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THE MOLECULAR BASIS OF HUMAN DISEASE

Impaired mitophagy in T regulatory cells is involved in breakdown of self-tolerance and development of autoimmune responses

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Introduction

Immune homeostasis

Immune homeostasis is a term used to describe a great number of mechanisms that co-operate to ensure that the number, diversity and function of lymphocytes is maintained, both during periods with and without inflammation. Under physiological conditions, homeostatic mechanisms act to maintain the balance between effector and regulatory cells, thereby ensuring efficient pathogen elimination while preventing immunopathology and autoimmune disease [1]. This homeostatic equilibrium is accomplished by a combination of soluble cytokine signals and interactions between various cell types. Among the different immune cell types, regulation of T cell homeostasis is the best understood. Studies in the last 2 decades demonstrated that T cells alter their homeostatic requirements as they emigrate as naïve T cells from the thymus to secondary lymphoid tissues, differentiate into T effector cells after antigen stimulation, and persist as memory cells [2].

As conventional $CD4^+$ T cells, most $CD4^+Foxp3^+$ regulatory T (Treg) cells are generated in the thymus, although they display a different T cell receptor (TCR) repertoire that is skewed for the high-affinity recognition of self-antigens [3]. Tregs have been shown to suppress effector T cells through multiple mechanisms of action, including the production of immunosuppression cytokines such as interleukin (IL)-10 or tissue growth factor-β (TGF-β) and via antigen-presenting cells (APCs) or direct contact with effector T cells [4]. In contrast with the detailed understanding of conventional T cell homeostasis, the mechanisms that maintain the functionally diverse Treg cell pool in various tissue sites remain poorly understood.

IL-2 was characterized as T cell growth factor which promotes the expansion of antigen-activated T cells in an autocrine manner. This cytokine is produced in secondary lymphoid tissues, mainly by activated $CD4^+$ and $CD8^+$ T cells [5]. IL-2 is consumed primarily by cells that express the high affinity form of IL-2 receptor. High affinity signaling is made by association of CD25 (also known as IL-2Rα), which increases the affinity of IL-2R for the cytokine by 10-100 fold. Signal transduction occurs through activation of both Janus kinase (Jak)/signal transducer and activator of transcription Stat pathway, as well as phospoinositide 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) pathways which are activated via phosphorylation of the signaling adaptor Shc [6]. Although any cell types including $CD4^+$, $CD8^+$ T cells and NK cells can upregulate CD25 expression upon activation, Foxp3 directly promotes CD25 expression

and consequently, Tregs are unique to constitutively express the high affinity IL-2 receptor. Treg cell homeostasis is dependent on both the levels of IL-2 production and the rate of IL-2 consumption under steady state conditions [1]. The important role of IL-2 in Treg cell homeostasis and development was revealed from studies on IL-2 or CD25 deficient mice. Surprisingly, these mice exhibited autoimmune manifestations and instead of immunodeficiency, that was expected, the animals also demonstrated massive lymphoproliferation, hyper-active T cell responses, splenomegaly and develop inflammatory disease within 6-8 weeks of life [1]. The apparent lack of Tregs in these knock out mice led to conclusion this autoimmune phenotype that was observed was due to decreased Tregs and their inability to control self-reactive, inflammatory responses. However, the role of IL-2 in controlling Treg cell numbers and activity is much more complicated as there are finding also indicating that peripheral homeostasis of Treg cells is at least partially IL-2 independent.

Treg cells in autoimmunity

One of the major issues in immunology is to understand how the immune system is able to discriminate between non-self and self, inhibiting autoimmune responses but also allowing the immune responses against foreign antigens. One of the mechanisms that is involved in self-tolerance is the T-cell mediated active suppression of self-reactive lymphocytes [7],[8]. Recent years, there has been accumulating interest in regulatory T cells (Tregs) in many fields of immunology. This interest is based on the improved understanding that normally the immune system produces this $CD4^+$ T cell subpopulation which is specialized for suppressive function and that an abnormality in the function or number of these cells can be the primary cause of autoimmune and other inflammatory diseases [8]. The recognition of regulatory cells, originally termed as suppressor T cells, resulted from experiments performed on the 1960s and 1970s, which demonstrate the induction of suppressor T cells capable of down-regulating antigen-specific T cell responses [9].

Most, if not all, Treg cells are produced by the thymus as a functionally distinct and mature T cell population and cannot be induced from naïve T cells by antigen exposure in the periphery [8]. These endogenous (natural), best characterized, Treg cells express the CD25 molecule (IL-2 receptor α-chain) [10], but also they specifically express Foxp3 (forehead box P3), which is a transcription factor critical for their development and function [11]. The $CD4^+Foxp3^+CD25^+$ Treg cells are essential for preventing multiorgan autoimmunity throughout lifespan [8], [12]. The importance of Treg cells is exemplified by patients with the immunodeficiency syndrome IPEX (‘immunodysregulation polyendocrinopathy enteropathy X-linked’) and mice of the scurfy strain, each of which

lack functional Foxp3 and suffer from severe systemic autoimmunity [13],[14]. Interestingly, numbers of circulating Treg cells appear to be mostly normal in patients with autoimmunity. $CD4^+$ $Foxp3^+$ Treg cell numbers from the peripheral blood of patients with multiple sclerosis (MS) and type I diabetes (T1D) demonstrated no difference compared to heathy subjects [15],[16]. However, most studies of patients with systemic lupus erythematosus (SLE) showed decreased numbers of circulating Treg cells that was correlated with disease activity [17],[18]. These findings are limited only to peripheral blood of patients and this is not necessary indicative of the Treg abundance at the sites of inflammation. For example, Treg numbers were increased in the synovial fluid of rheumatoid arthritis (RA) patients and in the lamina propria of patients with inflammatory bowel disease (IBD) [19],[20]. Nevertheless, in many cases Treg cells isolated from autoimmune patients exhibit functional defects in *in vitro* suppression assays [1].

Treg metabolism

The interest on the field of immunometabolism is growing at an exponential rate and focuses on distinguishing the metabolic pathways of T cell subsets. This idea is of great importance as it reveals the exciting possibility of targeting and regulating the function of specific cell subsets.

Resting T cells are metabolically inert and require little energy generation or expenditure to fulfill their demands. However, upon activation, their energy needs increase substantially. Most initial studies of T cells have focused on naïve T cells, effector T cells (T_{eff} cells) and memory T cells (T_{mem} cells), which have both shared distinct and shared metabolic features. For this reason, increasing attention has been focused on regulatory T cells (T_{reg} cells), as these cells have their own signaling and metabolic "signature" that can dictate and drive their function and stability [14].

Upon activation, naïve T cells that differentiate towards T_{eff} cell lineage shift from catabolic metabolism to an anabolic state. This is driven predominantly by the use of aerobic glycolysis, known as the "Warburg effect", which is orchestrated via the mTOR-dependent nutrient-sensing pathway [21],[22],[23]. As an immune response resolves, cells that remain and/or transit into the memory pool shift to a catabolic state and rely mainly on lipid oxidation which is promoted by increased mitochondrial biogenesis and regulated by signaling via the AMP-activated kinase AMPK [24]. Thus, $CD4^+$ T_{eff} cells are dependent on glucose transporter Glut1 and "Warburg effect" for proliferation and inflammatory functions, fact that was proven by inhibiting glycolysis or by deletion of Glut1 leading to impaired Teff cell function *in vivo* [25]. In contrast, Tregs cells act

independently of Glut1. These cells have been shown to utilize mainly a distinct metabolic program based on mitochondrial oxidation of lipid and pyruvate. Although Tregs play a crucial role in autoimmunity, inflammation resolution and healing, the mechanisms that govern Treg metabolism and how these pathways affect their proliferation and function remain poorly understood [26].

Autophagy

Autophagy is a general term that describes the process via which cytoplasmic material and organelles reach lysosomes for degradation. Among the three types of autophagy, which are the macroautophagy, microautophagy and chaperone-mediated autophagy, macroautophagy is the most extensively studied [27]. Macroautophagy (hereafter referred as “autophagy”) constitutes a multi-step process through which organelles or portions of cytoplasm are engulfed within double-membrane vesicles, known as autophagosomes. Autophagosomes mature and fuse with lysosomes, so called autophagolysosomes, in order to be degradated [28]. In more detail, upon induction of this pathway, isolated membrane called phagophore elongates and encloses the cargo, which results in the formation of autophagosome. The outer membrane of the autophagosome fuses with the lysosome, leading to the degradation of the enclosed materials [27] **(Figure A)**. Autophagy not only maintains cellular homeostasis in an environment of endogenous distress [29] but also plays a pivotal role in controlling immune responses [30]. Thus, autophagy is upregulated when cells are exposed to dangerous environmental cues, such as physical, chemical, or metabolic and therefore constitutes a universal response to stress [29]. Moreover, defective autophagy is associated with diverse range of disease states, including neurodegeneration, cancer and autoimmunity [31].

The execution of autophagy requires a set of gene products known as the Atg (Autohagy-related) proteins. More than 30 Atg proteins are conserved from yeast to mammals and represent the core machinery of the membrane dynamics that drive autophagy [32]. Autophagy induction is regulated with the essential autophagy gene Beclin-1 (yeast Atg6) and the activity of class III PI3K. Two ubiquitin-like conjugation pathways involving the Atg3, Atg5, Atg7, microtubule-associated protein light chain 3 (LC3), Atg10, and Atg12 are required for the formation of the autophagosomes [33]. The identification of the autophagy machinery has facilitated the detection of autophagy, through LC3-based assays, as well as the ability to manipulate the autophagy pathway, through knockout of specific genes or the expression of dominant negative proteins for the pathway. The importance of autophagy is best demonstrated in mice lacking Atg5 and Atg7, which succumb to starvation as neonates [34],[35]. The autophagy pathway

can also be experimentally manipulated with agents that regulate autophagosome formation or the subsequent degradation steps [27] (**Figure B**).

