with a huge infiltration of circulating monocytes and subsequent M1 polarization in obese adipose tissue.

1.2.3 Regulatory T cells (Treg)

Regulatory T cells (Treg) cells are a small subset of T cells, usually constituting only 5-15% of the peripheral $CD4^+$ T cell compartment in mice and humans (*Sakaguchi et al., 2008;Thornton and Shevach, 1998*). Treg cells are important in the control of the inappropriate immune responses that characterize autoimmunity and allergy. In general, Treg cells control effector T cell responses and also influence the activities of cells of the innate immune compartment. These cells, characterized as $CD4^+$ $CD25^+$ $Foxp3^+$, are therefore a well known anti-inflammatory T cell subset (*Procaccini et al., 2011*). The proportion of Treg cells among $CD4^+$ T cells is relatively high in adipose tissue compared with spleen, lymph nodes, and lung. The number of Treg cells is decreased in adipose tissues of obese mice models such as *ob/ob, db/db* mice lacking of leptin and leptin-receptor respectively (*Deiuliis et al., 2011*). Depletion of Treg cells in mice by diphtheria toxin (DT) aggravates adipose tissue inflammation and insulin resistance (*Bapat et al., 2015*). On the other hand, expansion of Treg cells in mice induced by IL-2 injection, attenuates adipose tissue inflammation and improves insulin sensitivity, in part through IL-10-mediated suppression of the conventional T cell proliferation. Notably, VAT

Treg cells have been shown to display adipose tissue-specific T cell receptor (TCR) repertoires compared with splenic Treg cells but the identities of the antigens specific to VAT Treg cells remain to be explored. One of the distinct characteristics of Treg cells residing in VAT is a high level of PPARγ expression as compared to Treg cells in other tissues (*Cipolletta et al., 2012*). PPARγ KO mice specifically in Foxp3-expressing cells, show a significant decrease in the number of VAT Treg cells and a consequent increase in adipose tissue inflammation (*Hamaguchi et al., 2012*). On the other hand, treatment with TZD, a PPARγ agonist, induces an increase in VAT Treg cells followed by a reduction of inflammation in adipose tissue, indicating an important role of PPARγ in the accumulation and phenotype of adipose tissue Treg cells.

1.2.4 $CD8^+$ T cells

CD8 T cells recognize peptide antigens loaded by MHC class I molecules on antigen presenting cells and participate in proinflammatory cytokine secretion and cytolysis of target cells. It has been reported that the number of CD8 T cells is elevated in obese adipose tissue. Nishimura and collegues have shown that the percentage of $CD8^+$ T cells in stromal vascular cells (SVCs) is increased upon 2 weeks of HFD feeding whereas macrophage infiltration is induced after 6 weeks of HFD feeding (*Nishimura et al., 2009*). Furthermore, elevation of $CD44^+$ $CD62L^+$ (effector memory marker) CD8 T cells and a decrease in $CD44^-$ $C62L^+$naïve $CD8^+$ T cells is observed in obese adipose tissues(*Yang et al., 2010*). Interestingly, the presence of $CD8^+$ T cells with a distinct TCR repertoire in obese adipose tissue has been reported, suggesting the presence of an adipose tissue-specific response of $CD8^+$ T cells during obesity obesity. Consistent with the above observation, depletion of CD8 T cells by injection of anti-CD8 antibody into diet-induced obesity (DIO) mice results in a sharp decrease in the levels of pro-inflammatory cytokines such as IL-6 and TNF-α, associated with a concomitant increased glucose tolerance and insulin sensitivity, independently from obesity (*Nishimura et al., 2009*). Moreover, in co-culture experiments of $CD8^+$ T cells with macrophages, $CD8^+$ T cells have been shown to induce macrophage differentiation from monocytes and to sustain pro-inflammatory cytokine secretion, confirming the critical role of $CD8^+$ T cells in the control of macrophage polarization and activation (*Nishimura et al., 2009*).

1.2.5 B lymphocytes

B cells are key lymphocytes in the adaptive immune response, especially the humoral immune response. B cells not only produce antibodies but also may act as antigen presenting cells. In early obesity, an increase in the number of immunoglobulin G (IgG^+ $CD19^+$ B cells) in VAT has been reported, indicating accumulation of class switched mature B cells in obese adipose tissues (*Winer et al., 2011*). Confirming a key role of B cells in the control of obesity, B cell deficiency has been shown to reduce VAT-resident M1 macrophages and $CD8^+$ T cell-mediated IFN-γ expression, leading to an improvement in glucose tolerance during obesity. Additionally, transplantation of MHCI- or MHCII-deficient B cells suppresses IFN-γ expression in both $CD4^+$ T cells and $CD8^+$ T cells in obese mice, indicating that MHC I and MHC II molecules on B cells would affect adipose tissue inflammation by modulating T cell activity in adipose tissues. Furthermore, IgG produced by B cells induce clearance of apoptotic and necrotic debris through antibody-mediated fixation of complement proteins involved in the phagocytosis of macrophages. Recently, an adipose regulatory B cell population called “Breg” has been reported to play an intermediate antiinflammatory role in adipose tissue inflammation *via* the production of IL-10 (*Nishimura et al., 2013*). Nevertheless, it is necessary to delineate more precisely which subtypes of B cells are involved in the adipose tissue inflammation and identify the regulatory mechanisms underlying the B cell-mediated immune response in adipose tissues during obesity.

1.3 TALE Family

1.3.1 TALE proteins

The tri-amino acid loop extension proteins (TALE), best known as transcription factors family, are responsible for the regulation of important processes, such as growth and differentiation during vertebrate embryogenesis. The genes encoding these proteins are highly preserved ancestrally and they are highly conserved in both vertebrates and plants, constituting a link in the evolution of the two systems (*Gehring et al. 1994*). TALE proteins have a domain that binds highly

preserved DNA sequence of about 60 amino acids called “homeodomain”. This region consists of three alpha helixes and an N-terminal arm. Homeodomain interacts with DNA through one of the three alpha helix that specifically bind certain bases to the major DNA groove. Instead, the N terminal portion binds to the bottom of the DNA. In the middle of the first two alpha helix there is an extension of three amino acids, virtually represented by proline (P) - tyrosine (Y) - proline (P) in position 24-26. This domain has been implicated in important protein-protein interactions needed for the fundamental aspects of development. The superfamily of TALE proteins consists of two subclasses: PBC and MEIS, whose cooperative function is crucial for transcriptional regulation. Many players of these two families, has been shown to be of fundamental importance to Hox-mediated development programs (*Moens and Selleri, 2006; Chan et al., 1994*).

1.3.2 Pbx1 protein

Pbx1 (pre-B cell leukemia transcription factor) is an ubiquitous molecule belonging to the PBC family. Several studies in the past years have identified this proto-oncogene as one of the loci affected by translocation t(1;19), found in a large cohort of children affected by pediatric pre-B-cell acute lymphoblastic leukemias (*Kamps et al., 1990*). However, in mice, the individual contribution of each member of the PBC family seemed to be different because, while the single mutant Pbx1 had a wide range of malformations, the same cannot be said forPbx2 and Pbx3 mutants. This evidence suggests an important role for Pbx1 in embryonic development. Recent genetic studies indicate the role of TALE homeodomain protein Pbx1 in the development and function of pancreatic islets. Pbx1, indeed, is required for pancreatic cell growth and differentiation. $Pbx1^{-/-}$ embryos exhibit pancreatic hypoplasia and marked defects in exocrine and endocrine cell differentiation before death at embryonic day (E) 15 or E16 (*Jonsson et al., 1994*). Pbx1 has been shown to play an absolutely non-marginal role in pancreatic insulin secretion in mature mice and the absence of its regulatory activity leads to inadequate levels of circulating insulin and impaired glucose tolerance (*Selleri et al., 2001; Kim et al., 2002*). In these animals PDX-1 levels are strongly reduced, indicating that Pbx1 is also important for its expression.

1.3.3 Prep1 protein

Pbx-regulating-protein 1 (Prep1) is a 64 kDaubiquitary transcription factor mapping on the chromosome 21q.22.3 which plays a key role in early development, genomic stability, hematopoiesis. Indeed, Prep1 forms DNA-independent dimeric complexes with the Pbx homeodomain transcription factor, enhancing target specificity and regulatory function (*Berthelsen et al., 1998*). Prep1 could be localized both in cytoplasm and in nucleus and the heterodimerization with Pbx1 appears to be essential to translocate Prep1 into the nucleus to bind DNA target. Moreover, Prep1 dimerization prevents nuclear export and the proteasomal degradation of Pbx1, prolonging its half-life (*Berthelsen et al., 1999*) (Figure 2). Formation of dimeric complex drastically increases binding affinity of Prep1/Pbx1 to the DNA and also broadens the DNA target selectivity of Pbx1 to include sequences containing the TGACAG sequence. Prep1 and Pbx1 can form ternary complexes with PDX-1 to regulate somatostatin gene transcription (*Goudet et al., 1999*). The role of Prep1 has been in part clarified by the generation of mutant mice. Nevertheless, Prep1 null embryos result in early lethality before gastrulation (E7.5) (*Fernandez-Diaz et al., 2010*). An insertion of a retroviral vector in the first intron of the Prep1 gene results in a hypomorphic mutation (*Prep1*$^{i/i}$) that exhibits variable penetrance and expressivity. *Prep1*$^{i/i}$embryos die between E17.5 and P0 and display a profound alteration in the hematopoietic development (*Ferretti et al., 2006*). However, a small percentage of the *Prep1*$^{i/i}$embryos are born alive and subsequently live a normal length-life. The mice escaping embryonic lethality show T-cell development anomalies with a decreased number of hematopoietic precursors (*Penkov et al., 2005*). In addition, *Prep1*$^{i/i}$feature an impairment of erythropoiesis and angiogenesis accompanied by liver hypoplasia, decreased hematocrit, anemia, and delayed erythroid differentiation together with a decrease in capillary formation. *Prep1* deficiency affects the expression of both TALE class partners Pbx and Meis, both required for embryonic (*Azcoidia et al., 2005; Di Martino et al., 2001*).

