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REVIEW

Regulatory T cells, mTOR kinase, and metabolic activity

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Abstract The field that links immunity and metabolism is rapidly expanding. Apparently, non-immunological disorders such as obesity and type 2 diabetes have been linked to immune dysregulation, suggesting that metabolic alterations can be induced by or be a consequence of an altered self-immune tolerance. In this context, a key role is played by signaling systems acting as metabolic "sensors" linking energy/nutritional status to regulatory T (Treg) cell functions. We propose that a dynamic/oscillatory activity of intracellular metabolism, through mTOR modulation, might represent a shift in understanding the molecular mechanisms governing Treg cell tolerance. In particular, the decision between Treg cell proliferation and hyporesponsiveness arises from their ability to probe the extracellular milieu and, modulating the metabolic intracellular signaling, to determine different qualitative and quantitative functional outcomes.

Keywords Metabolism · Immune tolerance · mTOR · Treg cells

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Immune system and metabolism: a common route in the control of immune tolerance

The question of self-non-self discrimination, and thereby immunological tolerance, is as old as our appreciation of the receptor diversity in the immune system. Initially, negative selection of self-reactive lymphocytes by clonal deletion in primary lymphoid organs, enhanced by promiscuous expression of ectopic antigens in these organs, attracted attention. It became clear, however, that such central deletion mechanisms were some of many used to achieve hyporesponsiveness. Additional "recessive mechanisms", such as deletion and/or anergy in the periphery, were also discovered, but it became clear that even these additional mechanisms were insufficient to account for self-tolerance and that "dominant immune regulation" played a major role. The discovery of the transcription factor forkhead box P3 (FoxP3), as the master switch enabling regulation, has lead to the identification of regulatory T (Treg) cells, whose role is suppression of the functions of other adaptive and innate immune cells [1, 2]. These cells play a central role in the control of autoreactive clones and the major characteristic of Treg cells is that they are naturally anergic in vitro to T cell receptor (TCR)-mediated activation and their suppressive function is closely related to this state [3, 4]. For these reasons, Treg cells are difficult to expand in vitro, an aspect that limits their potential clinical application in autoimmunity and transplantation.

Recent evidence has shed fundamental insights concerning the emerging frontier of immunometabolism in the context of self-non-self discrimination. It has emerged that certain supposedly non-immune disorders such as obesity, type 2 diabetes, metabolic syndrome, and atherosclerosis, implicate the pathological involvement of the immune

system in their pathogenesis; indeed a key role of adipokines and both innate and adaptive immune cells in the regulation of fat inflammation and glucose homeostasis has been recently proposed.

Further evidence for a pivotal role of inflammation in metabolic disturbances comes from clinical studies using either anti-inflammatory approaches or biological agents (i.e., blockade of IL-1R1 activation, inhibition of the NF-κB pathway with salicylate derivatives, metformin, thiazolidinediones, and statins) [5–10] that target specific pro-inflammatory cytokine pathways to improve parameters of glucose control, thus reducing evidence of systemic inflammation.

For example, metformin, which activates the AMP-activated protein kinase (AMPK), has anti-inflammatory properties as it inhibits T cell-mediated immune responses and the production of Th1 or Th17 cytokines, while inducing generation of IL-10-secreting Treg cells [7]. Statins—inhibitors of the 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA)-reductase—inhibit cholesterol synthesis and thus lower cholesterol levels but, more interestingly, they also dampen autoreactive immune responses by promoting the release of Th2 cytokines and by reducing pro-inflammatory cytokines such as leptin and TNF-α [10]. For these reasons, statins are widely utilized in type 2 diabetes and insulin resistance, and have been found highly effective as therapeutic agents for autoimmune disorders, such as experimental autoimmune encephalomyelitis (EAE).

On the one hand, it has emerged that these supposedly non-immune pathologies result in the involvement of regulatory T cells (Treg), on the other hand, Treg cell function is also controlled at different levels by internal metabolic processes. Since the local environment reflects the metabolic state of the host, Treg cells might need to be able to rapidly adjust their activity depending on what would be most expedient for the overall economy of the host. In this context, the adipocyte-derived hormone leptin, which reflects the metabolic/nutritional status of the host, can provide a "prototypic" example of the link among Treg cell metabolism, "external/environmental signals" (i.e., nutrient availability) and regulation of T cell tolerance [11–16]. In more recent times, dissection of the molecular underpinnings of the immune tolerance/metabolic cross-talk has become a priority, as testified by the growing number of reports related to this issue. Indeed, molecules known to be involved in the metabolic processes have been shown to be key players in the control of T cell tolerance. Of particular interest is the mammalian-Target-of-Rapamycin (mTOR), which together with the AMP-activated protein kinase (AMPK) and the Foxo-family proteins have been described as the dominant elements linking metabolism and self-tolerance at intracellular level [17–19].

Analyses of transcripts from particular immune subpopulations and cell-specific inactivation or overexpression of the proteins controlling these pathways should yield exciting discoveries in this emerging field and it is imperative to define whether mechanisms initiated by such metabolic shifts might serve as important therapeutic targets for the treatment of metabolic disorders.