Within the field of immunology, there is an expanding role for autophagy, in both innate and adaptive immune system [36]. Autophagy is required for the clearance of intracellular pathogens, as well as the MHCII cross-presentation of endogenous antigens [28],[37]. The role of autophagy has also been demonstrated in T lymphocytes [38],[39]. Evidence reveal a complex role for autophagy in T cell survival and function, however, the exact functional consequences of autophagosome formation in T cell has not been fully understood [39]. Treg specific deletion of Atg5 or Atg7 leads to compromised stability of Treg cell lineage [40]. Autophagy plays also a pivotal role in immune tolerance. Self-antigen presentation on MHC-II of both thymic epithelial cells and dendritic cells can lead in the maintenance of central and peripheral tolerance in the $CD4^+$ T cell compartment. Supportingly, thymic epithelial cells demonstrate great amount of autophagosomes under steady state [41] and more autophagosomes are found in newborns that in adult mice. These findings associate with the notion that T cell selection and central tolerance of $CD4^+$ T cells are most active at young age [42]. Moreover, autophagy is upregulated in immature dendritic cells [37], which are implicated in peripheral tolerance induction [43].

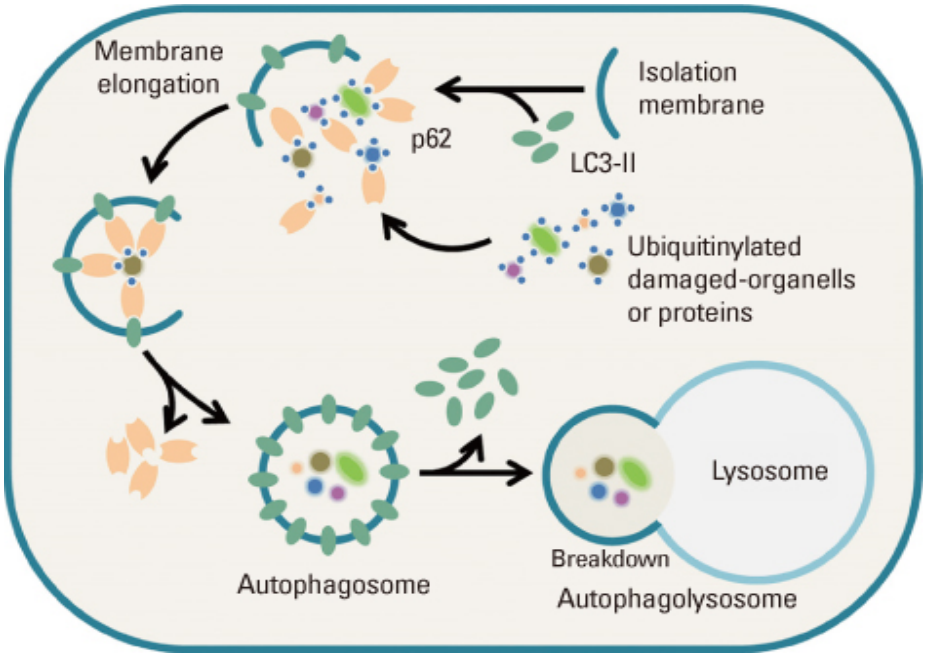

Figure A: Schematic diagram of the steps of autophagy

Autophagy is initiated by the formation of the isolation membrane. Ubiquitinylated proteins may be directly targeted for degradation via the autophagic pathway. The p62 protein interacts with ubiquitinylated-damaged proteins in cells. The complex is then selectively tied to the autophagosome through the interaction between p62 and light chain 3-II (LC3-II). When the outer membrane of the autophagosome fuses with a lysosome, it forms an autophagolysosome. Finally, the sequestered material is degraded inside the autophagolyosome and recycled.

mTOR Signaling

The regulation of autophagy is governed by the mechanistic target of rapamycin (mTOR) pathway, which negatively regulates this process (**Figure B**). mTOR is a serine/threonine protein kinase that mediates vital cellular function such as translation, cell growth and metabolism [44]. This pathway involves two distinct complexes: the mTORC1 (mTOR complex 1), which contains the scaffolding protein RAPTOR (regulatory associated protein of mTOR) and is sensitive to the immunosuppressant rapamycin, and mTORC2 which has a distinct scaffolding protein, RICTOR (rapamycin-insensitive companion of TOR) [45]. Starvation inhibits mTORC1 activity, therefore autophagy can be induced in order to recycle intracellular components to provide a source of energy. Inhibition of mTORC1 and subsequent induction of autophagy is correlated with reduced phosphorylation of two mTORC1 downstream effectors: the pS6K (ribosomal protein S6 kinase-1) and 4E-BP1 (translation initiation factor 4E-binding protein-1) [33], [44].

Emerging evidence highlight that mTOR pathway also direct T-cell fate decisions by integrating diverse environmental inputs, such as immune signals and metabolic cues [45]. Whereas the major responsibility of mTOR in T cells is the promotion of cell cycle progression, studies have also established mTOR pathway as a fundamental determinant for cell fate decision both under steady-state and after antigen recognition. This pathway seems to affect diverse processes in T cells, including immune receptor signaling, metabolic programs, and migratory activity. Although mTOR is thought to deliver essential signals for effector $CD4^+$ T cell activation and differentiation, is acknowledged as a crucial negative regulator of Treg cell differentiation as mTOR inhibition induces *de novo* Foxp3 expression and expansion of preexisting natural Treg cells. On the other hand, is has been shown that mTORC1 is fundamental for Treg suppressive activity. Collectively, it is obvious that this pathway may play an important but also complex role in immune tolerance [46].

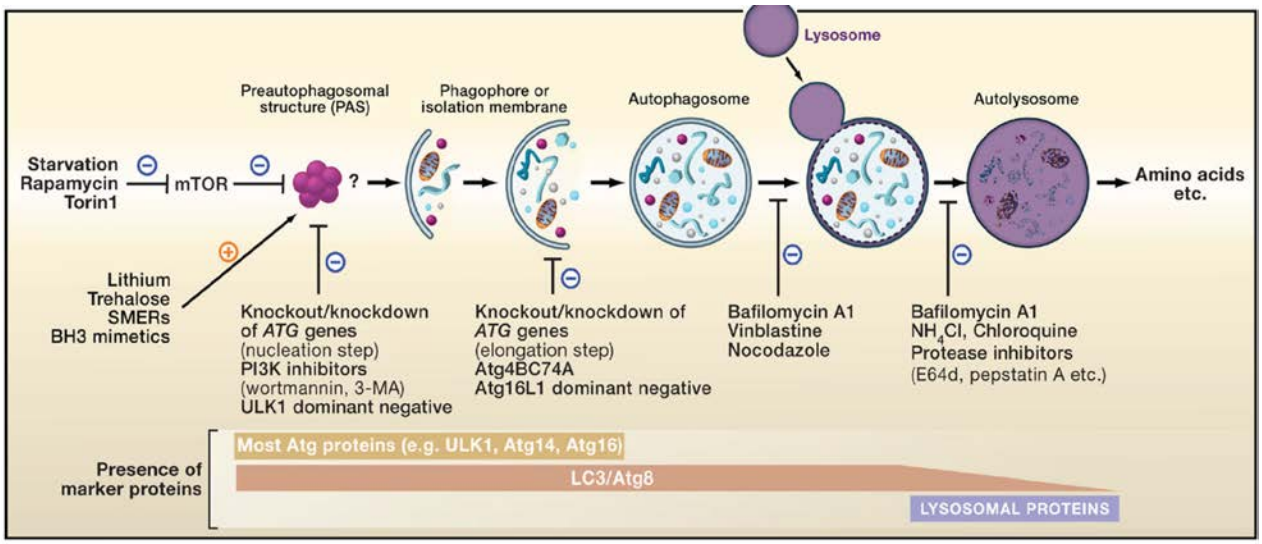

Figure B. The Process of Macroautophagy

A portion of cytoplasm, including organelles, is enclosed by a phagophore or isolation membrane to form an autophagosome. The outer membrane of the autophagosome subsequently fuses with the lysosome, and the internal material is degraded in the autolysosome. In yeast, autophagosomes are generated from the preautophagosomal structure (PAS), which has not yet been identified in mammalian cells. A partial list of treatments and reagents that modulate autophagy are indicated. Notably, lithium may also inhibit autophagy through mTOR activation. Atg proteins that have thus far been identified on isolation membranes include ULK1/2, Atg5, Beclin 1, LC3, Atg12, Atg13, Atg14, Atg16L1, FIP200, and Atg101.

Mitochondrial role

After symbiosis of proteobacteria in pre-eukaryotic cells, mitochondria became essential for eukaryotic cells. Functional mitochondria are critically important in many facets of cellular function, integrity and survival [47]. They not only produce ATP though electron transport chain (ETC) system, but also participate in many cellular metabolic pathways such as lipid metabolism, amino acid synthesis, iron-sulfur cluster biogenesis, and regulation of apoptosis [48]. However, oxidative phosphorylation (OXPHOS) in mitochondria, which is responsible for the supplementation of cellular ATP and the control of cell homeostasis, is also associated with the production of reactive oxygen species (ROS) that damages lipids, proteins and mitochondrial DNAs [49]. Excessive ROS production will disrupt the mitochondrial membrane potential, eventually leading to abnormal cell function and cell death [50]. Therefore, dysfunctional mitochondria, with impaired membrane potential must be properly eliminated.

Mitophagy

Given the pivotal role of mitochondria, their amount and activity need to be tightly controlled in order to properly adapt the cells to the energy metabolic status. Therefore, it is critical for the cells to clear dysfunctional mitochondria in a timely manner [49].

Mounting evidence implicates the autophagy process in mitochondria removal, so called mitophagy [51]. Significant progress has been made in identifying how the mitochondrial serine/threonine kinase PINK1 and the E3 ligase Parkin cooperate to eliminate damaged mitochondria, a mechanism that relies on the orchestrated crosstalk between ubiquitin phosphorylation signaling and autophagy [48]. This consists a multistep signaling event that culminate in engulfment of damaged mitochondria within autophagosomes and degradation by lysosomes.