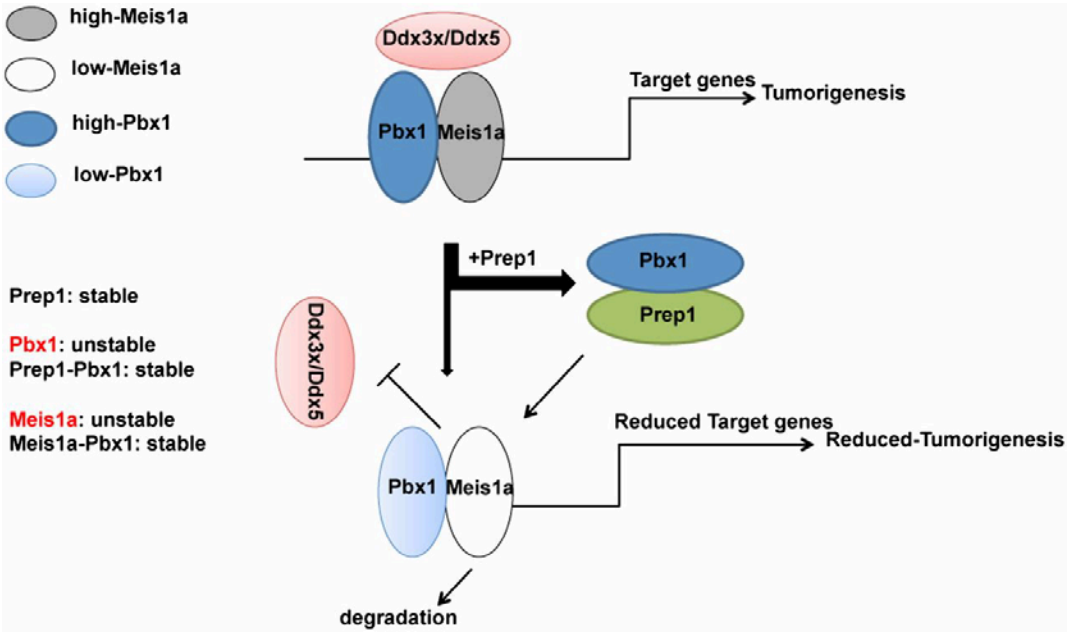


Figure 2.Model of how Prep1 attenuates Meis1a action. Prep1 and Pbx1 interaction determines Meis1a levels and the extent of tumor formation. Specifically, Prep1 attenuates Meis1a tumorigenic activities by squelching Pbx1 from Pbx1–Meis1a complexes, leading to proteasome-dependent degradation of Meis1a.

1.3.4 Prep1 and metabolic alterations

Recent scientific evidence has shed light on an important role of Prep1 in pancreas development, glucose homeostasis and regulation of insulin activity in muscle and liver (*Oriente et al., 2008; Oriente et al., 2011*). Prep1-hypomorphic mice show smaller pancreatic islets but with a normal architecture. However, these mice are characterized by hypoplasia of pancreatic insulae, accompanied by significantly reduced levels of absolute insulin (basal and post-load). In the hypomorphic mice (*Prep1*$^{i/i}$) there was also a reduction in pancreatic Pbx1 expression, underlining the idea that Prep1 could act hierarchically upstream and indirectly on the development of the pancreas, by controlling Pbx1 levels. These mice exhibit protection against streptozotocin-induced diabetes and increased insulin sensitivity with improved ability to metabolize glucose. *Prep1* deficiency in animal models improves insulin signaling in liver increasing hepatic glycogen content and decreasing glucose output and triglyceride levels. Analysis of the initial steps in insulin signaling in the *Prep1*-deficient mouse liver revealed increased tyrosine phosphorylation of both insulin receptor and the major insulin-receptor-substrate (IRSs) (*Oriente et al., 2008; Oriente et al., 2011*). Taken together, all these data can suggest that Prep1 could be considered a new

gene involved in the pathogenesis of type 2 diabetes and insulin resistance. In agreement with these findings, a recent paper showed that serum triacylglycerols and liver expression of fatty acid synthase (FAS) were significantly decreased in Prep1 hypomorphic heterozygous (*Prep1*$^{i/+}$), as compared with their non-hypomorphic littermates. Moreover, other studies, showed that despite the treatment with a methionine- and choline-deficient diet (MCDD) induced steatosis in both *Prep1*$^{i/+}$ and non-hypomorphic control mice, the levels of alanine aminotransferase, the intracellular triacylglycerol content, the liver steatosis, inflammation and necrosis were significantly less evident in *Prep1*$^{i/+}$mice, indicating that *Prep1* silencing can protect mice from MCDD-induced steatohepatitis (*Oriente et al., 2013*).

2. AIM OF STUDY

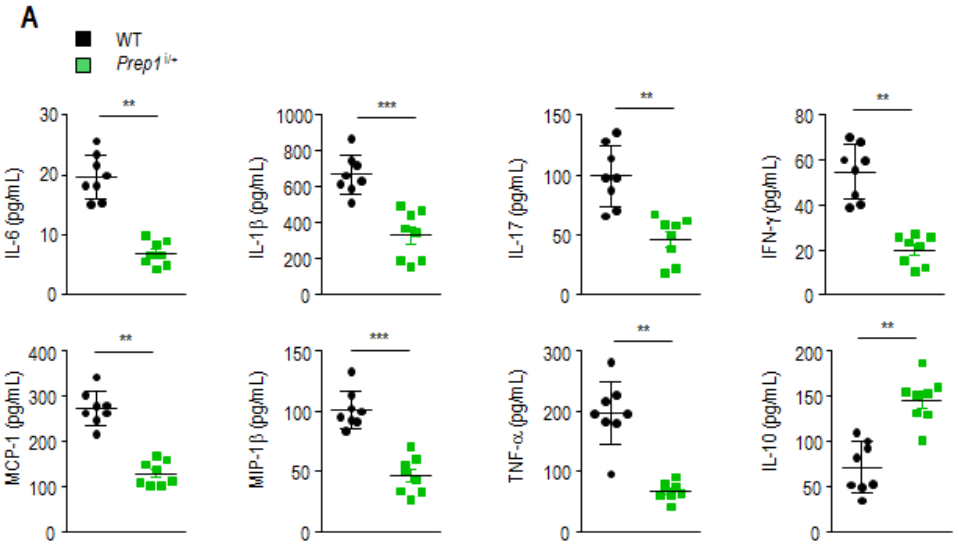
The radical change in the lifestyle of Western countries observed in recent years has inevitably promoted the emergence of dysmetabolic conditions. In this regard, it has become necessary to identify new lifestyle-enhancing markers, possibly acting in tandem, on one side on the resolution of obesity/insulin resistance, and on the other side on the immune system functions. As in recent years, immunometabolism is becoming a matter of growing interest in the scientific community, the identification of possible novel factors, working at the interface between these two disciplines, becomes of crucial importance.Prep1 is a homeodomain transcription factor involved in the regulation of key processes such as organogenesis and major developmental pathways. Since recent evidence have highlighted the pathological involvement of the immune system in the regulation of altered metabolic conditions, and given the recent findings showing that *Prep1* inhibition increased insulin sensitivity, protecting from streptozotocin-induced diabetes, the purpose of this study is to evaluate the role of Prep1 in the control of immune system functions. More specifically, we will investigate how and whether *Prep1* down-regulation may affect the functional activity of the main immune cells, in physiological settings and in condition of metabolic pressure. Finally, our ultimate goal will be to identify Prep1 as a potential novel therapeutic target for immune-mediated metabolic disorders.

3. RESULTS

3.1 Immunophenotype of *Prep1*-deficient mice

Starting from recent data showing the link between metabolic alterations and the immune system functions, and from the previous experimental evidence demonstrating that hypomorphic mice for *Prep1* are protected from liver damage (*Oriente et al., 2013*), we first measured the secretion of several cytokines in the sera of in *Prep1*$^{i/+}$ mice by comparing them with those observed in their littermate controls (WT mice). We found that *Prep1* hypomorphic mice displayed a reduced amount of different pro-inflammatory cytokines (IL-6, IL-1β, IL-17, IFN-γ, MCP-1, MIP-1β and TNF-α) and an increased concentration of one of the best characterized anti-inflammatory cytokines, such as IL-10 (Figure 3A). Next we compared the basal immunophenotype of the spleens of *Prep1*$^{i/+}$mice and their littermate controls. Flow cytometric analyses revealed no significant differences in the spleens of the two groups of mice, in terms of percentage of the different immune cells subsets (data not shown).

However, significant differences were observed in the percentage of immune cells infiltrating the liver. Indeed, *Prep1*$^{i/+}$mice displayed significant lower percentage of CD45$^+$ cells (a common leukocyte antigen) associated with a reduction in T cell compartment (CD3$^+$, CD4$^+$ and CD8$^+$ cells) and increased percentage of B cells (Figure 3B).