Metabolic control of T cell functions

T cells switch between highly proliferative states (i.e., developing thymocytes and antigen-activated T cells) and quiescent states (i.e., naive, memory, and anergic T cells). These conditions are finely regulated by signals that, once delivered through T cell receptor (TCR) and cytokine receptors, induce the activation of different intracellular metabolic pathways [20]. For example, during T cell receptor (TCR) stimulation, signals from growth factors and cytokines such as Interleukin (IL)-2 or IL-7, together with ligation of co-stimulatory molecules, such as CD28, lead to an increase in glucose uptake and glycolysis through induction of phosphoinositide-3-kinase (PI3K)-dependent activation of Akt [21, 22]. This kinase promotes glucose metabolism by stimulating the localization of the glucose transporter Glut1 to the plasma membrane of T cells, thus facilitating glucose uptake. Overexpression of Glut1 leads to increased glucose uptake and glycolysis, and transgenic expression of Glut1 specifically in T cells determines increased T cell proliferation, survival, and cytokine production [23]. On the contrary, failure of effector T cells to properly upregulate glucose metabolism results in decreased cytokine production, proliferation, and can lead to apoptosis of these cells [24–26]. Finally, if T cells survive to this condition of metabolic stress, inhibition of metabolism during T cell activation can induce cell anergy [27]. Interestingly, the effects of Akt on glucose metabolism in lymphocytes are antagonized by the inhibitory receptor CTLA-4, suggesting that antagonists of T cell activation may function in part by disrupting glucose metabolism [20, 28, 29]. In this context, it is important to remind that T cells use glucose as their primary fuel source for generation of adenosine triphosphate (ATP) and it appears to be particularly necessary for cell survival, size, activation, proliferation, and cytokine production [30, 31].

T cell activation is also accompanied by an increased rate of protein synthesis, which supports cell growth and effector functions. Downstream of TCR and CD28, Akt controls the activation of the mammalian Target of Rapamycin, mTOR, a key regulator of protein synthesis in T cells [32, 33]. mTOR modulates the rate of protein synthesis by regulating both the availability of amino acids and the process of cap-dependent translation. Phosphorylation of the components of the translational machinery (i.e., the translation inhibitor 4E-BP1, the translation initiation

factor EIF2B, and the ribosomal p70 S6 kinase) by mTOR promotes the initiation of cap-dependent translation [34] and all these events are inhibited by rapamycin, a specific inhibitor of mTOR pathway (Fig. 1). The importance of the mTOR pathway for T cell activation is testified also by the finding showing that rapamycin induces a condition of immunosuppression, through the induction of a G1 cell cycle arrest in proliferating T lymphocyte. Although altered metabolic needs and activity certainly follow changes in signaling and proliferation rate, evidence is now emerging that the regulation of T cell metabolism is also intimately linked to T cell function and differentiation. In each stage of the life of a T cell, whether it is naive, activated, antigen-experienced, or anergic, cell metabolism must be matched to the function of that particular T cell. Is now evident that in addition to mTOR, several other metabolic pathway have been demonstrated to be involved in T cell function and in the control of T cell tolerance.

In this context several cytokines/hormones, such as leptin or adiponectin play a central role in the modulation of immune responses. Indeed, the latter induces the secretion of some anti-inflammatory cytokines, such as IL-10 and IL-1RA (receptor antagonist), by human monocytes, macrophages and dendritic cells and suppress the production of INF-γ [35]. Several data suggest that adiponectin is a negative T cell regulator. In particular, although a small percentage of T cells express adiponectin receptor (ADIPOR) on their surface, a great amount of T cells store ADIPORs within clathrin-coated vesicles and these receptors colocalized with Cytotoxic T-Lymphocyte Antigen 4 (CTLA4) molecules. After stimulation of T cells, the expression of both ADIPORs and CTLA-4 was upregulated. Interestingly, it was observed that the addition of adiponectin results in a significant diminution of antigen-specific T cell proliferation and cytokines production [36]. A paper by Tsang et al. [37] suggests that the immunomodulatory effect of adiponectin on immune response could be at least partially mediated by its ability to alter dendritic cell functions. Indeed, adiponectin-treated dendritic cells show a lower production of IL-12p40 and a lower expression of CD80, CD86 and histocompatibility complex class II (MHCII). Moreover, in co-culture experiments of T cells and adiponectin-treated dendritic cells, a reduction in T cells proliferation and IL-2 production and an higher percentage of $CD4^+CD25^+FoxP3^+$ Treg cells was observed [37], suggesting that adiponectin could also control regulatory T cell homeostasis.