PINK1 acts as a molecular sensor of mitochondria health by constantly surveying mitochondrial status until it detects damage. After loss of mitochondria membrane potential (Δψm), PINK1 accumulates on the outer mitochondria membrane (OMM) where it signals for the recruitment and activation of Parkin [47]. Ubiquitin that is linked to proteins on the OMM can be used by PINK1 as a substrate to initialize the pathway. So, after accumulating on damaged mitochondria, PINK1 phophorylates OMM ubiquitin at S65 (pS65-Ub). Parkin was shown to exhibit high affinity for pS65-Ub and this drives Parkin recruitment from the cytosol to the OMM [52]. PINK1 also phosphorylates Parkin at S65 of the N-terminal ubiquitin-like (UBL) domain leading to the activation of Parkin's ligase activity [53],[47]. Once fully activated, Parkin conjugates ubiquitin chains on OMM proteins, which provide more substrates to PINK1 to phosphorylate and leads subsequent rounds of recruitment and activation [47] (**Figure C**). Mitophagy is most commonly induced in vitro using the uncoupler CCCP (carbonyl cyanide chlorochenyl hydrazone), which disrupts/reduces the mitochondrial membrane potential (Δψm) [54].

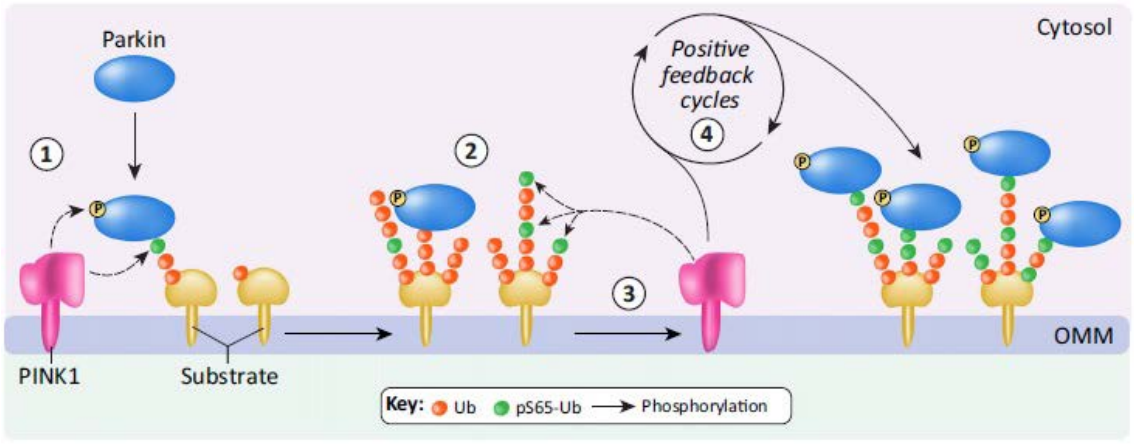

Figure C: Rapid Parkin recruitment for induction of mitophagy
To recruit and activate Parkin, PINK1 mediates S65 phoshorylation of both Parkin and ubiquitin linked to outer mitochondrial membrane (OMM) proteins (1). Activated Parkin conjugates ubiquitin onto OMM proteins (2), thereby providing more substrate for PINK1 to phosphorylate (3). This additional ubiquitin phosphorylation by PINK1, drives more rounds of Parkin recruitment and ubiquitination (4). These events lead to a positive feedback cycle that continues, resulting in rapid recruitment and activation of Parkin that amplifies the molecular signals and drive mitophagy [47].

Multiple Sclerosis and Experimental Autoimmune Encephalomyelitis

Approximately 2.5 million people worldwide suffer from multiple sclerosis (MS), a chronic neuroinflammatory disease of the spinal cord and brain, which is a common cause of serious physical disability in adults [55]. The average age of disease onset is 30 years and 25 years after diagnosis, 50% of patients require permanent use of a wheelchair [56]. MS patients are grouped into three categories: 85% are initially diagnosed with a relapsing-remitting disease and of which 50-60% progress to secondary progressive MS. A percentage about 10% present primary progressive MS, which symptoms are exhibited early and worsen without remission [57]. The clinical manifestations are heterogenous and include visual and sensory disturbances, fatigue, pain, motor impairments and cognitive deficits [55]. The variation in the presentation of the condition correlates with the spatiotemporal dissemination of lesion sites of pathology within the central nervous system (CNS) [58]. These lesions are caused by immune cell infiltration across the blood-brain barrier (BBB) which promotes inflammation, demyelination, gliosis and neuroaxonal degeneration, leading to impaired neuronal signaling [59]. The disease is considered to be autoimmune, initiated by

autoreactive T cells that respond against CNS autoantigens, the nature of which remains unknown. Specifically, T lymphocytes directed against myelin proteins play a crucial role in the pathogenesis of MS. This notion is proved not only by studies in animal models but also from evidence obtained from MS patients [60].

The animal model used for the study of MS pathogenesis is the well-characterized mouse model, called experimental autoimmune encephalomyelitis (EAE), which closely resembles the disease [61]. EAE is induced in susceptible mice upon immunization with one a number of myelin antigens emulsified in complete Freund's adjuvant along with intraperitoneal injection of pertussis toxin. Upon entering the CNS, T cells are reactivated by activated antigen presenting cells (APC), which present major histocompatibility complex (MHC) class II-associated myelin peptides, leading to inflammatory processes and subsequent demyelination and axonal damage [61]. Depending upon the strain of the mouse used and the immunization protocol, EAE may be acute, chronic progressive or relapsing-remitting, which consist the 3 types of MS disease [62]. The clinical characteristics of EAE include paralysis that starts from the tail and hind legs and then progresses towards the upper limbs. Importantly, EAE can also be induced by adoptive transfer of activated myelin specific $CD4^+$ T cells into naïve syngeneic hosts indicating that this autoreactive T cells are necessary and sufficient to induce EAE [60].

The cascade of event leading to MS pathogenesis is initiated by the activation of myelin specific T cells in the periphery after recognition of myelin antigens presented by APCs. Upon activation, T cells exit from the lymphoid organs and migrate to the CNS by crossing through the BBB. Into the CNS, local APCs present myelin peptides to activated T cells, triggering their effector function. Consequently, T cells proliferate and produce inflammatory molecules including cytokines, such as interferon (IFN)-γ, tumor necrosis factor (TNF)-α, IL-2 and IL-17, and chemokine. The two major pathogenic effector T cell subsets in MS are the T helper type 1 (Th1) and Th17 cells [60]. Th1 are the main producers of IFN-γ, which was found in increased levels in the spinal cord of EAE mice and in the cerebrospinal fluid of MS patients [63]. However, studies have demonstrated that IFN-$\gamma^{-/-}$ mice that lack Th1 cells can also develop EAE, indicating that these cells are not the only pathogenic cells responsible for the pathogenesis of the disease. In addition to this, a distinct $CD4^+$ T cell subset was described as Th17 cells due to their secretion of IL-17. These cells also produce IL-21 and TNF-α, which contribute to the development of MS [60].

Once self-reactive T cells escape in the periphery, additional mechanisms of tolerance must be functional to respond. Autoimmunity is thought to develop upon disruption of any of these mechanisms. As already mentioned, Tregs play a crucial role in maintaining immune homeostasis and self-tolerance, and their dysfunction is associated with the

appearance of multiple autoimmune diseases, such as Type 1 diabetes, psoriasis, GBS, and others [64]. Although it is not frequently reported that MS patients acquire significant differences in the number of circulating Treg cells, compared to healthy controls, Tregs from these patients seem to have lower suppressive capabilities. This indicates that functional defect in these cells may contribute the pathogenesis of MS [65],[66]. Moreover, a genetic analysis of over 14.000 MS patients reveled abnormalities that are involved in Treg IL-2 signaling, CD25 and CD127 [67]. The dysfunction of Treg was also correlated with decreased expression of Foxp3 [68] and more importantly brain biopsies from MS patients showed that 30% of lesions lacked Foxp3 expression Furthermore, the biopsies revealed high levels of a cellular apoptotic pathway receptor, Fas, on Tregs, which indicates increased susceptibility to apoptosis [69]. More evidence for the role of Tregs in MS development has been obtained from the EAE mouse model. Adoptive transfer of $CD4^+$ $CD25^+$ Tregs reduced the onset and the severity of the disease in MOG-immunized mice, revealing their protective effects which appear to be mediated by IL-10, as IL-$10^{-/-}$ Tregs failed to ameliorate the disease [70],[71]. In addition to this, administration of anti-CD25, which results to Treg depletion, lead to increased severity of EAE and mortality [72]. Tregs has also been shown to play an important role in the remission of EAE. Tregs increase in the CNS at the peak of the disease, but are unable to suppress autoreactive T cells due to high levels of inflammatory mediators [73]. Collectively, Treg cells have the potential to suppress myelin specific T cell responses, but might be restricted in the CNS or undergo apoptosis.

Materials and Methods

A. Mice

Foxp3gfp.KI and *Foxp3cre*.KI mice (C57BL/6 background), kindly provided by Dr. Alexander Rudensky (Department of Immunology, Memorial Sloan-Kettering Cancer Center, New York, USA), *CD4cre*.KI were obtained from the Jackson Laboratory and *Atg5*$^{fl/fl}$ mice kindly given from Dr. Noboru Mizushima (Department of Biochemistry and Molecular Biology, Graduate School and Faculty of Medicine, University of Tokyo, Tokyo, Japan). All procedures were in accordance to institutional guidelines and were approved

by the Greek Federal Veterinary Office. All mice used in the experiments were female 6-10 weeks old.

B. *In vivo* immunization protocol

Mice were immunized by subcutaneous (s.c) injections at the base of the tail, with 100 μg MOG_{35-55} emulsified (1:1) in complete Freund's adjuvant (CFA, Sigma Aldrich) and sacrificed 9days post-injection. For the experimental autoimmune encephalomyelitis (EAE) induction: mice were immunized with 200 μg Mog_{35-55} emulsified (1:1) in CFA, plus 4 μg/ml Mycobacterium. Emulsion was injected s.c over three sites (i.e. 50 μl per site): one along the midline of the back between the shoulders, and two on either side of the midline on the lower back. Additionally, mice received intraperitoneally (i.p.) 300 ng pertussis toxin (Sigma Aldrich), on day 0 and day 2, in order to break down the blood-brain barrier, as shown below (**Figure D**):

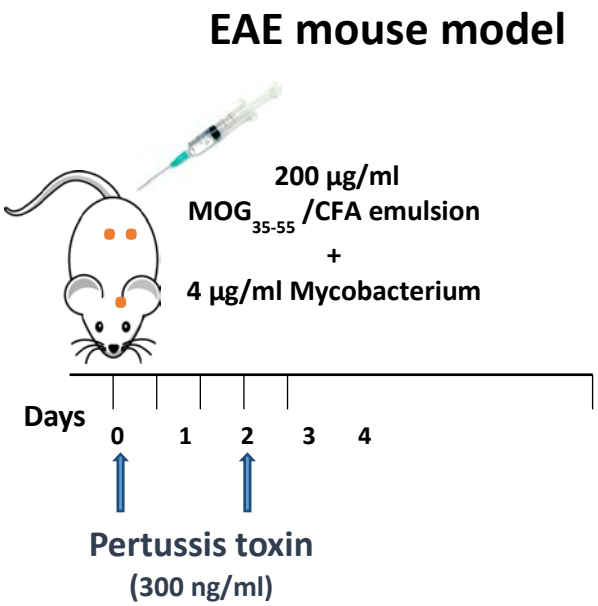

Figure D: Protocol of EAE induction in mice.