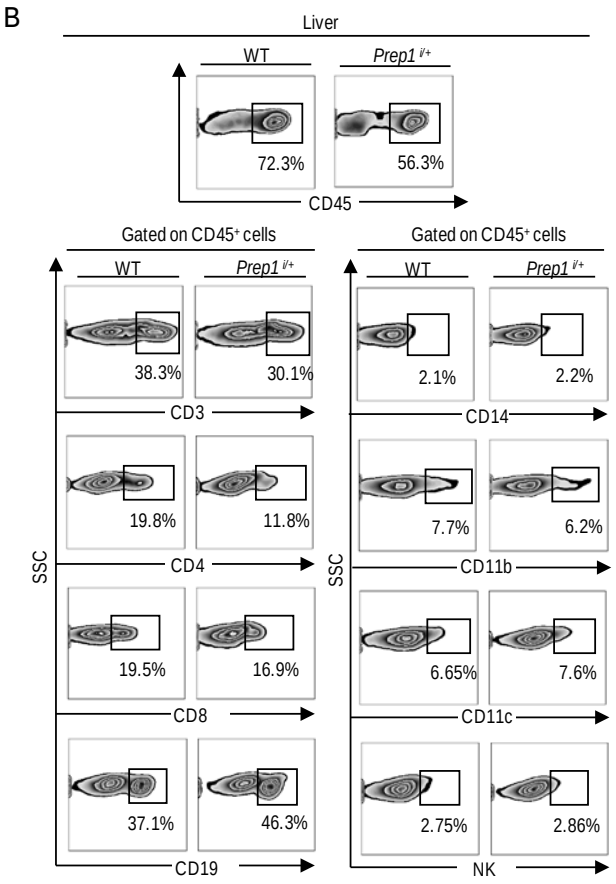


Figure 3. Cytokine production and liver immunophenotype of *Prep1*$^{i/+}$ mice. (**A**)The graphs show IL-6, IL-1b, IL-17, IFN-γ, MCP-1, MIP-1b, TNF-a and IL-10 production in the sera of WT (black columns) and *Prep1*$^{i/+}$(green columns) mice. Data are shown as mean ± S.E.M. (n= 8 mice/group; $^{**}p < 0.001$, $^{***}p < 0.0001$). (**B**) Cytofluorimetric plots showing *ex vivo* immunophenotype (CD3, CD4, CD8, CD19, CD11b, CD11c, CD14 and NK) of the livers from *Prep1*$^{i/+}$mice and their control littermates. One representative out of three independent experiments.

3.2 Prep1 deficiency associates with functional impairment of T cell compartment

We next analyzed whether Prep1 inhibition could be associated with a functional alteration of T cell responses. To this purpose, we isolated splenocytes from both groups of mice (*Prep1*$^{i/+}$and WT) and we analyzed their proliferative profile. We found a reduced proliferative capability in *Prep1*$^{i/+}$ mice upon TCR-mediated activation (with anti-CD3, 2C11 mAb) compared to WT mice, as indicated by a reduced thymidine incorporation (Figure 4A). In accordance with reduced proliferation, T cells from *Prep1*$^{i/+}$mice also secreted significantly lower levels of proinflammatory cytokines, including IL1-α, IL1-β, IL-6, IL-17, IFN-γ, TNF-α, MCP-1, MIP1-α, and MIP1-β in cell culture

supernatants with respect to WT mice (Figure 4B). This cytokine profile was accompanied by a reduced expression of classical cell surface activation markers on $CD4^+$ T cells in *Prep1*$^{i/+}$mice. Indeed, the expression of CD25, CD54 (ICAM-1), CD49d (VLA-4), CD71 and MHCII was significantly lower in T cells of *Prep1*$^{i/+}$mice as compared to WT controls (Figure 4C),thus providing a clear indication of the possible key role of Prep1 in the control of activation and function of the immune system.

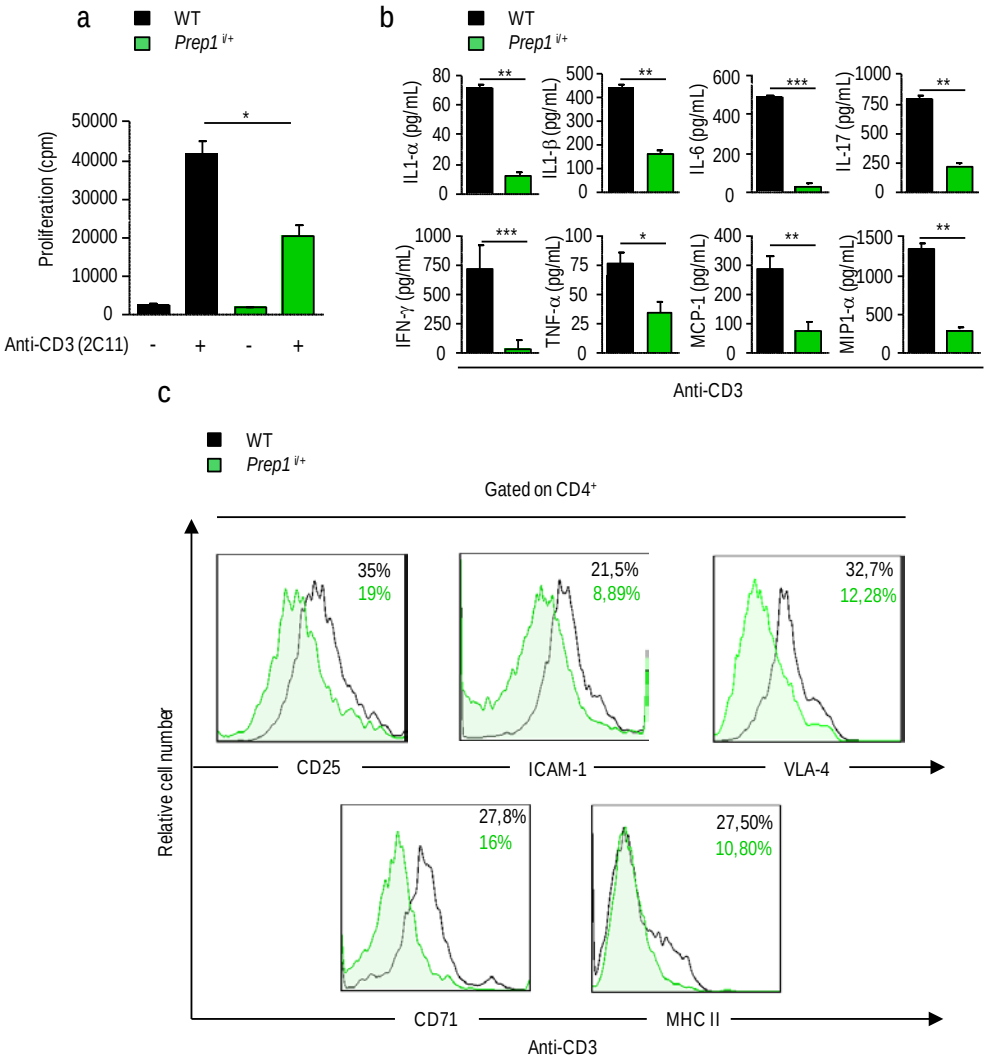


Figure 4. Analysis of T cell functions in *Prep1*$^{i/+}$mice. (**A**) Proliferative response (evaluated as thymidine incorporation) of T lymphocytes isolated from WT (black columns) and *Prep1*$^{i/+}$(green columns) mice, stimulated or not with anti-CD3 for 48 hours. Data are presented as mean ± S.E.M. of three independent experiments (*$p < 0.005$). (**B**) Graphs show the secretion of IL-1a, IL-1b, IL-6, IL-17, IFN-γ, TNF-a, MCP-1, MIP-1a, in cell supernatant derived from T cells isolated from WT (black columns) and *Prep1*$^{i/+}$(green columns) mice, stimulated with anti-CD3 for 48 hours. The data are shown as mean ± S.E.M of three independent experiments (***$p < 0.0001$; **$p < 0.001$; *$p < 0.01$). (**C**) Cytofluorimetric analysis of the expression of the main activation markers (CD25, CD54, CD49d, MHCII, CD71, MHC II) expressed by T cells from WT (black histogram) and *Prep1*$^{i/+}$(green histogram) mice, stimulated with anti-CD3 for 36 hours. One representative plot out of three independent experiments.

3.3 Biochemical events in T cells from *Prep1*$^{i/+}$ mice

In order to identify the molecular mechanism associated with impaired Tcell proliferation observed in *Prep1*$^{i/+}$mice, we analyzed the biochemical events in 12 hours cultured splenocytes from both groups of mice, in the presence or absence of TCR-mediated stimulation. The substantial amount of ERK1/2 phosphorylation and the low level of the cell cycle inhibitor p27^{kip1} indicated the high proliferative status of TCR-stimulated T cells from WT mice (Figure 5A). Conversely, upon TCR stimulation, T cells from *Prep1*$^{i/+}$mice showed a reduced activation of ERK1/2 and increased levels of p27^{kip1}, suggesting a possible cell cycle arrest in these cells (Figure 5A). Since it has been demonstrated that mTOR (mammalian-target-of rapamycin) kinase complex supports differentiation and proliferation of T cells (*Laplante and Sabatini, 2010; Procaccini et al., 2010; Procaccini et al., 2012*), we next evaluated its activity in terms of phosphorylation of mTOR itself and of its downstream target, the ribosomal protein S6. The amount of phosphorylated mTOR in the two groups of mice was almost the same, while a significant decrease in S6 phosphorylation has been observed in *Prep1*$^{i/+}$ T cells, both in basal conditions and upon anti-CD3 stimulation, thus suggesting a reduced mTOR kinase activity associated with impaired proliferation of T cells in those mice where the expression of *Prep1* was reduced (Figure 5B).

As we previously shown a reduced production of pro-inflammatory cytokines in *Prep1*$^{i/+}$ mice, we also analyzed the activity of STAT5, a key element in IL2/IL2-Receptor signal transduction, in T cells of both groups of mice. In WT mice there was a strong phosphorylation of STAT5 upon TCR engagement, while T cells from the *Prep1*$^{i/+}$mice showed low levels of p-STAT5 even after TCR-mediated stimulation (Figure 5C). In addition, we also found that inhibition of *Prep1* compromised the phosphorylation of STAT3, another downstream molecule involved in inflammatory signaling, both in basal conditions and after-TCR-mediated stimulation (Figure 5C).