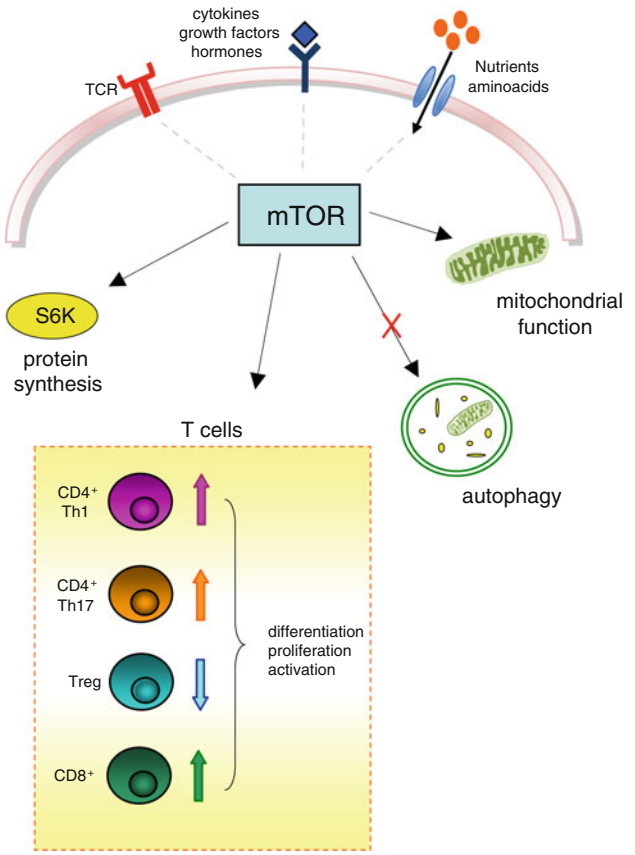

Fig. 1 Schematic model of the effects of mammalian target of rapamycin (mTOR) signaling in T cells. Environmental cues, including TCR and costimulatory molecule engagement (CD28), nutrients, growth factors, amino acids, insulin and different cytokines (IL-1, IL-2, IL-12, leptin) stimulate the activity of the phosphatidylinositol 3-kinase (PI3K) signaling cascade, promoting the activation of mTOR complex. mTOR exerts pleiotropic effects: acting through downstream S6K, it induces protein synthesis, survival, proliferation. mTOR sustains mitochondrial function, thus supporting the increase in the cellular metabolic function and inhibits the autophagic process. More interestingly in T cells, mTOR activity promotes both $CD8^+$ and $CD4^+$ effector T cell generation, activation and proliferation. Sufficient mTOR activity induces effector $CD4^+$ T helper subsets. On the contrary, a complete or strong block of the signaling (through rapamycin) prevents the generation of these effector cells, while it promotes Treg differentiation and the generation of memory cells in $CD8^+$ T cell compartment

mTOR role in the orchestrating of lymphocytes functions

mTOR is an evolutionarily conserved 289-kDa serine/threonine protein kinase inhibited by rapamycin [17, 38, 39], that directly influences T cell differentiation and proliferation by integrating environmental cues including nutrients, energy stores and growth factors [40–42]. Briefly, mTOR is activated in response to growth factors and cytokines and can modulate cellular metabolic pathways. This kinase operates in two distinct signaling complexes [43, 44]; mTORC1 contains the scaffolding protein Raptor, as well as the subunits mLST8, proline-rich AKT substrate (PRAS40) and Deptor [33]. Its activation is achieved through signaling by the kinases PI(3)K, 3-Phosphoinositide-dependent kinase 1 (PDK1) and Akt.

This complex promotes phosphorylation of the translational regulators S6 Kinase (S6K1) and Eukaryotic translation initiation factor 4E-binding protein-1 (4E-BP1) and is believed to have a central role in regulating cellular growth and proliferation, by modulating metabolic intracellular pathways, including glycolysis, the oxidative arm of the pentose phosphate pathway, and de novo lipid biosynthesis. This is also achieved through the activation of a transcriptional program affecting expression of the gene encoding for the transcription factor HIF1-α, and the sterol regulatory element-binding proteins (SREBP1 and SREBP2) [45, 46]. On the other side, mTORC2 consists of mLST8, the scaffolding protein Rictor, a critical adaptor protein for mTORC2, the subunits mSIN1 and Protor and regulates actin cytoskeleton, activating Akt through phosphorylation of Ser473 [44, 47]. mTORC2 has been shown to function as an important regulator of the cytoskeleton through its stimulation of F-actin stress fibers, paxillin, RhoA, Rac1, Cdc42, and protein kinase C α (PKCα) [44] and it appears to be regulated by insulin, growth factors, serum, and nutrient levels [47]. Originally, mTORC2 was identified as a rapamycin-insensitive entity, as acute exposure to rapamycin did not affect mTORC2 activity or Akt phosphorylation [44]. However, subsequent studies by Sarbassov et al. [48] have shown that, at least in some cell lines, chronic exposure to rapamycin, while not affecting pre-existing mTORC2s, promotes rapamycin inhibition of free mTOR molecules, thus inhibiting the formation of new mTORC2.

Besides its well-established role in T cell activation and proliferation, mTOR has recently been shown to serve a crucial function in the induction of anergy and recent data have reinforced this notion by showing that the anergic status in T cells is critically due to a failure of the Akt-mTOR pathway to upregulate nutrient transporters and activate glycolytic pathways, in the absence of an appropriate costimulus [33].