Red dots indicate the 3 sites of the emulsion subqutaneous injections. Pertussis toxin is injected intraperitonially.

Progression of the disease was evaluated daily according to the grading system for clinical assessment of EAE [74]:

Score 0: Normal mouse; no overt signs of disease

Score 1: Limp tail or hind limb weakness but not both

Score 2: Limp tail and hind limb weakness

Score 3: Partial hind limb paralysis

Score 4: Complete hind limb paralysis

Score 5: Moribund state; death by EAE: sacrifice for humane reasons

Mice were sacrificed when the disease scored 4 and their cervical LNs were isolated.

C. Cell sorting

For isolation of Teff and Treg cells, mice LNs were isolated. Cells were stained with conjugated antibodies to mouse: CD4 (GK15), CD25 (3C7), GITR (DTA-1) (Biolegent). $CD4^+$ $Foxp3^+$ and $CD4^+Foxp3^-$ or $CD4^+CD25^+$ and $CD4^+CD25^-$ cells were sorted on a FACS ARIA III (BD Biosciences). Cell purity was above 95%.

D. Flow cytometry

Single-cell suspensions were prepared from tissues and live cells (7AAD, BD Biosciences) and were stained with conjugated Abs to mouse CD4 (GKI.5), CD25 (3C7), GITR (DTA-1; BioLegend), pAkt (S473, SDRNR), pS6 (S235/236, cupk43k), p4E-BP1 (T36/T45, V3NTY24; eBioscience Inc.). Cells were also stained with TMRE (ENZ-52309), Mitosox (M36008), Mitotracker (M22425), Lysotracker (L7528) in concentrations of 100 nM, 5 μM, 100 nM and 100 nM respectively and JC-1 (1:100, eBiosciences). Dyes were added to the cells in the well plate, and after incubation according to manufacturer's protocol, cells were centrifuged at 1.800 rpm, for 7 minutes, at 4 ^oC. After centrifugation, cells re-suspended in 5% PBS-FBS (Fetal Bovine Serum) and transferred in flow cytometry tubes. Then, the cells were subjected to flow cytometry in FACS ARIA III (BD Biosciences). Analysis was performed with FlowJo software.

E. *In vitro* co-culture experiments for mitophagy assesment

For proliferation and death assay, $1x10^6$ sorted Treg cells ($CD4^+$ $Foxp3^+$ or $CD4^+CD25^+$) were labeled with the division-tracking dye CellTrace (C34557) according to the manufacturer's protocol, then co-cultured with beads coated with monoclonal antibody (mAb) to the invariant signaling protein CD3 plus mAb to CD28, at a ratio of 1 bead per 4

cells (11456D), in the presence of IL-2 (5.000 U/ml) and treated either with 2,5 μM CCCP (Tocris Bioscience) or with its control Dimethyl Sulfoxide (DMSO). After 96 h cells were stained (7-AAD) and subjected to FACS.

- Proliferation protocol in brief: Tregs + aCD3/aCD28 beads + IL2 + CCCP or DMSO

For induction assay, $1x10^6$ sorted Teff cells ($CD4^+$ $Foxp3^-$ or $CD4^+CD25^-$), labeled with the division-tracking dye CellTrace (C34557) according to the manufacturer's protocol, co-cultured with beads coated with mAb to the invariant signaling protein CD3 plus mAb to CD28, at a ratio of 1 bead per 4 cells (11456D), in the presence of IL-2 (20 U/ml) and 2ng/ml TGF-b, and treated as above either with 2,5 μM CCCP (Tocris Bioscience) or with DMSO. After 96 h cells were stained (7-AAD) and subjected to FACS.

- Induction protocol in brief: Teffs + aCD3/aCD28 beads + TGF-b + IL2 + CCCP or DMSO

F. ATP Detection Assay

Cells were sorted and $1x10^5$ cells/well were plated in a 96well-plate. ATP was measured according to the manufacturer instructions (ATPlite Luminescence Assay System, Perkin Elmer).

G. Confocal microscopy

For autophagy immunofluorescence, $1x10^6$ sorted Teff and Treg cells were seeded in coverslips pretreated with poly lysine, fixed with 4% PFA for 15 min in room temperature followed by 10 min of fixation with ice cold methanol in -20^o C, washed twice with PBS and ice cold methanol. Cells were permeabilized by using 0.1% saponin (Sigma-Aldrich) and stained with mouse anti-LC3 antibody (1:20, 5F10 nanoTools), rat anti-Lamp-1 (1:400, 1D4B Santa Cruz Biotechnology), rabbit anti-p62 (1:500, MBL), or rabbit anti-phospho-ubiquitin (Ser65) (1:200, Millipore) and mouse anti-Tom20 (1:100, Calbiochem) followed by incubation with Alexa fluor® 555 anti-mouse IgG (1:500, Molecular Probes), Alexa fluor® anti-rabbit IgG (1:200, Molecular Probes), Alexa fluor® 488 anti-rat IgG (1:250, Molecular Probes). For visualization of the nuclei Dapi (Sigma-Aldrich) was used. Samples were coverslipped with moviol and visualized using inverted confocal live cell imaging system Leica SP5.

Puncta of LC3/cell and puncta of p62/cell were calculated using a macro developed in Fiji software.

Colocalization of Tom20-pSer65Ub puncta/cell and Tom20-Lamp-1 puncta/cell were calculated using cross-correlation analysis with velocity software [75].

H. Foxp3 intracellular staining

For Foxp3 intracellular staining, cells were fixed and stained using Foxp3 staining Set (anti- Foxp3 clone: 150D, eBioscience Inc.) according to manufacturer instructions

I. Metabolic Assay

OCR was measured using a 96-well XF or XFe extracellular flux analyzer (EFA) (Seahorse Bioscience).

J. Statistical analysis

Statistical analysis was performed using unpaired t-test in GraphPad Prism v5 software. Data are presented as means ± S.E.M. P value < 0.05 was considered as indicative of statistical significance.

Results

1. Gating strategy followed for the isolation of Treg cells

In our experiments two subtypes of Treg cells were isolated depending on the mouse strain. When Foxp3YFP mice were used Tregs were sorted as $CD4^{+}Foxp3^{+}$ (**Figure 1a**). whereas, in the case that animals didn't have any endogenous tag for the intracellular Foxp3 marker, Tregs were sorted as $CD4^{+}CD25^{+}GITR^{+}$ T cells (**Figure 1b**).

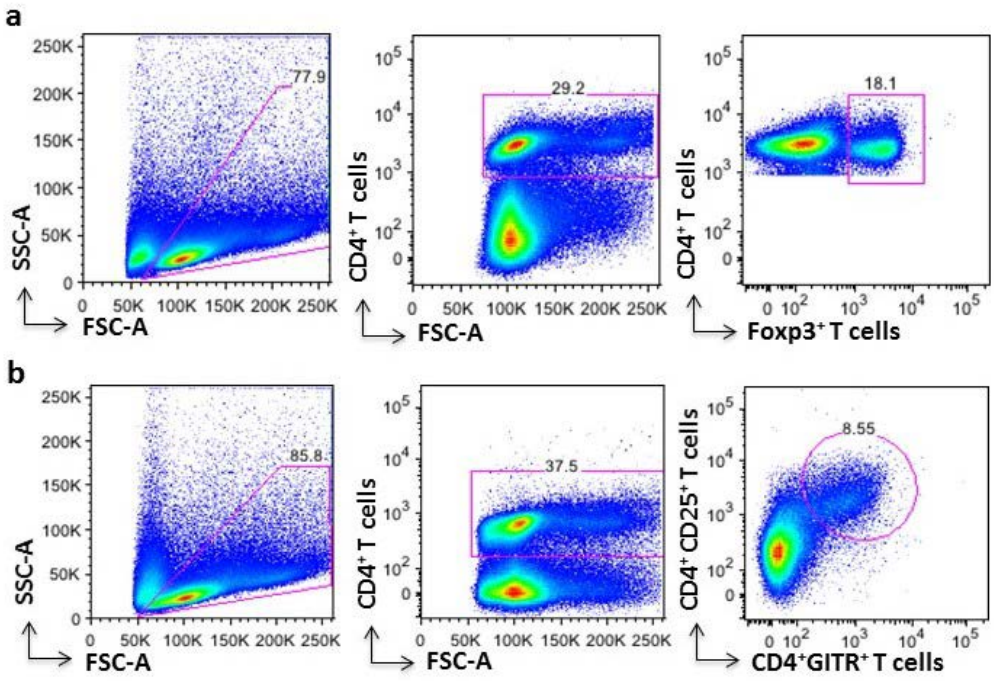

Figure 1: Gating strategy to select T regulatory cells

(a) $CD4^+Foxp3^+$ Treg cells from Foxp3yfp.KI mice or (b) $CD4^+CD25^+GITR^+$ Treg cells from $CD4creAtg5^{fl/fl}$ or C57BL/6J mice.

2. Mitochondrial characterization

It is known that during an autoimmune pathology T regulatory cells play a dominant role. Series of studies have associated the altered Treg numbers or/and function with the development of autoimmune responses [76]. Moreover, Treg cell function rely on mitochondria metabolism for their efficient suppressive function. Thus, we hypothesized that during an autoimmune response Treg cells might exhibit mitochondrial dysfunction.