Autophagy is the basic catabolic mechanism which ensures cell survival during reduced energy availability (ie. starvation) by maintaining cellular energy levels (*Riffelmacher et al., 2017*).Current evidence indicates that a fine tuning of autophagy has a major role in enabling differentiation and activation of immune cells. Given the functional difference of T cells from WT and *Prep1*$^{i/+}$mice, we

also analyzed the involvement of the autophagic process in this phenomenon. The conversion of microtubule associated protein light chain 3 (LC3-I) into LC3II (autophagic flux), a well-known marker of autophagy activation, has been analyzed by western blotting in unstimulated and TCR-activated T cells from *Prep1*$^{i/+}$mice and their littermate controls. As shown in Figure 5D, unstimulated splenocytes from *Prep1*$^{i/+}$mice displayed significant higher levels of LC3II lipidation, when compared to WT mice (Figure 5D). TCR stimulation decreased LC3II lipidation both in WT and *Prep1*$^{i/+}$mice (Figure 5D).To exclude that the observed phenomena might be due to an altered lysosomal proteases degradation, we confirmed the involvement of *Prep1* inhibition on the autophagic flux also in the presence of lysosomal protease inhibitors (NH_4Cl/leupeptin) (Figure 5D). In this case, we observed that the levels of LC3II were always higher in T cells from *Prep1*$^{i/+}$ mice, even though TCR engagement reduced LC3II lipidation, thus suggesting that impaired activation of T cells from these mice might be due to the activation of the autophagic pathway.

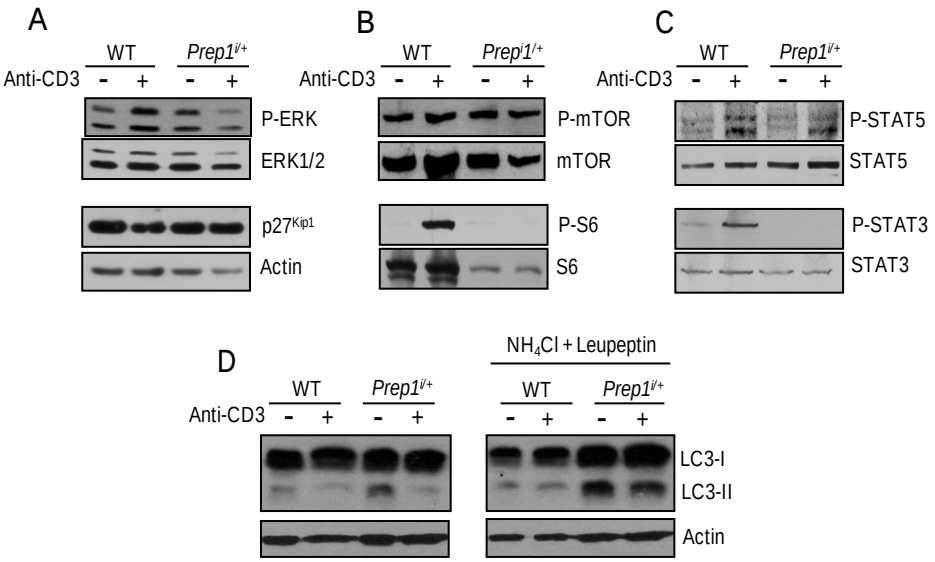


Figure 5. Intracellular pathways altered by *Prep1* inhibition in T cells. Western blot analysisfor P-ERK 1/2, ERK1/2, p27Kip1 and actin (**A**), p-mTOR, mTOR, p-S6 and S6 (**B**), p-STAT5, STAT5, p-STAT3 and STAT3 (**C**) in T cells isolated from WT and, *Prep1*$^{i/+}$ mice (n= 3 mice/group), in the presence or absence of anti-CD3 stimulation for 12 hours. One representative out of two independent experiments. (**D**) Immunoblot for LC3I/II normalized on actin in T cells isolated from WT and, *Prep1*$^{i/+}$ mice, stimulated or not with anti-CD3 for 12 hours, in the presence or absence of specific lysosomal protease inhibitors (NH_4Cl/leupeptin). One representative out of two independent experiments.

3.4 Impaired mitochondrial and glycolytic activity in T cells from *Prep1*$^{i/+}$ mice

It has been recently demonstrated that intracellular metabolism plays a key role in controlling immune processes. Particularly, the idea that the mitochondrial metabolism is crucial to sustain T cell proliferation/activation is becoming increasingly popular (*Naik et al., 2011; West et al., 2011; Perl et al., 2004*). Therefore, in order to investigate whether the alteration of *Prep1* may affect the bioenergetic profile and the mitochondrial function of T cells, we measured the oxygen consumption rate (OCR) (an indicator of oxidative phosphorylation) in purified CD4$^+$ T cells in both groups of mice, by cell flux analyzer (Seahorse), upon TCR-mediated stimulation (Figure 6A) CD4$^+$T cells from *Prep1*$^{i/+}$mice showed a reduced OxPhos (oxidative phosphorylation) when compared to littermate controls, as indicated by the significant decrease in basal OCR and ATP-linked OCR (Figure 6B). The same holds true also for the maximal respiratory capacity (MRC) (obtained upon stimulation with carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone (FCCP), which again was also significantly inhibited in CD4$^+$ T cells from *Prep1*$^{i/+}$mice (Figure 6B), thus suggesting that functional alteration of T cell compartment associates with a reduced mitochondrial activity.

In addition, to assess whether *Prep1* inhibition was also associated with a decreased glycolytic capacity (the alternative route used by T cells to generate ATP), we also evaluated the extracellular acidification rate (ECAR) (an indicator of glycolysis engagement) in the same above mentioned experimental conditions. CD4$^+$ T cells from *Prep1*$^{i/+}$mice**,** displayed decreased glycolysis both at the baseline (after stimulation with glucose) and under maximal conditions (upon oligomycin stimulation) (Figures 6C and 6D). Also the glycolytic capacity was reduced in *Prep1*-deficient mice. Taken together, these data suggest that the metabolic pathways (mitochondrial respiration and glycolysis), which would sustain T cell activation and proliferation, were significantly impaired in the absence of Prep1 activity.

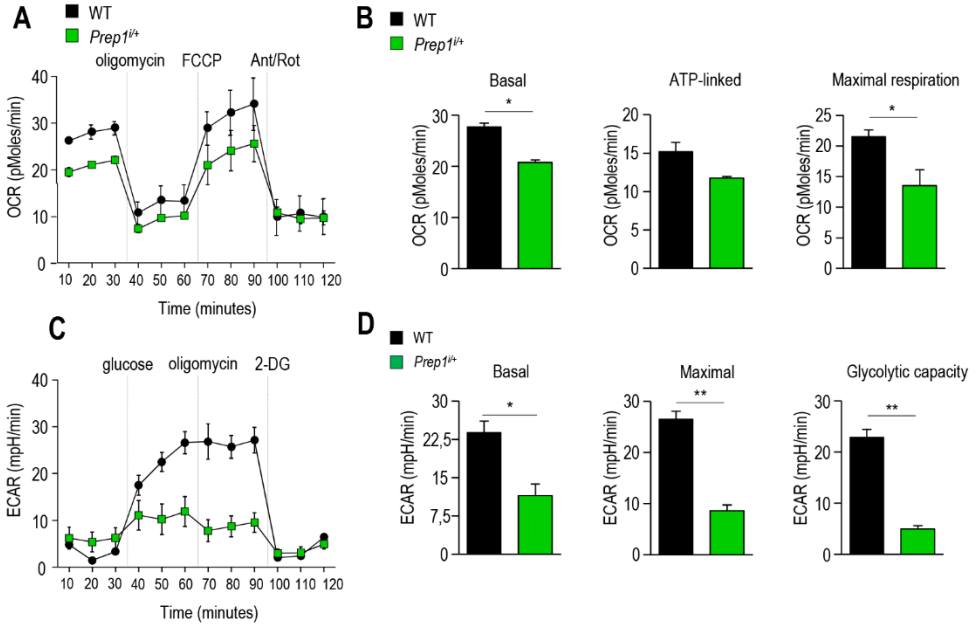


Figure 6. Intracellular metabolic alterations induced by Prep1 inhibition in CD4+ T cells. (A) O_2 consumption rate (OCR), measured in real time, in $CD4^+$ T cells stimulated with anti-CD3/CD28-microbeads and purified from WT (black circles) and $Prep1^{i/+}$ mice (green squares). OCR was measured under basal conditions and in response to indicated mitochondrial inhibitors: oligomycin, carbonylcyanide-4- (trifluoromethoxy)-phenylhydrazone (FCCP), Antimycin A and Rotenone (Ant/Rot), using an XFe-96 Extracellular Flux Analyzer. One representative out of two independent experiments. The data are shown as mean ± S.E.M. of triplicates. (B) Indices of mitochondrial respiratory function: basal OCR (before addition of oligomycin), ATP-linked OCR (calculated as the difference of basal rate and oligomycin rate), maximal respiration (calculated as the difference of FCCP rate and antimycin/rotenone rate) were calculated from the kinetic profiles of anti-CD3/CD28-stimulated $CD4^+$ T cells from WT (black columns) and $Prep1^{i/+}$ (green columns) mice. Data are expressed as mean ± S.E.M. of three measurements, each of them in triplicates (*$p< 0.01$). (C) Extracellular acidification rate (ECAR), measured in real time, in $CD4^+$ T cells stimulated with anti-CD3/CD28-microbeads and purified from WT (black circles) and $Prep1^{i/+}$ mice (green squares). ECAR was measured under basal conditions and in response to glucose, oligomycin and 2-DG. One representative out of two independent experiments. The data are shown as mean ± S.E.M. of triplicates. (D) Indices of glycolytic pathway activation: basal ECAR (in the presence of glucose), maximal ECAR (after oligomycin injection) and glycolytic capacity (calculated as the difference of oligomycin-induced ECA and 2-DG-induced ECAR) were calculated from the kinetic profiles of anti-CD3/CD28-stimulated $CD4^+$ T cells from WT (black columns) and $Prep1^{i/+}$ (green columns) mice. Data are expressed as mean ± S.E.M. of three measurements, each of them in triplicates (**$p< 0.001$).