Data from literature suggest that there are multiple lineages of $CD4^+$ T cells that include helper subsets (Th1, Th2, Th17) and a regulatory subset (Treg) [49, 50] and emerging evidence suggests that mTOR activity regulates development and differentiation of these $CD4^+$ T cell subsets (Fig. 1). Delgoffe et al. have shown that mTOR-deficient $CD4^+$ T cells fail to differentiate into Th1, Th2 and Th17 subsets upon activation and this phenomenon was due to a decreased phosphorylation of STAT, as well as an insufficient induction of lineage specific transcription factors [51]. On the other hand, mTOR-deficient $CD4^+$ T cells rather differentiate towards a $FoxP3^+$ regulatory (Treg) phenotype [51] (Fig. 1). Moreover, another recent study by the same group has shown that mTORC1 and mTORC2 differentially regulate the generation of $CD4^+$ T helper subsets. More in detail, abrogating mTORC1 activity by deletion of Rheb, an upstream activator of mTORC1, resulted in a failure of $CD4^+$ T cells to develop to either Th1 or Th17 cells, while differentiation into Th2 remained intact upon in vitro stimulation. On the contrary, Rictor-deficient T cells, with the consequent block of mTORC2, differentiated into Th1 and Th17 cells but lost their ability to differentiate into Th2 cells [52].

During the past few years, considerable progress has been made in understanding the role of the mTOR pathway also in $CD8^+$ T cell responses. Indeed it has been shown that mTOR activity regulates trafficking of these cells by altering expression of cell surface receptors (CD62L and CCR7), which are important for the ability to home to secondary lymphoid tissues [53, 54]. Moreover, it is now well established the central role played by mTOR in memory $CD8^+$ T cell differentiation [55–61] (Fig. 1).

Rapamycin has long been considered an immunosuppressive agent due to its antiproliferative effects on immune cells, and it is currently used as a component of antirejection regimens in transplantation. Nevertheless, several paradoxes concerning rapamycin immunobiology still remain unsolved. In particular, emerging evidence suggests that under certain circumstances, rapamycin can exert immunostimulatory effects. Recently Araki et al. [60] have demonstrated that rapamycin treatment during the T cell expansion phase increased the quantity of memory $CD8^+$ T cells by increasing the number of memory precursor effector cells and by reducing apoptotic cell death during the contraction phase. These discoveries have implications for the development of novel vaccine regimens. The explanation for this phenomenon could be linked to the ability of rapamycin to enhance fatty acid oxidation (FAO) in responding T cells, thereby enhancing memory T cell differentiation, as the transition from glycolysis to FAO was recently shown to be critical for effector to memory transition in $CD8^+$ T cells [61]. Another possible mechanism by which rapamycin may augment the generation of T cell memory is decreasing the expression of T-bet, which is highly expressed in effector T cells, and promotes expression of Eomesodermin [59], which is highly expressed in memory T cells.

Recent reports have also shown that rapamycin can also enhance immune responses by modulating cytokine production. Macrophages and myeloid DCs (mDCs) treated with rapamycin produce larger amounts of IL-12 and less IL-10 upon stimulation with Toll-like receptor (TLR) ligands or bacteria compared with cells without rapamycin treatment [62, 63]. In addition, mTOR appears to regulate antigen presentation in macrophages and DCs by modulating autophagy [64], a lysosomal degradation pathway. Rapamycin-induced autophagy of DCs enhances the ability of the DCs to prime T cells in vitro [64]. Taken together, these data suggest that exposure to rapamycin may produce different outcomes depending on the cell cycle and metabolic state of a given cell or population.

Several groups have shown that the mTOR pathway is required for development and maturation of B cells, as well [65–67]. Indeed inhibition of mTORC2-dependent Akt activity, due to genetic Sin1 deletion, prevents B cell development [66] and similar results were obtained by Zhang et al. [65] who showed that inhibition of mTOR transcription increased the number of peripheral B cells with altered phenotypes and impaired function. Interestingly, hyperactivation of mTOR signaling also impairs B cell maturation, as indicated by the accumulation of immature B cells and the loss of marginal zone B cells in TSC1 (inhibitor of mTORC1) conditional knockout mice [67].

Unsolved issues in Treg cell biology: a key role for metabolism?