A. Mitochondrial function

In order to examine mitochondrial function, we performed Seahorse analysis to measure Oxygen Consumption Rate (OCR) of Tregs and Teffs from MOG_{35-55}/CFA-immunized and naïve animals. Tregs isolated from immunized animals exhibited the higher OCR from all other groups (**Figure 2a**). As the major product of OXPHOS is ATP we measured also intracellular ATP levels of $CD4^+Foxp3^+$ Tregs and $CD4^+Foxp3^-$Teff isolated from the inguinal lymph nodes of naïve Foxp3YFP mice, MOG_{35-55}/CFA-immunized

Foxp3YFP animals that were euthanized 9days post-immunization or EAE Foxp3YFP mice with disease score 4. Surprisingly, the high OXPHOS did not positively correlate with the levels of ATP production in Tregs from immunized animals (**Figure 2b**).

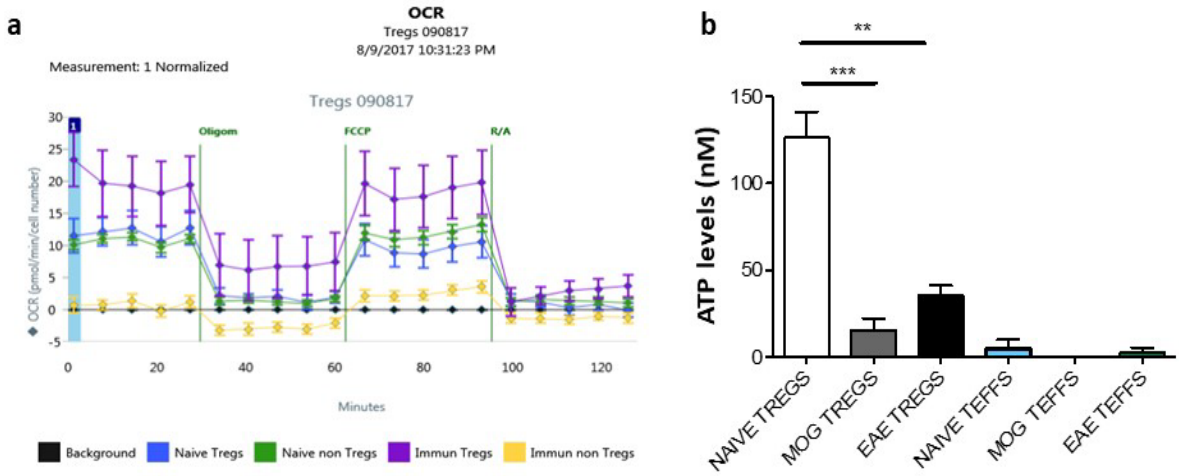

Figure 2: Increased OXPHOS but reduced ATP production by Foxp3^{+} Tregs during priming of EAE

a) Oxygen Consumption Rate of Tregs and Teffs from MOG_{35-55}/CFA-immunized and naïve animals detected by Seahorse Flux Analyzer b) Luminescent ATP detection assay in CD4^{+}Foxp3^{+} Tregs and CD4^{+}Foxp3^{-}Teff isolated from the inguinal lymph nodes of naïve Foxp3YFP mice, MOG_{35-55}/CFA-immunized Foxp3YFP animals that were euthanized 9days post-immunization or EAE Foxp3YFP mice with disease score 4.

B. Transmission Electron microscopy to visualize mitochondrial integrity

To visualize the mitochondrial phenotype of CD4^{+}Foxp3^{+} Tregs and CD4^{+}Foxp3^{-}Teff cells we used Transmission Electron Microscopy. It was obvious that damaged mitochondria were accumulated more in Treg cells isolated from MOG_{35-55}/CFA-immunized Foxp3YFP animals (**Figure 3**).

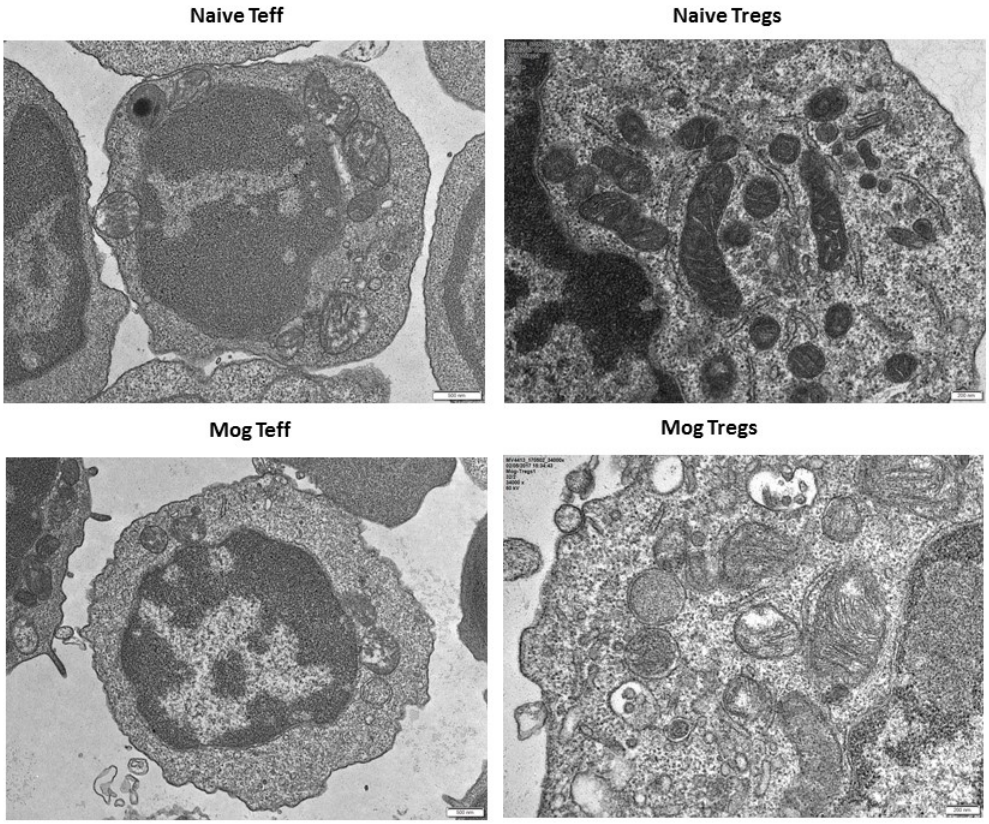

Figure 3: Depiction of damaged mitochondria in Tregs during EAE by transmission electron microscopy

C. Mitochondrial characteristics during an autoimmune response

As mitochondria metabolism is essential for Treg cell function it was necessary to examine critical characteristics of mitochondrial status. For this purpose, $CD4^+Foxp3^+$ or $CD4^+CD25^+GITR^+$ Tregs, from the inguinal lymph nodes of naïve, MOG_{35-55}/CFA-immunized animals that were euthanized 9days post immunization or EAE Foxp3YFP and C57BL/6J mice with disease score 4, were stained with TMRE, Mitosox or JC-I. TMRE indicates mitochondria membrane potential and Mitosox is a mitochondria superoxide indicator. JC-I dye also exhibit potential-dependent accumulation in mitochondria, but with a more specific way, as it shifts fluorescence emission after mitochondria depolarization. In detail, mitochondrial depolarization is indicated by a decrease in the red/green fluorescence intensity ratio. Our data demonstrate that Tregs from both day9 immunized animals (**Figure 4a**) and EAE animals with disease score 4 (**Figure 4b**) had lower mitochondrial membrane potential compared to naïve, indicated by TMRE (**Figure**

4a, b) and JC-1 staining (**Figure 4c**) and higher mitochondrial superoxide production noted after Mitosox staining (**Figure 4a, b**).

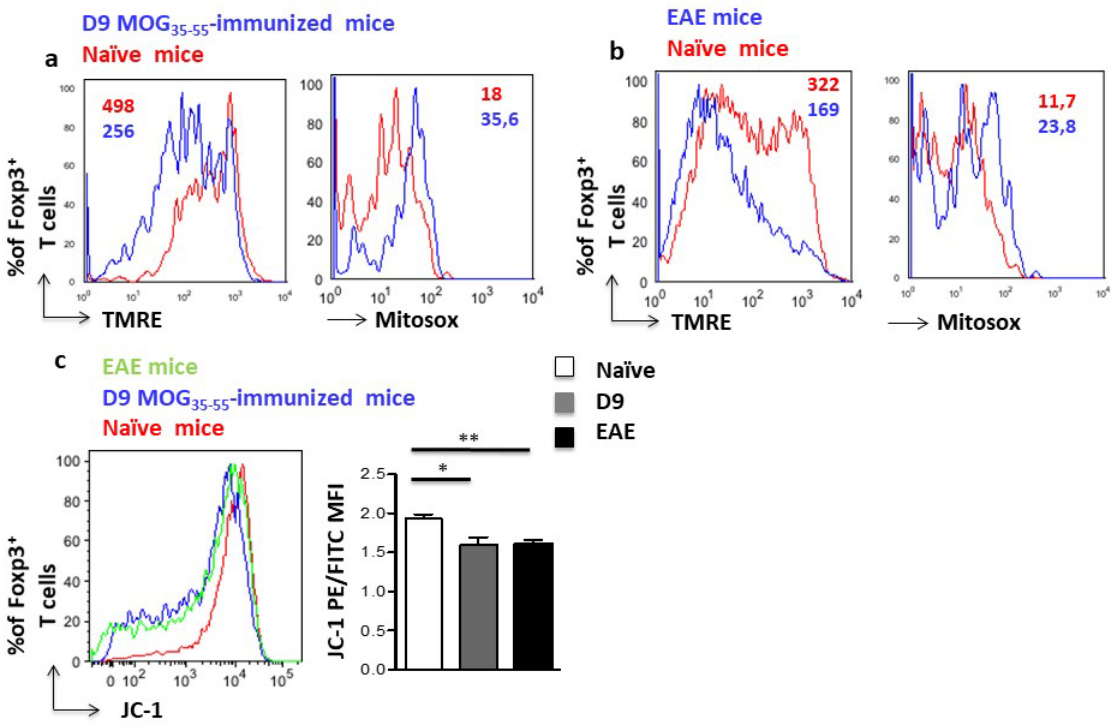

Figure 4: Impaired mitochondrial status in Treg cells during EAE

(a,b) Flow cytometry analysis based on mitochondria membrane potential (TMRE) and mitochondria superoxide production (Mitosox) staining of $CD4^+Foxp3^+$ Tregs isolated from inguinal lymph nodes of naïve, MOG_{35-55}/CFA immunized animals that were euthanized after 9 days and EAE Foxp3YFP mice with disease score 4. (c) JC-I staining, analyzed by flow cytometry, of $CD4^+CD25^+GITR^+$ Tregs isolated from inguinal lymph nodes of naïve, 9 days MOG_{35-55}/CFA immunized and EAE (disease score 4) C57BL/6J animals.