3.5 Increased suppressive activity of Treg cells from *Prep1*$^{i/+}$ mice

In order to understand whether functional alterations of conventional T cells (Tconv) from hypomorphic mice (*Prep1*$^{i/+}$) were counterbalanced by an increased activity of regulatory T cell compartment ($CD4^+CD25^{high}FoxP3^+$), we analyzed this specific cell subset in both groups of mice. *Ex vivo* analyses revealed that *Prep1*$^{i/+}$mice had a higher percentage of $CD4^+FoxP3^+$ than that observed in WT mice (Figure 7A). Moreover Treg cells from *Prep1*$^{i/+}$mice showed an enhanced *in vivo* proliferative profile, as testified by the increased expression of the nuclear proliferation antigen (PCNA) as compared to Treg cells from control mice (Figure 7A). Since it has been previously shown that mTOR kinase plays a key role in the metabolic control of Treg cell responsiveness (*Procaccini et al., 2010*), we also analyzed the phosphorylation of the ribosomal protein S6, as readout of mTOR kinetic activity, in Treg cells of both experimental groups. We found that the higher proliferative rate of Treg cells in *Prep1*$^{i/+}$mice associated with increased mTOR engagement (Figure 7A), as suggested by the increased phosphorylation of S6. It is now well accepted that one of the prominent features of Treg cells is represented by their strong proliferative capacity *in vivo* (*Vukmanovic-Stejic et al., 2006*), despite they are anergic to *in vitro* TCR-mediated stimulation (*Li et al., 2005*), a condition that associates with an optimal suppressive activity. Here we found that, Treg cells from *Prep1*$^{i/+}$mice were able to suppress the proliferation of autologous $CD4^+CD25^-FoxP3^-$ T cells (Tconv) in co-culture assays, more efficiently than Treg cells from WT mice (Fig. 7B). In addition, in *Prep1*$^{i/+}$ mice, Treg cells showed not only a greater ability to suppress Tconv proliferation, but also an enhanced ability to inhibit proinflammatory cytokines secretion by Tconv cells. As shown in Figure 7C, IL-6, IL-12, IL-17, IFN-γ produced by Tconv cells were better inhibited by Treg cells from *Prep1*$^{i/+}$ mice than by Treg cells from littermate controls in co-culture experiments (Figure 7C).

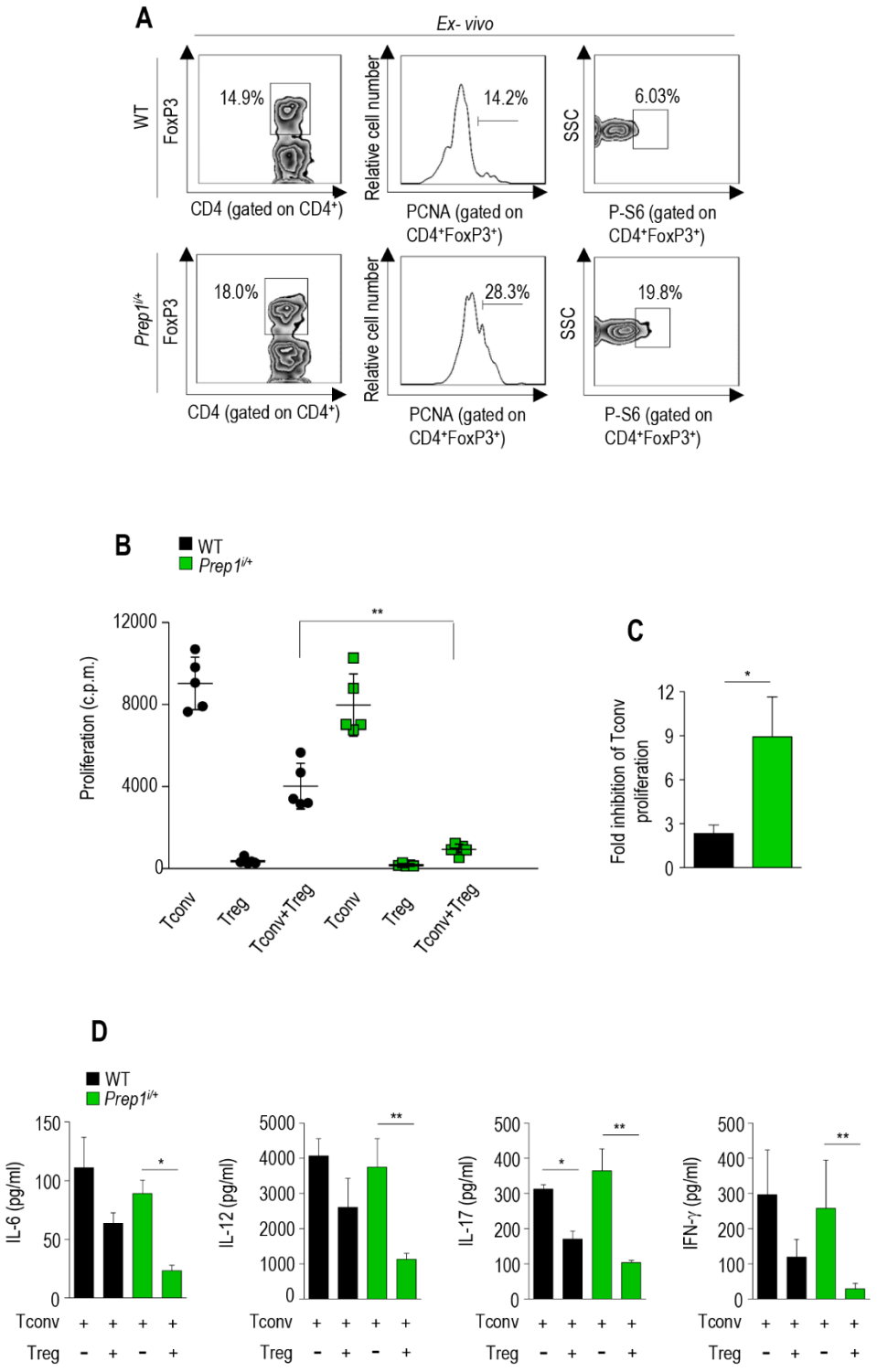


Figure 7. Treg cell frequency and function in *Prep1*$^{i/+}$mice. (A) Cytofluorimetric plots showing the percentage of CD4$^+$FoxP3$^+$ cells (Treg), PCNA and p-S6 expression in Treg cells from the spleens of WT and *Prep1*$^{i/+}$ mice (one representative out of three independent experiments). (B) *In vitro* proliferation of purified Tconv, Treg cells and of both cell types in co-culture (at the ratio of 1:1) from WT and *Prep1*$^{i/+}$ mice upon anti-CD3/CD28 mAb stimulation. Data are shown as mean ± S.E.M (one representative out of two independent experiments, each of them performed on a pool of 3 mice/group, $^{**}p < 0.001$). (C) Fold inhibition of Tconv proliferation and IFN-γ, TNF-a, IL-6, MIP-1a, MCP-1 and IL-1b production (D) by Tconv cells purified from WT (black column) and *Prep1*$^{i/+}$ (green column) mice in co-culture with autologous Treg upon anti-CD3/CD28 mAb stimulation. Data are shown as mean ± S.E.M (one

representative out of two independent experiments, each of them consisting of a pool of 3 mice/group, *p< 0.01; **p< 0.001).

3.6 *Prep1* deficiency protects from high fat diet-induced alterations

To evaluate the role of Prep1 in the protection of liver inflammation and metabolic disorders during a condition of metabolic stress, *Prep1*-deficient mice were fed with a high-calorie diet (HFD). The mice were followed for 18 weeks and their weight gain has been evaluated over time. We found that *Prep1*$^{i/+}$mice showed a lower weight gain as compared to WT control mice, when feeding with a HFD (Figure 8A).

In line with these findings, we also found lower levels of serum triglycerides (TG) (Figure 8B) in *Prep1*$^{i/+}$mice fed with HFD versus WT mice group. Since HFD has been associated with impaired glucose tolerance and type 2 diabetes development, we next evaluated the effect of the *Prep1* deficiency on glucose tolerance. WT mice fed a HFD displayed glucose intolerance compared with *Prep1*$^{i/+}$mice, which showed a significant improvement in glucose tolerance (Figure 6C). These data suggest that Prep1 deficiency may play a protecting role from the development of glucose-associated abnormalities and the pathological fat accumulation observed during HFD.