Regulatory T cells (Tregs) that express the transcription factor forkhead box P3 (FoxP3) are essential for the maintenance of dominant self-tolerance and the immune homeostasis [68, 69]. Tregs dysfunction (for example, owing to *FoxP3* gene mutation) can cause fatal autoimmune disease, immunopathology, and allergy [70–73]. $FoxP3^+$ Tregs, most of which are $CD4^+$ T cells that express high levels of CD25 (the interleukin-2 (IL-2) receptor α-chain), can suppress the activation, proliferation, and effector functions, such as cytokine production, of a wide range of immune cells, including $CD4^+$ and $CD8^+$ T cells, natural killer (NK) and NKT cells, B cells, and antigen-presenting cells (APCs) such as dendritic cells (DC) in vitro and in vivo [74–79]. This unique ability to control immune responses makes $FoxP3^+$ Tregs central players in keeping at bay autoimmune disease, immunopathology and allergy, as well as in maintaining allograft tolerance and fetal–maternal tolerance during pregnancy [1, 80–84]. In humans and mice, naturally occurring Tregs show classical surface markers of activated T cells and possess a highly proliferative profile in vivo. In striking distinction, Tregs are anergic in vitro as they fail to proliferate in response to T cell receptor (TCR) ligation. Classically, this in vitro condition is reversible upon TCR stimulation in presence of high doses of interleukin-2 (IL-2) in cultures. Rapamycin is an immunosuppressive drug that also promotes the expansion of Tregs in long-term cultures in the presence of supraphysiologic concentrations of IL-2 (1,000–2,000 IU/ml) [85, 86] (Fig. 1). This approach carries apparently an inherent paradox, since IL-2 activates mTOR, while rapamycin selectively inhibits mTOR [39]. Despite that Treg cells are hyporesponsive to antigenic stimulation in vitro, they have an intact proliferative potential, as testified by their highly proliferative profile in vivo. This dichotomous capacity has raised a number of unresolved issues as summarized below:

1. Why do Treg cells have a highly proliferative profile in vivo but are hyporesponsive/anergic to TCR-induced proliferation in vitro?
2. Why do Treg cells show an activated surface phenotype and why are they hyporesponsive?
3. Why do current strategies to improve their in vitro expansion need the addition of high doses of interleukin-2 (IL-2) (which mTOR kinase pathway) together with rapamycin, a specific inhibitor of mTOR kinase;
4. How can it be explained that rapamycin, which is a strong inhibitor of cell growth and proliferation (commonly used to block tumor cell growth and kidney transplant rejection), is used for expansion of already-hyporesponsive cells such as Treg cells?

These specific aspects of Treg cell biology can be partly explained in light of metabolic regulation.

The current view on Treg cell metabolism in the control of their anergic state has been considered often in a "static" manner, as a series of recent studies support a "conventional" and not "dynamic" view of Treg cell biology. More in detail, the definition of ex vivo "Treg cell anergy" must be clearly distinguished by that condition of T cell anergy artificially inducible in vitro (i.e., by TCR ligation in the absence of costimulation). Indeed, while an anergic T cell is characterized by functional inactivation and reduced mTOR activity (in line with what is known from the literature) [27], Treg cells, even though are in vitro hyporesponsive to TCR-stimulation, at the same time show an activated surface phenotype (i.e., high expression of activation surface markers such as CD25, CD39, CD71, CTLA-4), an active metabolic machinery as suggested by high amounts of ATP, cAMP, and leptin, an adipocyte-derived cytokine. Also, most of the knowledge on Treg cell biology comes from studies on either specific knock-out or transgenic models for intracellular molecules involved in the control of T cell metabolism. Despite that this approach is of great value, it shows intrinsic limitations, which are mainly ascribed to the possibility to activate "compensatory loops" related to the constitutive ablation of such metabolic-related pathways and to the lack of a "dynamic" control of different metabolic processes, which are strictly connected with Treg cell functions. From these considerations comes the need of simply more physiologic systems able to study how modulation of metabolic functions impacts the control of T cell tolerance.

The metabolic demands of Treg cells functions: a role for mTOR

Data emerging from the literature has revealed that Treg cells are characterized by a specific metabolic signature that governs their responsiveness to antigenic stimulations

[13, 15, 51, 87, 88]. It has recently been shown that effector T cells and Treg require distinct metabolic programs to support their specific functions. More specifically, Th1, Th2, and Th17 cells express high surface levels of the glucose transporter Glut1 and are highly glycolytic. Treg, in contrast, express low levels of Glut1 and have high lipid oxidation rates in vitro, thus suggesting that $CD4^+$ T cell subsets require distinct metabolic programs that can be manipulated in vivo to control Treg and Teff development in inflammatory diseases [89]. A very recent paper has also shown that blocking glycolysis promoted Treg cell generation, through the transcription factor hypoxia-inducible factor 1α (HIF1α), whose induction required mTOR pathway activation [90]. Confirming these results, Dang et al. [85] have shown that HIF-1 enhances Th17 development through direct transcriptional activation of RORγt, and concurrently, it attenuates Treg development, by binding FoxP3 and targeting it for proteasomal degradation. In addition, mice with HIF-1α-deficient T cells are resistant to induction of Th17-dependent experimental autoimmune encephalitis associated with diminished Th17 and increased Treg cells [91], thus suggesting again the importance of metabolic cues in T cell fate determination.

Recent data have also shown the involvement of mTOR in the differentiation of Treg cells. In this cellular subset, mTOR represents a negative regulator of TCR-dependent FoxP3 expression [92], of de novo Treg cell differentiation [93], and of Treg cell lineage commitment [51]. Several other reports provided further evidence for the key role of mTOR signaling in the control of Treg differentiation, by showing that PD1/PD-L1 interaction can, through inhibiting the Akt-mTOR axis, sustain the generation of Tregs [94], or that sphingosine 1-phoshate (S1P)-mediated activation of the Akt-mTOR axis impairs Treg cell suppressive activity [95].