3. Study autophagy of Treg cells

Three principal methods are presently used to monitor the number of autophagosomes, including electron microscopy, light microscopy detection of the subcellular localization of LC3, and biochemical detection of the membrane-associated form of LC3.

The assessment of autophagosome number by electron microscopy requires specialized expertise, therefore is becoming increasingly replaced by fluorescence microscopy and biochemical methods. As mentioned above, LC3 is a marker of autophagosomes (**Figure A**). Among the four LC3 isoforms, LC3B is mostly used. After synthesis, LC3 is processed

at its C terminus by Atg4 and becomes LC3-I. LC3-I is subsequently conjugated with phosphatidylethanolamine (PE) to become LC3-II, which associates with both the inner and outer membrane of the autophagosome. Thus, endogenous LC3 that is visualized by fluorescence microscopy either as punctate structures or as diffuse cytoplasmic pool represent autophagosomes [27].

This method is useful to evaluate the number of autophagosome but is not always an indicator of the level of autophagic activity. Indeed, accumulation of autophagosomes is not indicative of autophagic induction as it may represent either increased generation of autophagosomes and/or a block in the autophagosomal maturation and the completion of the pathway. Thus, simply by measuring autophagosome, by LC3, one cannot distinguish between bona fide induction of autophagy and impairment of the completion of the process. Therefore, the need of measuring "autophagic flux" appears. There are several assays that provide measurement of the "autophagic flux". The one we used, is based on the measurement of a specific substrate that is preferentially degraded by autophagy, which is p62 (also known as SQSTM1/sequestome 1). p62 binds directly to LC3, so it is incorporated into autophagosomes and efficiently degraded by autophagy [77]. Thus, the total cellular levels of p62 are inversely correlated with autophagic activity.

A. Autophagy in Tregs during an autoimmune response

As mentioned above, mitophagy is the selective degradation of defective mitochondria by autophagy. Therefore, since we characterized impaired mitochondrial status of Treg cells during an autoimmune response it was critical to study the autophagic pathway in these cells.

$CD4^+Foxp3^+$ Tregs were sorted from inguinal lymph nodes of Foxp3YFP naïve, MOG_{35-55}/CFA immunized animals that were euthanized after 9 days and EAE mice with disease score 4 and stained for the autophagy markers, LC3, p62 and Lamp-1 (**Figure 5a**). Whereas Treg cells from immunized animals showed higher numbers of autophagosome formation (**Figure 5b**), p62 levels were also higher (**Figure 5c**) indicating an impairment in the completion of the pathway.

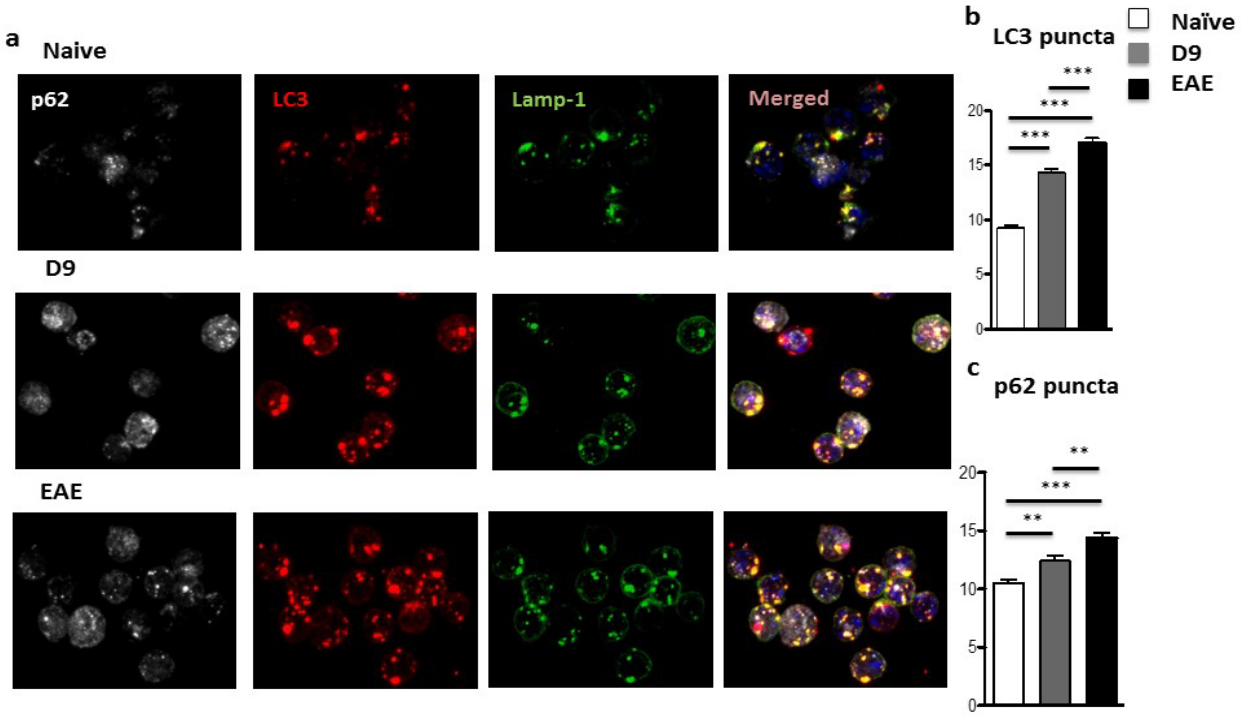

Figure 5: Impaired completion of autophagy pathway in Foxp3^{+} Tregs during priming of EAE

a) Representative figure by confocal microscopy from LC3/Lamp-1/p62 stained cells for assessment of autophagy pathway in Foxp3^{+} Tregs isolated from naïve Foxp3YFP mice, MOG_{35-55}/CFA-immunized Foxp3YFP animals that were euthanized 9days post-immunization or EAE Foxp3YFP mice with disease score 4. Calculation of (b) LC3 puncta/cell and (c) p62 puncta/cell of Foxp3^{+} Tregs isolated from the same groups as (a).

B. Study autophagy signaling pathway in Tregs during EAE

We further studied the PI3K-AKT-mTOR signaling pathway which is responsible for the inhibition of autophagy (**Figure 6b**). We performed intracellular staining and analysis by flow cytometry for foxp3 and the pathway-related molecules, p-Akt, p-4EBP1 and p-S6 of inguinal lymph nodes of naïve and MOG_{35-55}/CFA-immunized C57BL/6J animals that were euthanized 9days post-immunization. Treg cells that were isolated from immunized animals exhibited lower mean fluorescent intensities of all p-Akt, p-4EBP1 and p-S6 (**Figure 6a**), meaning that the mTOR pathway is less activated in these cells.

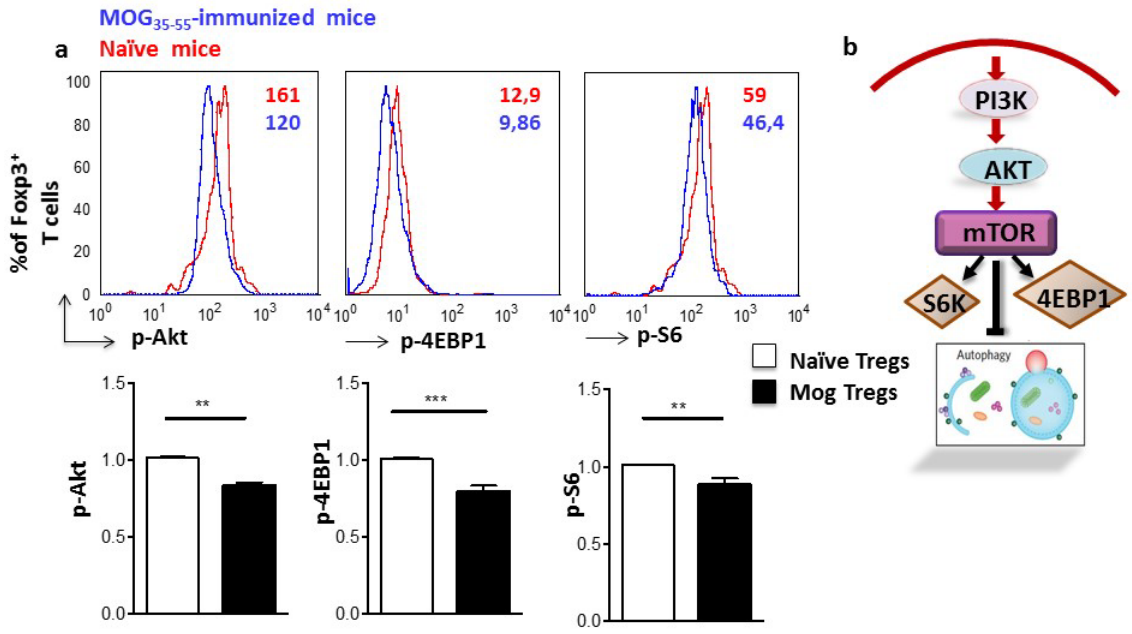

Figure 6: Reduced mTOR signaling in Foxp3^{+} Tregs during EAE

(a)Phosphorylation levels of Akt, 4EBP1 and S6 in Foxp3^{+} Tregs isolated from draining lymph nodes of naïve or MOG_{35-55}/CFA immunized mice were determined by flow cytometry. Mean fluorescent intensity (MFI) is shown in each plot. (b) PI3K-AKT-mTOR signaling pathway. (data are representative of 4 independent experiments)

C. *In vivo* autophagy deletion in Foxp3^{+} Treg cells

Aiming to investigate whether autophagy is implicated in Treg mechanism of action, we examined Foxp3creAtg5$^{fl/fl}$ mice lacking autophagy in their Foxp3^{+} Treg compartment. In order to generate these mice, mice expressing a YFP-Cre fusion in the Foxp3 locus (Foxp3YfpCre) were crossed with Atg5 flox/flox mice, carrying loxP-flanked Atg5 (Atg5$^{fl/fl}$). These Foxp3creAtg5$^{fl/fl}$ mice had altered development compared to Foxp3cre mice (**Figure 7a**). We detected decreased percentages of Treg cells in mice that lacked autophagy (**Figure 7b**), increased death in the Treg cell compartment (**Figure 7c**) and high mitochondrial superoxide production (**Figure 7d**), assessed by 7-AAD and Mitosox staining, respectively, analyzed by flow cytometry.