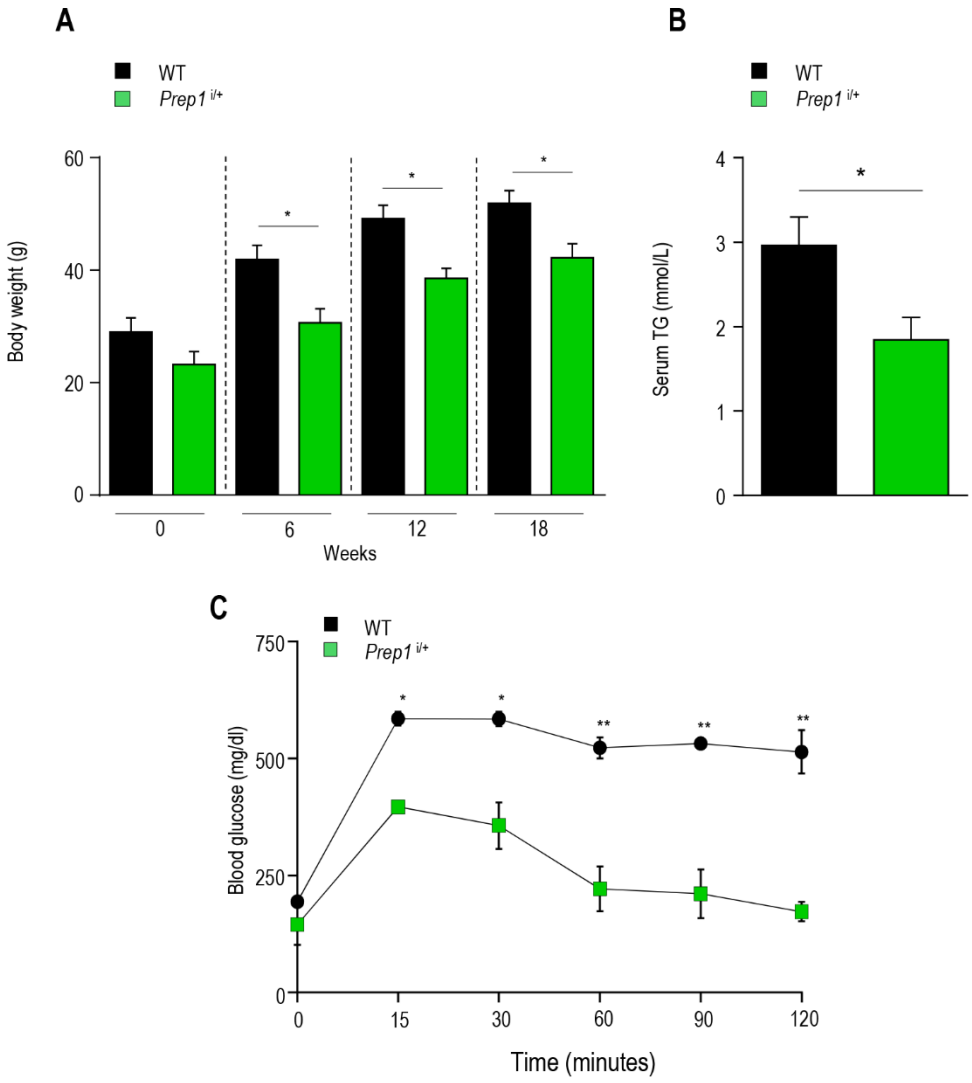


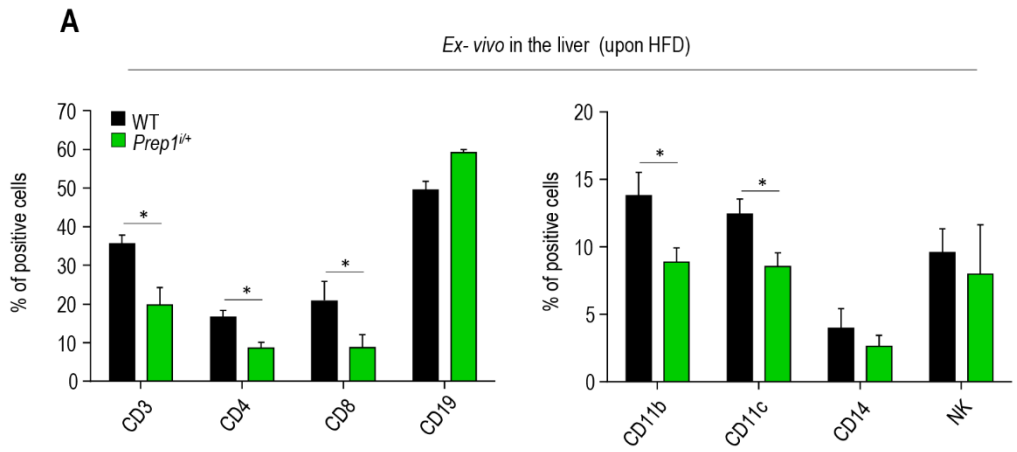
Figure 8. Metabolic alteration of *Prep1*$^{i/+}$ mice undergoing a HFD. (**A**) body weight (in grams) of WT and *Prep1*$^{i/+}$ mice fed with a high fat diet (HFD), followed for 18 weeks. Data are shown as mean ± S.E.M (n = 4 mice for HFD, **p*< 0.01). (**B**) Serum triglyceride content in WT (black columns) and *Prep1*$^{i/+}$ mice (green columns) fed with a HFD for 18 weeks. Data are shown as mean ± S.E.M (n = 4 mice/group for HFD, **p*< 0.01). (**C**) Glucose tolerance test (GTT) (panel) performed in WT and *Prep1*$^{i/+}$mice fed with HFD at week 18. The blood glucose values in milligrams per deciliter are shown as mean ± S.E.M (n= 4 mice/group for HFD, **p*< 0.01; ***p*< 0.001).

3.7 *Prep1* deficiency protects from high fat diet-induced inflammation

Since the immune system has been shown to play a central role in the pathogenesis of obesity and its-associated alterations, we also analyzed changes in liver inflammation caused by Prep1 deficiency upon HFD feeding. First, we found a decreased percentage in T cell compartment ($CD3^+$, $CD4^+$, $CD8^+$) infiltrating the liver of Prep1hypomorphicas compared to WT littermate controls

(Figure 9A) and a tendency to increase the percentage of B cells ($CD19^+$). Moreover a decreased accumulation of macrophages has been detected in the liver of *Prep1*$^{i/+}$mice fed a HFD, as indicated by the reduced percentage of $CD11c^+$ and $CD11b^+$ cells (macrophage markers) (Figure 9A), whereas no significant differences have been detected in $CD14^+$ and NK cells, between the two groups of mice.

The reduced inflammatory infiltrates observed in the liver of *Prep1*$^{i/+}$ mice undergoing a HFD, possibly responsible for the protection to liver steatosis, were accompanied by an increased percentage of Treg cells, which also exhibited a higher proliferative profile (increased positivity for PCNA) sustained by a greater mTOR activation (as evidenced by increased phosphorylation of ribosomal protein S6) (Figure 9C). Finally, the anti-inflammatory phenotype observed in *Prep1*$^{i/+}$mice, has been also confirmed by the findings showing a reduced secretion of pro-inflammatory cytokines (IFN-γ, TNF-α, IL-17) and increased production of anti-inflammatory cytokines (such as IL-10) in the sera of these mice, feeding a HFD, as compared to WT mice (Figure 9C). Taken together, these data suggest a potential protective role exerted by *Prep1* deficiency in immune cells during metabolic disorders.



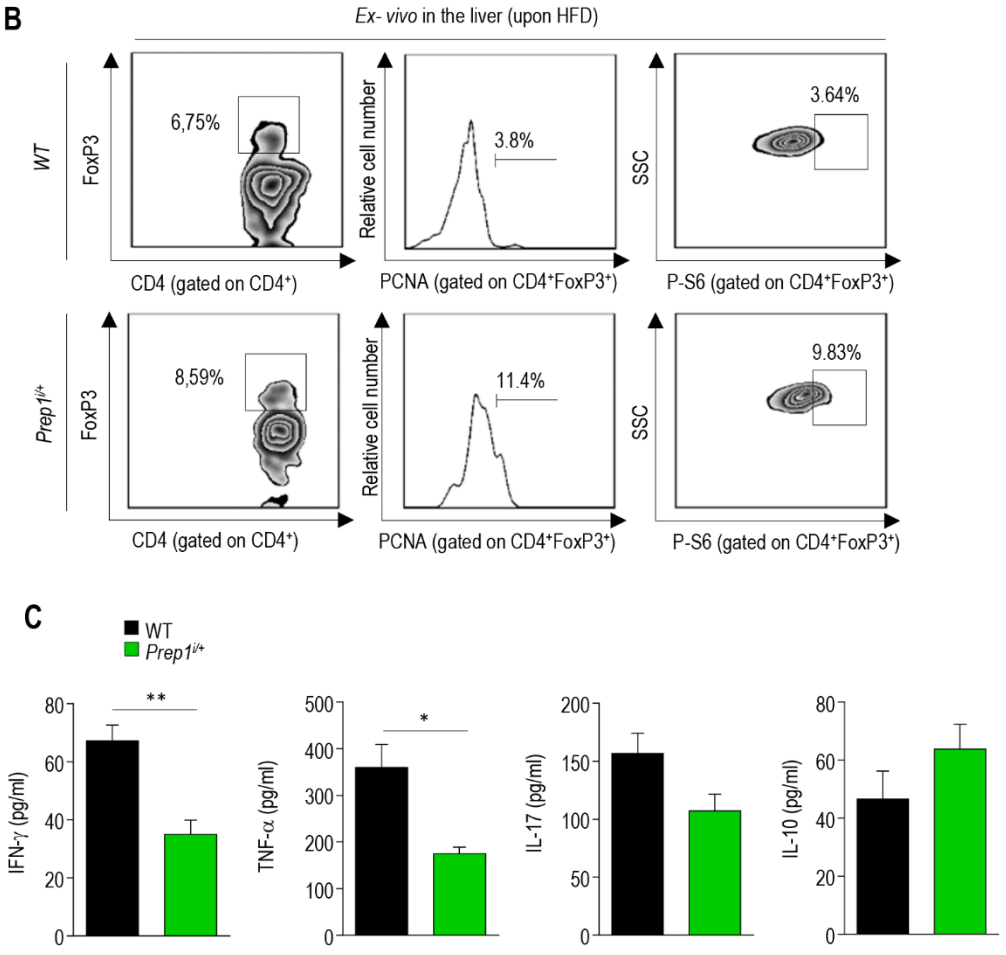


Figure 9. Hepatic inflammation and liver immunophenotype of *Prep1*$^{i/+}$ mice upon HFD. (**A**) Cytofluorimetric immunephenotype (CD3, CD4, CD8, CD19, CD11b, CD11c, CD14, NK) of livers from WT (black columns) and *Prep1*$^{i/+}$(green columns) mice undergoing a HFD (one representative out of three mice/group). Data are shown as mean ± S.E.M. (n= 3 mice/group, **p*< 0.01).(**B**) Flow cytometric plots showing the percentage of CD4$^+$FoxP3$^+$ cells (Treg) and the expression of PCNA and p-S6 in Treg cells from the liver of WT and *Prep1*$^{i/+}$ mice undergoing a HFD (one representative out of three mice/group). (**C**) Serum levels of IFNγ, TNF-α, IL-17, IL-10 WT (black columns) and *Prep1*$^{i/+}$(green columns) mice fed with a HFD. Data are shown as mean ± S.E.M. (n= 4 mice/group; **p* < 0.01).