The link between mTOR signaling and Treg cell differentiation and function has recently been extended in studies using rapamycin, which inhibits mTOR activity. As previously mentioned, Treg cells are in vitro anergic to TCR-mediated stimulation and their suppressive capacity is related to this state of hyporesponsiveness [3, 4]. Thus, Treg cells are difficult to expand in vitro, condition which limits their potential clinical application in autoimmunity and transplantation. However, Battaglia et al. [85] have shown that chronic addition of rapamycin, an inhibitor of mTOR, together with high-dose IL-2, was able to expand murine Treg cultures and consistently increases the yield of FoxP3-expressing cells with suppressive function. Similar findings were reported in studies using human Treg culture systems and in vivo administration of rapamycin preferentially preserved suppressive function in mice Treg cells [86].

Several hypotheses for the mechanism by which rapamycin regulates the expanding capability of Tregs have been developed. Strauss et al. [96] reported that Treg cells are resistant to rapamycin-induced apoptosis, since they upregulate the anti-apoptotic (bcl-2, bcl-xl), downregulate the pro-apoptotic (bad) proteins, and increase the expression of phosphatase and tensin homolog (PTEN). In another paper, Zeiser et al. [97], have also shown that Treg cells are more resistant than Teff to rapamycin treatment and the authors linked this condition to an increased usage of the signal transducer and activator of transcription 5 (STAT5) pathway over the PI3K pathway in response to IL-2 by Treg cells. This is in line with recent data indicating that STAT5 activation is sufficient to promote the IL-2Rβ-dependent development of $CD4^+FoxP3^+$ Tregs and that Stat5a/b directly regulate FoxP3 [98, 99]. More recently, it has been proposed that Tregs might be selectively expanded in the presence of rapamycin through the up-regulation of particular proteins such as Pim2, a serine/threonine kinase that overlaps functionally with Akt and mTOR, [100]. A different study suggested that rapamycin is able to induce a transient Treg phenotype in T effector cells [101]. However, these strategies to improve Treg expansion in vitro expansion are puzzling because they require high-dose IL-2 (which activates the mTOR pathway) together with rapamycin, which inhibits mTOR kinase activity. How can rapamycin, a strong inhibitor of cell growth and proliferation (commonly used to block tumor cell growth and kidney transplant rejection), induce Treg cell expansion in vitro?

Leptin-mTOR activity in the control of Treg responsiveness

There is a growing understanding of how host metabolism can affect the immune system. A recent study has described another important link between host energy status and immune function by showing that leptin, a hormone that is mainly produced by adipocytes and controls food intake and energy expenditure, can affect the generation and the proliferative capacity of Treg cells, acting as a negative signal for their own homeostasis [15]. Freshly isolated Treg cells produced leptin and expressed high amounts of the long-signaling form of the leptin-receptor (LepR); in vitro leptin neutralization during anti-CD3 and anti-CD28 stimulation resulted in marked Treg cell proliferation, thus confirming the negative control that leptin exerts on this cellular subset. Indeed chronic leptin- and leptin-receptor deficiency, are characterized by increased percentage, absolute number, and suppressive function of Treg cells that return to levels comparable to wild-type mice only after leptin replacement [15]. In an experimental model of atherosclerosis, supplementation of Treg-cell-deficient lymphocytes with Treg cells from *db/db* mice induces a

significant reduction of lesion size and a marked inhibition of interferon (IFN)-γ production, compared with supplementation by Treg cells from wild-type mice [102, 103]. Interestingly, in relapsing-remitting multiple sclerosis (RRMS) patients an inverse correlation between serum leptin and percentage of circulating Treg cells was also observed. Moreover, treatment of WT mice with soluble LepR fusion protein (LepR:Fc) increased the percentage of Tregs and ameliorated the clinical course and progression of disease in relapsing-experimental autoimmune encephalomyelitis (R-EAE), an animal model of RRMS [104]. Taken together, all these findings confirm an inverse correlation between leptin secretion or adipose tissue amounts and the frequency of Treg cells in physiologic and pathologic conditions. Taken together, these data indicate that leptin could be considered the molecular link between obesity and reduced number and probably impaired function of Treg cell observed in this condition.