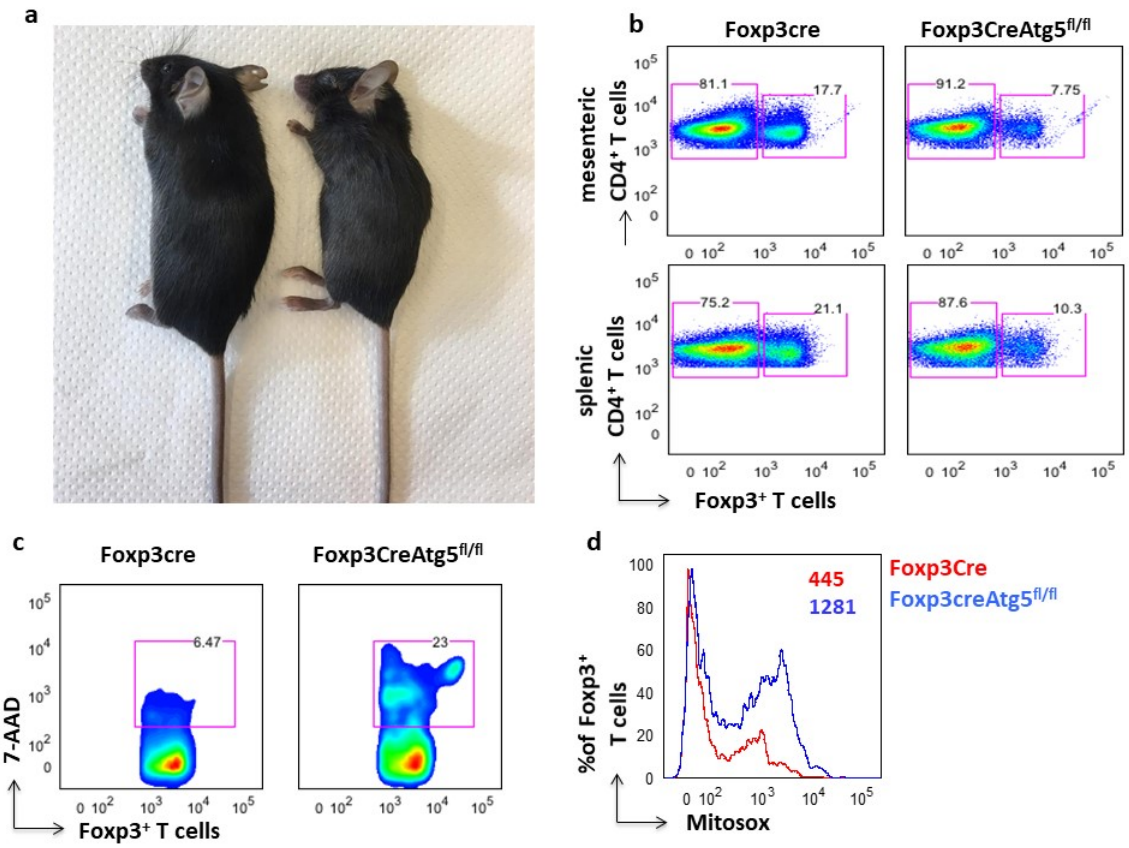

Figure 7: Increased death and ROS in Tregs that lack autophagy

(a) Foxp3cre (left) and Foxp3creAtg5$^{fl/fl}$ at the same age (b) Decreased frequencies of Foxp3^{+} Tregs in the spleen and lymph nodes of Foxp3creAtg5$^{fl/fl}$ mice. (c) Increased death detected with 7-AAD staining and (d) mitochondrial ROS accumulation in Foxp3^{+} Tregs from naïve Foxp3creAtg5$^{fl/fl}$ mice.

D. Examination of mitochondria status in CD4^{+}CD25^{+}GITR^{+} Tregs during an autoimmune response

In order to further study the mitochondrial characteristrics in the Treg compartment during an autoimmune response we examined MOG$_{35-55}$/CFA immunized C57BL/6J and CD4creAtg5$^{fl/fl}$ animals that were euthanized 9 days post-immunization. CD4creAtg5$^{fl/fl}$ mice were generated by crossing mice expressing a Cre fusion in the CD4 locus (CD4Cre) with Atg5 flox/flox mice, carrying loxP-flanked Atg5 (Atg5$^{fl/fl}$). We observed decreased frequencies in CD4^{+} T cells that lack autophagy (**Figure 8a**), whereas not such difference in Foxp3^{+} Treg population (**Figure 8b**). In addition, CD4^{+}CD25^{+}GITR^{+} Tregs, from the immunized autophagy-deficient mice, exhibited higher levels of mitochondria

superoxide production (Mitosox) and mitochondrial mass (Mitotracker) (**Figure 8c**). We also evaluated the ATP production from $CD4^+CD25^+$ Tregs and $CD4^+CD25^-$ Teffs from naïve C57BL/6J and $CD4creAtg5^{fl/fl}$ mice. Autophagy-deficient Tregs produced lower levels of ATP compared to wild type (**Figure 8d**).

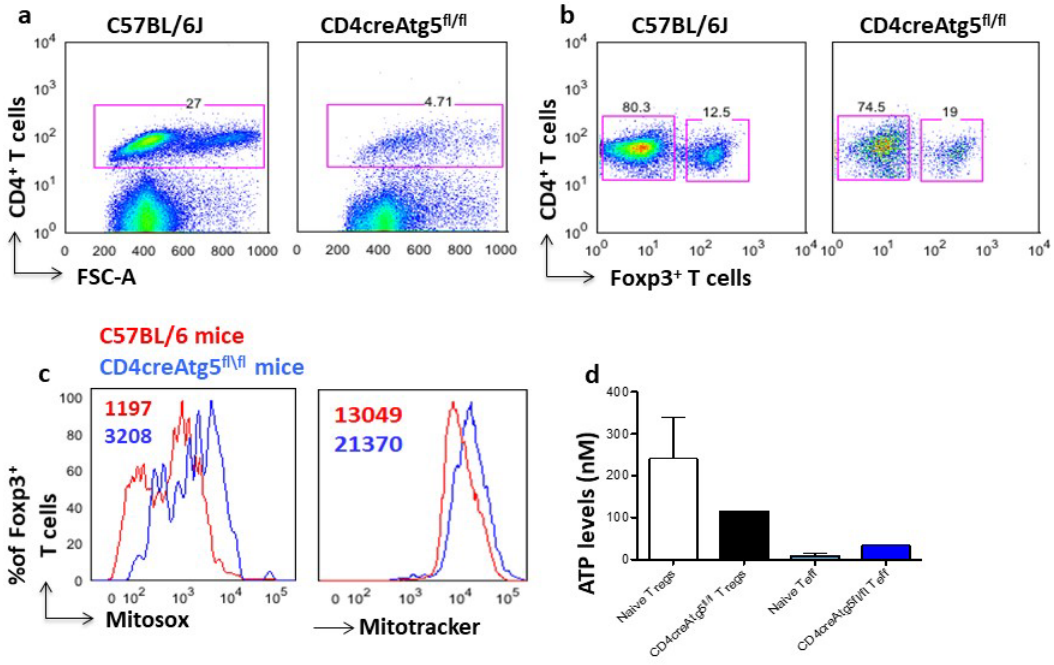

Figure 8: Dysfunctional mitochondria in autophagy-deficient Tregs in autoimmunity

(a) Decreased frequencies of $CD4^+$ T cells and (b) $CD4^+$ $Foxp3^+$ T cells in the inguinal lymph nodes of MOG_{35-55}/CFA immunized C57BL/6J and $CD4creAtg5^{fl/fl}$ mice. (c) Flow cytometry analysis in $CD4^+CD25^+GIRT^+$ Tregs from inguinal lymph nodes of MOG_{35-55}/CFA immunized C57BL/6J and $CD4creAtg5^{fl/fl}$ mice for mitochondrial ROS (MitoSox), and mitochondrial mass (Mitotracker). (data are representative of 3 independent experiments) (d) ATP production of isolated $CD4^+CD25^+GIRT^+$ Treg and $CD4^+CD25^-$ T effector cells from inguinal lymph nodes and spleen of naïve C57BL/6J and $CD4creAtg5^{fl/fl}$ mice.

4. Study mitophagy in Treg cell during EAE

After observing impaired autophagy of Treg cell during EAE, we sought to examine the operation of the mitophagy pathway in the same concept.

A. Mitophagy in Tregs during autoimmune response

For this purpose, we applied colocalization measurements of Tom20 and pSer65-Ub which denotes the initiation of the mitophagy pathway and Tom20 with lamp-1 which indicates the mitochondria within the lysosomes (**Figure 9a**) in . Both Teff cells and Treg cells from EAE mice showed higher numbers of mitochondria that are targeted for mitophagy (**Figure 9b**). Interestingly, only Mog-activated Treg cell showed higher accumulation of damaged mitochondria within the lysosomes (**Figure 9c**).