4. DISCUSSION

Immuno-metabolism is an emerging field of investigation working at the interface between historically distinct disciplines: immunology and metabolism (*Mathis and Shoelson, 2011*). Metabolic abnormalities are characterized by a strong increase in the secretion of pro-inflammatory cytokines that favor the migration of inflammatory cells from the periphery to the inflammatory tissues. On the contrary, inflammation associated with metabolic disorders is accompanied by a drastic decrease in the number and/or in the function of regulatory T cells (Treg cells), a specific cell subset that control immune tolerance. In this context, the identification of genes and molecular mechanisms involved in the susceptibility and pathogenesis of metabolic disorders is becoming crucial. However, the identity of the genes and factors influencing the risk of inflammation, working on immune cell functions, have been only partially discovered and described, so far. In this thesis, we identified a new player, whose modulation is able to improve both the metabolic and the immune system functions. Specifically, we found that the homeodomain Prep1 transcription factor is able to control liver inflammation and insulin resistance by acting on different immune cell subsets, modulating their functions. It has been observed that *Prep1* down-regulation is able to reduce the expansion, activation and pro-inflammatory cytokines production by T lymphocytes, both in physiological settings and during a condition of metabolic pressure, such as high fat diet (HFD). Recently, Oriente and colleagues have demonstrated that *Prep1*-ipomorphic mice (*Prep1*$^{i/+}$, characterized by reduced expression of the functional protein Prep1) displayed a peculiar phenotype characterized by an increased insulin sensitivity in skeletal muscle accompanied by protection from streptozotocin-induced diabetes (*Oriente et al., 2008*). In addition, mice expressing a reduced percentage of *Prep1* appeared to be protected from triglycerides (TG) accumulation and hepatotoxic damage, caused by inadequate methionine/choline diet (MCDD) (*Oriente et al., 2013*).

In the present study, we show that *Prep1*$^{i/+}$ mice are characterized by a general anti-inflammatory condition, in which we found decreased activity of conventional T lymphocytes (Tconv) and an increased regulatory activity exerted by regulatory T cells (Treg). In line with this assumption, lower levels of

the major proinflammatory cytokines have been detected in the sera of *Prep1*$^{i/+}$ mice when compared with those found in WT control mice. In particular, *Prep1*$^{i/+}$ mice exhibited a reduced secretion of IL-1β, IL-6, IL-17, TNF-α, IFN-γ, MCP-1, MIP1-β in line with recent findings showing that pro-inflammatory cytokines can be considered as co-inducers of insulin resistance.

Indeed, it has been widely demonstrated that recombinant TNF-α administration decreases insulin sensitivity (*Hotamisligil and Spiegelman, 1994; Kern et al., 1995*). In addition, we found a decreased secretion of the chemotactic monocytic factor-1 (MCP-1) (*Wu et al., 2007*), thus supporting the hypothesis that reduced release of proinflammatory cytokines in the microenvironment does not sustain the generation of a chemiotactic gradient responsible for the migration of immune cells into inflamed tissues. In contrast, *Prep1* inhibition induced significantly higher levels of IL-10, one of the most representative anti-inflammatory cytokine.

Furthermore, we also showed that *Prep1*$^{i/+}$ mice exhibited a reduced accumulation of immune cells in the liver, as compared to WT animals and this reduction mainly affects T cell compartment. Our data, therefore, could give to Prep1 a very important role in the control of T cell fate, function and recruitment, and reinforce the notion that T cells are key components in the progression of inflammatory condition associated with metabolic alterations. Indeed, the decrease in their number and/or function relieves adipose tissue inflammation and alleviates insulin resistance in obese mice. Therefore, it is clear that the imbalance between the effector T cells and regulatory T cells in favor of the former is able to affect metabolic functions, prolonging and intensifying the inflammatory state in dysmetabolic syndromes. A clear beneficial role in metabolic alterations is covered by Treg cells; in fact several scientific papers have shown that Treg cells reduce adipose tissue inflammation and insulin resistance (*Feuerer et al., 2009; Winer et al., 2009*). We then wondered whether *Prep1* inhibition could affect the functional activity of immune cells, and we mainly focused our attention on T cell compartment. We found that T cells isolated from *Prep1*$^{i/+}$mice exhibited a significantly reduced proliferative capacity as compared to their littermate controls, together with an impaired capability to secrete pro-inflammatory cytokines in cell culture supernatants. The same trend has been observed for the main surface markers

expressed by activated T cells, (such as IL-25, ICAM-1, VLA-4 and MHC II), as their induction resulted strongly compromised by inhibition of Prep1 activity. Therefore we could speculate that *Prep1* deficiency renders T cells hyporesponsive to TCR-mediated stimulation, thus representing a possible target to modulate T cell activity. The protective role provided by Prep1 inhibition is further demonstrated by the increased proliferative capacity and suppressive activity of Treg cells isolated from the *Prep1*$^{i/+}$mice, supported by a prominent activation of the mTOR pathway.

To get insight on the possible biochemical events associated with altered T cell functions in *Prep1*$^{i/+}$mice, we analyzed the main pathways involved in cell cycle progression, proliferation, inflammatory signal transduction and autophagy. We found a decreased phosphorylation of ERK1/2 and an increased accumulation of $p27^{Kip1}$. This negative cell cycle regulator has been shown to play a central role in the block of T cell clonal expansion, being therefore crucial for induction of anergy, determined by blockade of costimulatory pathways (*Boussiotis et al., 2000; Rowell et al., 2005*). In parallel, T cells from *Prep1*$^{i/+}$ mice displayed a compromised activation of the mTOR pathway, which we have previously demonstrated to be crucial for the control of T cell differentiation and proliferation (*Procaccini et al., 2010; Procaccini et al., 2012*). mTOR, indeed, has been shown to integrate different extracellular signals by acting as a nutrient availability sensor, able to determine the quality of T cell activation and cytokine production. In this context, the reduced expression of activation markers and the decreased production of pro-inflammatory cytokines in T cells from *Prep1*$^{i/+}$ mice could be partly due to the inhibition of mTOR kinase activity. These results are also in line with recent evidence showing that inhibition of mTOR decreases *in vitro* adipogenesis and shows anti-obesity effects in mice fed a HFD, lowering leptin and insulin levels and altering T cells functions (*Chang et al., 2009*), similarly to what has been observed in *Prep1*$^{i/+}$mice.

In addition, here we also provide evidence that Prep1 inhibition in T cells increases the autophagic process. Recent genetic and functional studies have shown that autophagy is an anti-inflammatory mechanism, as it dampens inflammasome activation and its absence (ATG 16L1 null mice) has been associated with increased production of IL- 1β and IL-18 in a mouse model of Crohn's disease (*Saitoh et al., 2008*). In light of these results, the sustained

activation of autophagy observed in T cells from *Prep1*$^{i/+}$mice is in line with their impaired proliferation and their switch towards an anti-inflammatory phenotype.

Recent reports have recently demonstrated that T cell function is governed by their specific metabolic requirements (*Procaccini et al., 2016*). Since Prep1 inhibition strongly affected T cell functional activity and since it has been shown that dysregulated mitochondrial activity could lead to aberrant T cell development (*Naik et al., 2011; West et al., 2011; Perl et al., 2004*), we also assessed the impact of Prep1 inhibition on the metabolic machinery governing T cell growth and function. More specifically, we analyzed the mitochondrial respiration and the activation of the glycolytic pathway in T cells from *Prep1*$^{i/+}$ mice and their littermate controls. Both these energy-producing pathways were impaired in the absence of Prep1 and a particular reduction has been observed for the glycolytic engagement. These findings demonstrate that Prep1 down-regulation, in mice, is able to modulate T cell function, by reducing the engagement of metabolic pathways. Therefore the manipulation of specific T cell metabolic routes or of the autophagic process, induced by Prep1 inhibition, may represent an attractive target for the development of new therapeutic approaches for all those diseases characterized by the hyperactivation of the immune system.