Other recent evidence has suggested that leptin can activate the mTOR pathway to regulate the proliferative capacity of Treg cells [13]. In initial experiments conducted in vitro, which authors showed that freshly isolated human Treg cells, displayed higher mTOR activity and an increased metabolic rate compared to purified effector T cells. Although Treg cells do not normally proliferate in response to in vitro TCR stimulation, transient inhibition of mTOR, through pre-treatment with rapamycin, led to robust proliferation of Treg cells following culture with CD3- and CD28-specific antibodies, while rapamycin has opposite effects on effector T cells. Extending these findings in vivo, the authors found that a single injection of rapamycin promoted Treg cell proliferation in resting and immunized mice. Additionally, in a model of experimental autoimmune encephalomyelitis (EAE), mice treated with rapamycin before EAE induction showed increased frequencies of Treg cells and decreased disease severity [13]. Interestingly, although decreased mTOR activity appeared to be necessary for the initial phases of Treg cell proliferation, Treg cells that were actively proliferating in vivo expressed high levels of phosphorylated mTOR. In other words, early/transient inhibition of mTOR activity overcomes Treg cell anergy, by reducing the threshold required for a Treg cell to engage the molecular machinery required for proliferation (increased activity of mitogen-activated protein kinases [MAPKs], degradation of cell cycle inhibitors, recruitment of transcription factors), mTOR activity appears necessary to sustain Treg cell proliferation [13]. Indeed, continuous treatment with rapamycin or shRNA-mediated silencing of mTOR expression failed to reverse Treg cell anergy in vitro. As previous work showed that leptin can be produced by and inhibits the proliferation of Treg cells [15], the authors predicted that this molecule might interact with the mTOR pathway. In support of this, the addition of leptin to cultures of TCR-activated, rapamycin-treated Treg cells led to increased activation of the mTOR pathway and prevented Treg cell proliferation. In addition, neutralization of leptin markedly reduced mTOR activity in cultured Treg cells, suggesting that autocrine production of leptin by Treg cells may promote their high mTOR activity in vitro. Finally, the authors examined the effects of acute starvation (which markedly reduces circulating levels of leptin and immune function) on the mTOR pathway and Treg cell function. Strikingly, starvation led to increased proportions of Treg cells in peripheral lymph nodes. Furthermore, Treg cells from starved mice showed markedly reduced mTOR activity and increased rates of proliferation in vitro compared with Treg cells from control animals [13]. Taken together, this study describes the leptin–mTOR signaling pathway as an important link between host energy status and Treg cell activity. The authors conclude that oscillating mTOR activity is necessary for Treg cell activation and suggest that this may explain why Treg cells are unresponsive to TCR stimulation in vitro, where high levels of leptin and nutrients may sustain mTOR activation [13]. In conclusion, these findings may help to explain why Treg cells proliferate in vivo but are anergic in vitro, and why for the expansion of Treg cells in vitro there is the need of high doses of IL-2 (which activates mTOR kinase) together with the inhibitor of mTOR kinase rapamycin for long-term cultures [105]. As the nutrient/energy sensing leptin-mTOR pathway sets the threshold for responsiveness of Treg cells, we hypothesize that the proliferating Treg cells in vivo [106] can sense changes in the microenvironment when cultured in vitro, through the leptin-mTOR pathway, which makes Treg cells unresponsive to TCR-mediated stimulation. More specifically, the authors hypothesize that the high proliferative rate in vivo of Treg cells would associate with continuous dynamic, "oscillatory" changes in mTOR activity depending on the fluctuations in the composition of the extracellular milieu—including amounts of leptin and nutrients such as amino acids, glucose, and lipids. Alternatively, in vitro-cultured Treg cells, after isolation, would be exposed to a "static" milieu of the culture media characterized by constantly high concentration of leptin and nutrients [107], which could sustain mTOR activation and thus inhibit its dynamic, "oscillatory" changes required for Treg cell proliferation. The acute, transient inhibition of mTOR (either with rapamycin or nutrient starvation) in culture would reset in vitro the "oscillatory" fluctuations in mTOR activity rendering Treg cells able to respond to TCR stimulation. This would explain why constant mTOR inhibition, either with chronic rapamycin treatment or mTOR gene silencing, does not allow these dynamic changes and inhibits Treg cell expansion. When IL-2 at very high concentration is

provided in vitro, the lowering of mTOR activity would be reversed and allow the dynamic changes required for Treg cells to expand.

Metabolic overload and autoimmunity

It is well established from literature that in more affluent countries, where increased metabolic overload and obesity are more frequent, chronic inflammation, obesity, cancer and autoimmunity are more common [108–113]. In this context, we focused our attention on the processes which lead to break of self-tolerance associated with metabolic overload. A primary link has been provided by the notion that obesity and type 2 diabetes are now being considered closed to autoimmune/chronic inflammatory disorders rather than classically metabolic/endocrine dysregulation. This is because of the presence of abundant immune cell infiltrates in the adipose tissue of obese individuals is considered a classical pathologic lesion in obesity. Although, the significance of these infiltrates is currently unknown, they result directly or indirectly from the attraction of immune cells towards adipocytes, particularly those immune cells belonging to the natural immune system (macrophages, neutrophils, natural killer cells and dendritic cells) [114, 115]. Attraction of immune cells by adipose tissue mainly relies on the production of adipocytokines and chemokines by adipocytes. Recent data in mice suggest that T cells in the adipose tissue can clonally expand locally [116], while macrophage infiltration and Th1 cytokine secretion account for insulin-resistance and chronic inflammation. Moreover, also Treg cells have been found preferentially localized in the adipose tissue of normal individuals. Their role in this context is still object of extensive investigations, but interestingly they appear massively reduced in obesity, further suggesting their possible role to control an autoimmune attack against adipose tissue [117]. Furthermore, adipose tissue Treg cells express (and are thought to secrete) an unusually high amount of the anti-inflammatory cytokine IL-10, which in lean mice could help to suppress adipose tissue inflammation. Winer et al. [118] have also shown that Treg cells are able to protect against insulin resistance and hyperglycemia.