Since it has been recently described that conditions of metabolic pressure (such as obesity or type 2 diabetes) can affect immune system functions, we have next investigated the role of Prep1 and of its inhibition on the regulation of immune processes in mice undergoing a hypercaloric diet (high fat diet, HFD), which induces obesity, insulin resistance and liver inflammation. We evaluated different parameters associated with dysmetabolic conditions (glucose tolerance test, body weight, TG content and inflammatory infiltrates in liver) in *Prep1*$^{i/+}$and age-matched WT mice fed a HFD. *Prep1*$^{i/+}$mice showed a reduced ability to gain weight overtime, upon HFD. Since obesity is a risk factor for the development of insulin resistance (*Esser et al., 2014*), the reduced body weight obtained through *Prep1* inhibition could represent an important tool for prevention of obesity and for the treatment of type 2 diabetes. The reduced body weight gain observed in *Prep1*$^{i/+}$mice also associated with an improvement in glucose tolerance. All these events have been also associated with a reduced liver inflammation, suggested by a strong reduction of immune cells infiltrating

the liver, especially those cells belonging to T-compartment ($CD4^+$ and $CD8^+$cells). Recruitment of immune cells in adipose tissue and/or liver is an important step in the development of insulin resistance during obesity induced by a high calorie diet. In light of these considerations, our findings in *Prep1*$^{i/+}$mice could suggest that targeting Prep1 might induce an improvement of dysmetabolic alterations, protecting against liver inflammation and altered glucose tolerance.

Our data are in agreement with recent findings showing that anti-inflammatory approaches (i.e. IL-1R blockade, NF-κB pathway inhibition with salicylated derivatives) aimed at targeting specific pro-inflammatory pathways, represent useful tools for improving parameters of glucose control, thus reducing the risk of systemic inflammation (*Ruan et al., 2009; Nath et al., 2009; Youssef et al., 2002 Zhang e Markovic-Plese, 2008*).

In conclusion, our findings have potential therapeutic implications: Prep1 could possibly provide a new platform for potential therapeutic manipulation of the immune system functions in order to control the progression of metabolic disorders, such as obesity and type 2 diabetes. This is an hypothesis worth testing in future studies.

5. MATERIALS AND METHODS

5.1 Mice

Prep1-targeted mice were generated by gene trapping by Lexicon Genetics (The Woodlands, TX, USA). The general phenotype of these mice has been previously described (*Ferretti et al., 2006*). In the experiments reported in this paper, *Prep1*$^{i/+}$ mice were backcrossed with wild-type (WT) C57BL/6J mice for 4 generations. All animal handling conformed to regulations of the Ethics Committee on Animal Use of H. S. Raffaele (IACUC permission number 207).
Hepatic tissue samples were collected rapidly from 8- to 10-week-old male C57BL/6-SV129 mice. Tissues were snap-frozen in liquid nitrogen and stored at −80°C for subsequent western blotting and real-time RT-PCR analysis, as previously described (*Oriente et al., 2011; Oriente et al., 2013*). All animals were kept in temperature and humidity controlled rooms, in a 12/12 h light/dark cycle, with lights on from 7:00 A.M. to 7:00 P.M. Food and water were provided *ad libitum*. All the experiments were performed in 8-10-weeks-old animals. The blood for serum biochemistry evaluation was collected with an intracardiac method, allowed to clot at room temperature and centrifuged for 10 min; the serum was then separated for shipment. Quantitative analysis of serum TG was performed with an ABX Pentra400 clinical chemistry analyzer (ABX-Horiba, Montpellier, France) according to the manufacturer's instructions. For the diet experiments, six to eight weeks old mice were fed a High fat diet (HFD) or a standard diet (SD) [60% kcal from fat or 10% kcal from fat, respectively; Research Diets, Brunswick, NJ) for different time periods up to 28 weeks. Body weight was recorded weekly.

5.2 Flow cytometric analysis

FITC-conjugated mAbs to CD4, CD11c, MHCII, NK, CD71, CD127; PE-conjugated mAbs to CD8, CD14, VLA-4 (CD49d), ICAM-1 (CD54); Cy5 conjugated mAbs to CD3, CD11b; and allophycocyanin-conjugated mAbs to CD45, CD25 were all purchased from BD Biosciences-Pharmingen, while allophycocyanin-conjugated mAbs to CD19 was purchased from Miltenyibiotec. Anti-FoxP3 (eBiosciences), PCNA (Chemicon International) and phosphor-S6

(Cell Signaling) antibodies have been used for intracellular staining, according to manufacturer's instructions. Mouse livers and spleen were dissociated into single-cell suspensions with gentleMACSDissociators (Miltenyibiotec) according to the manufacturer's instructions. Isolated spleens and livers were then prepared for flow cytometry by incubating cells with the appropriate Abs or control isotype–matched Abs followed by PBS washes. Flow cytometry experiments were performed with a FACSCanto (Becton-Dickinson, San Diego, CA) and analyzed by Flow-Jo software (Tree Star Inc., Ashland, OR)**.**

5.3 T cells purification, cultures and proliferation assay

Spleen cells were obtained from both strain of mice, dissociated into single-cell suspension, and cultured for proliferation assays in flat bottomed, 96- well microtiter plates (Falcon BD) at a density of 5×10^5 viable cells per well in a total volume of 200 μl RPMI-1640 medium (Invitrogen Corp.) supplemented with 2% FCS (Invitrogen Corp.), 2 mM L-glutamine (Invitrogen Corp.), 0.1 mM nonessential amino acids (Invitrogen Corp.), 1 mM sodium pyruvate (Invitrogen Corp.), 50 μM 2 mercaptoethanol (Sigma-Aldrich), and 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen Corp.). Cells were cultured at 37°C in 100% humidity and 5% CO2 in the presence or absence of either mouse anti-CD3/CD28 beads (Invitrogen Dynal) (0.5 bead/cell; 5×10^4 cells/well) in the case of highly purified T cells or with soluble anti- CD3 mAb stimulation (2C11, 0.5 μg/ml final concentration; BD Biosciences – Pharmingen) in the case of spleen-derived lymphocytes, being co-stimulation provided by the antigen-presenting cells (B cells) present in the assay.

Mouse conventional (Tconv) and regulatory T cells (Treg) were isolated with the Regulatory T Cell Isolation Kit (MiltenyiBiotec, Gladbach, Germany) and by AutomacsProeparator (MiltenyiBiotec) or by high-performance cell sorting (MoFlo, Dako). Cells were then cultured and stimulated as previously mentioned. The Treg:Tconv ratio in the suppression experiments was 1:1. After 48 hours cells were incubated for an additional 16 hours with 0.5 μCi/well of [^{3}H] thymidine (Amersham Pharmacia Biotech), harvested on glassfiber filters using a Tomtec (Orange) 96-well cell harvester, and counted in a 1205 Betaplate liquid scintillation counter (Wallac). Results obtained from triplicate cultures are expressed as mean c.p.m. ± S.E.M.

5.4 Cytokines measurement

Quantitative analysis IL-1α/β, IL-6, IL-17, INF-γ, monocyte chemoattractant protein 1 (MCP-1), macrophage inflammatory protein 1-alpha/beta (MIP-1 α/β), TNF-α, IL-10 on sera and cell supernatants were performed using the Bio-Plex multiplex Mouse Cytokine and Growth factors kit (Bio-Rad, CA, USA) according to the manufacturer's protocol.

5.5 Western blots and biochemical analyses

Total cell lysates and western blot analysis were performed as previously described (*De Rosa et al., 2007*). The antibodies used were the following: anti-p27Kip-1, anti-phospho-mTOR, anti-mTOR, anti-phospho-S6, anti-S6, anti-phospho-STAT5, anti-STAT5, anti-phospho-STAT3, anti-STAT3, anti-LC3B (all from Cell Signaling Technology, Beverly, MA); anti-ERK1/2, and anti-phospho-ERK1/2 (from Santa Cruz Biotechnology Inc., Santa Cruz, CA). The filters were also probed with a anti-actin antibody (Sigma) to normalize for the amount of loaded protein. All filters were quantified by densitometric analysis of the bands utilizing the program ScionImage (version 1.63 for Mac; Scion Corp. Inc.)

5.6 Metabolic assays and bioenergetic profiles

The metabolic profile has been evaluated in 12 hours-cultured purified T cells from *Prep1*$^{i/+}$mice and their littermate controls, in the presence anti-CD3/CD28 stimulation. Real-time measurements of oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were made using an XF-96 Extracellular Flux Analyzer (Seahorse Bioscience). Cells were plated in XF-96 plates (seahorse Bioscience) at the concentration of 2 x 10^5 cells/well and cultured for 12h in RPMI-1640 medium supplemented with 5% FBS serum. OCR was measured in XF media (non-buffered DMEM medium, containing 10 mM glucose, 2 mM L-glutamin, and 1 mM sodium pyruvate), under basal conditions and in response to 5 μM oligomycin, 1.5 μM of carbonylcyanide-4-(trifluoromethoxy)-phenylhydrazone (FCCP) and 1 μM of Antimycin and Rotenone (all from Sigma Aldrich). Indices of mitochondrial respiratory

function were calculated from OCR profile: basal OCR (before addition of oligomycin), ATP-linked OCR (calculated as the difference between basal OCR rate and oligomycin-induced OCR rate) and maximal OCR (calculated as the difference of FCCP rate and antimycin + rotenone rate). ECAR was measured in XF media in basal condition and in response to 10 mM glucose (from Sigma Aldrich). Experiments with the Seahorse system were done with the following assay conditions: 3 min mixture; 3 minutes wait; and 3 min measurement. Data are expressed as mean ±S.E.M.

5.7 Glucose tolerance test (GTT)

For glucose and insulin tolerance tests, mice were fasted overnight and then were injected with glucose (2 g·kg of body weight^{-1}) intraperitoneally. Venous blood was drawn by tail clipping at 0, 15, 30, 45, 60, 90, and 120 min without reclipping of the tails. Glucose levels were measured with an Accu-Chek glucose meter (Roche, Mannheim, Germany).

5.8 Statistical analysis

The unpaired *t*-test two tails or the Mann Whitney U test were used for unrelated two-group analyses, using GraphPad. For HFD experiments (body weight, serum TG, HLD, LDL, AST, ALT, GTT, ITT), two way Anova test was used to compare the mean differences between the different groups, followed by Sidak's multiple comparison tests. Results are expressed as mean ± S.E.M. *P* values < 0.05 were considered statistically significant.

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