Several studies have shown that calorie restriction (CR) without malnutrition prevents many age-associated, chronic diseases and prolongs the lifespan of mammals. Indeed dietary restriction causes metabolic and physiologic changes that have beneficial effects against obesity, insulin resistance, inflammation, oxidative stress and cardiovascular diseases [119–122]. A recent study by Galgani et al. [123] shows that nutritional status, through leptin, directly affects survival and proliferation of autoreactive T cells, modulating the activity of the survival protein Bcl-2, the Th1/Th17 cytokines secretion, and the nutrient/energy-sensing AKT-mTOR pathway. This is in line with the epidemiological evidence that susceptibility to autoimmune diseases, in some circumstances, correlates with increased body fat mass and higher body weight at birth [124, 125]. Moreover, other studies published by Piccio et al. [126] and our group, have shown that either nutritional deprivation or CR are able to profoundly modulate and reduce magnitude and disease score during EAE and the survival of chronically food restricted mice is higher than ad libitum fed mice, suggesting that nutritional and metabolic state influence the break of self-immune tolerance. Similar results have also been obtained in mice where, chronic rapamycin treatment, increased significantly their overall survival [127]. The precise mechanisms for these results are still not fully elucidated but it is well known that rapamycin treatment is able to induce pharmacologically a "frugal phenotype" similar to that observed in CR animals. Indeed, rapamycin, through mTOR inhibition, is able to dampen the metabolic overload through reduction of absorption of amino acids, glucose and also, to dampen the level of a series of pro-inflammatory adipocytokines produced by adipocytes, including leptin [128]. Recent reports have also confirmed this evidence in mouse models of autoimmunity in which rapamycin has been shown to improve disease curse and progression, particularly EAE and type 1 diabetes, by dampening Th1/Th17 responses and increasing regulatory T cell responses [129–131]. The fact that chronic leptin deficiency (*ob/ob* mice) or rapamycin-induced leptin deficiency can reduce the survival of autoreactive $CD4^+$ T cells indicates that the nutritional status can control survival of potentially autoreactive $CD4^+$ T cells—through leptin/mTOR.

Drugs that target nutrient-sensing pathways to obtain the health benefits of dietary restriction are realistic, but the effects of chronic administration require further study. For instance, rapamycin, the TOR inhibitor that extends mouse life span, is an immunosuppressant and may not produce an overall health benefit in humans living in an environment with pathogens. More testing of potential disadvantages is required and many open questions remain, but these seem really promising drug targets. Notably, in consideration that nutritional deprivation or CR reduce EAE, it could be suggested that manipulation of the leptin axis and in general of the nutritional status, could represent a new means to modulate T cell tolerance in autoimmunity.

Concluding remarks

Living cells continuously adjust gene expression patterns in response to the changing environment. A simple way of encoding the presence of a stress or stimulus is to shift the concentration of a signaling molecule from one steady-state level to another. In this context, leptin and nutrients

(i.e., amino acids and glucose) show intermittently periodic behaviors and pulsatile secretion in vivo associated with an oscillating energy status. These changes are able to activate intracellular metabolic sensors such as mTOR and consequently control the in vivo homeostatic proliferation of Treg cells. A recent report has indicated a new mechanism of cross regulation of metabolism and immunity by which immune-related genes can be activated by Foxo under normal physiological conditions in response to the oscillating energy status [132] and therefore mTOR kinase activity represents a novel target in the context of metabolic regulation of Treg functions.

Accelerating interest in the area of immunometabolism is being fuelled by the finding that obesity affects the immune system and promotes inflammation, and that obesity-induced inflammation potentially promotes a variety of chronic conditions and diseases. Moreover, drugs that have long been considered to affect metabolism or insulin actions are also strong immune modulators that reduce proinflammatory cytokines and increase the number and function of Treg cells and Th2/regulatory-type cytokine release, all important in the control of autoimmunity. Drugs like rapamycin (a potent inhibitor of the mammalian target of rapamycin, mTOR), able to induce reduction of effector T cell function and Th1/Th17 cytokine secretion as well as increase in the number of regulatory T cells, has been shown to control not only immune cell reactivity but also blunt the leptin-induced hypothalamic responses, thus further suggesting an involvement of immune response in the pathogenesis of obesity. Therefore it is conceivable to hypothesize that oscillations of mTOR activity could be considered the result of a wider network of interactions between intracellular signals and mTOR pathway itself in response to physiological adaptations to environmental changes. The multilevel interactions between the metabolic and immune systems suggest pathogenic mechanisms that may underlie many of the downstream complications of obesity. In this context, the study of such connections and of the possibility to modulate the energy status of Treg cells, might offer substantial therapeutic promise in the control of immune tolerance.

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