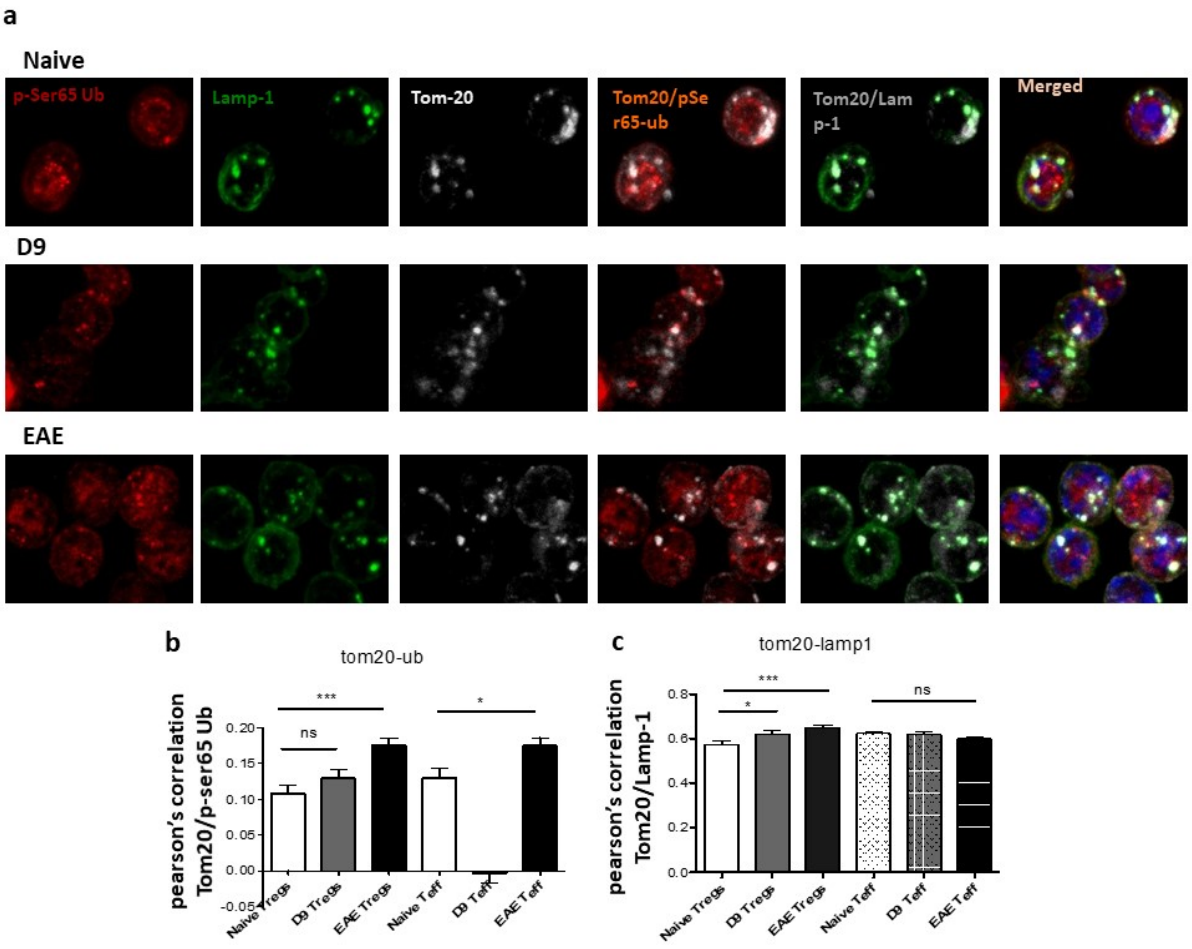

Figure 9: Accumulation of damaged mitochondria in the lysosomes of Treg cells during EAE

a) Representative figure by confocal microscopy from pSer65-ub/Lamp-1/Tom20 stained cells for assessment of mitophagy pathway in Foxp3^{+} Tregs isolated from naïve Foxp3YFP mice, MOG_{35-55}/CFA-immunized Foxp3YFP animals that were euthanized 9days post-immunization or EAE Foxp3YFP mice with disease score 4. (b) Colocalized pSer65-ub-Tom20 and (c) Tom20-Lamp-1 puncta/cell measured by Pearson correlation measurement in CD4^{+}Foxp3^{-} Teff and CD4^{+}Foxp3^{+} Treg cell from naïve Foxp3YFP mice, MOG_{35-55}/CFA-immunized Foxp3YFP animals that were euthanized 9days post-immunization or EAE Foxp3YFP mice with disease score 4.

B. Induction of mitophagy in Treg cells *in vitro*

To begin studying mitophagy in Treg cells we performed an *in vitro* assay using a chemical compound, called carbonyl cyanide m-chlorophenyl hydrazone (CCCP). CCCP is an ionophore that disrupts the mitochondrial proton gradient, thus triggering PINK1 accumulation and Parkin translocation, resulting in induction of mitophagy [48]. For this set of experiments, we cultured induced Tregs (iTregs) or natural Tregs (nTregs) that were isolated from total lymph nodes naïve Foxp3YFP mice as $CD4^+Foxp3^-$ Teffs or $CD4^+Foxp3^+$ Tregs, respectively. Cells were treated with DMSO or CCCP and after 4 days we assessed proliferation, with Cell Trace Violet (CTV) staining, and death using 7-AAD staining. It was obvious that nTregs could not handle CCCP treatment as iTregs, resulting in diminished proliferation capacity (**Figure 10a**) and increased cellular death (**Figure 10b**).

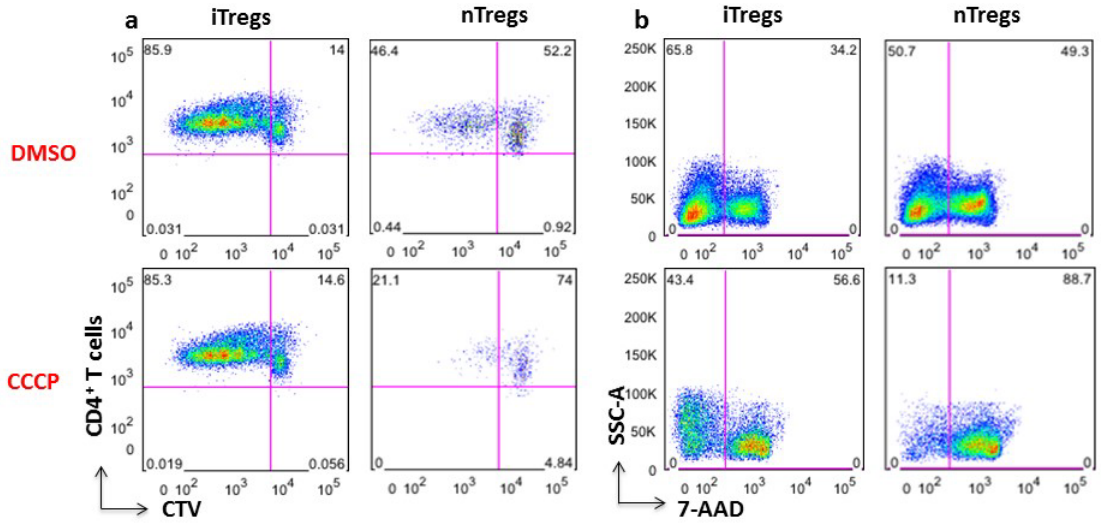

Figure 10: Diminished proliferation and induction of death upon mitochondria depolarization on natural Tregs but not induced Tregs

Teff ($CD4^+Foxp3^-$) and Treg ($CD4^+Foxp3^+$) cells were sorted from naïve Foxp3YFP mice and cultured for 4 days under DMSO or CCCP treatment. Teff were cultured in the presence of IL-2 and TGF-b in order to differentiate into induced Treg cells (iTregs). Natural Treg cells (nTregs)

were cultured in the presence of IL-2. Both cell types were stimulated with anti-CD3/CD28 microbeads. (a) Proliferation of CFSE-labeled iTreg and CFSE-labeled nTreg cells treated with DMSO or CCCP is shown. (b) representative flow cytometry for death of iTreg and nTreg cells after DMSO or CCCP treatment in vitro. (data are representative of 4 independent experiments)

Discussion

The breakdown of mechanisms that assure recognition of self and non-self by the immune system is a hallmark feature of autoimmune diseases. Thus, understanding this process has considerably expanded during the last years. The primary mechanism that leads to self-tolerance has termed as “recessive tolerance”, which is induced after thymic deletion of autoreactive T cells [78]. However, thymic selection is incomplete and self-reactive cells occur, even in healthy individuals. On the other hand, an additional mechanism for maintaining peripheral self- tolerance is “dominant tolerance”, which is mediated by regulatory T cells actively modulating immune responses [79].

In mice, lack of $CD4^{+}Foxp3^{+}$ Treg results in increased autoimmunity and adoptive transfer of Treg prevent and reverses the disease [80]. In humans, lack of functional Tregs also leads to aggressive autoimmunity including insulin-dependent diabetes, eczema and thyroiditis [81],[82]. These observations indicate that any defect in Treg number or function contribute to the development of human autoimmune disorders, including multiple sclerosis (MS). Treg cells have emerged as important players in the pathogenetic scenarios of CNS autoimmune inflammation [83]. Recent findings demonstrate that MS is also accompanied by dysfunction or impaired maturation of Treg cells, fact that makes these cells potential therapeutic targets in MS.

Mitochondrial integrity is essential for mammalian development and defects has been proven to contribute to neurodegenerative diseases and also to the pathogenesis of autoimmunity [84],[85]. Numerous studies have associated MS development with oxidative stress and mitochondria dysfunction [86],[87],[88],[89],[90]. Oxidative DNA damage has been observed in active MS lesions, in addition to impaired bioenergetics and metabolomic profile in MS patients [91],[92]. Moreover, MitoQ, a mitochondria-targeted antioxidant, was proven to delay disease progression and alleviate the pathogenesis of EAE [93].

Given the great importance of autophagy in the immune system [36] and specifically in T cell development and function [38, 39], along with the critical role of mitochondria in

Treg metabolism, we hypothesized that impaired mitophagy in these cells contribute to the breakdown of immune tolerance, leading to autoimmunity.

Our data demonstrate that although Treg cells isolated from both MOG_{35-55}/CFA-immunized Foxp3YFP animals that were euthanized 9days post-immunization (day 9 animals) and EAE Foxp3YFP mice with disease score 4 (**Figure 1**), exhibited high levels of OXPHOS (**Figure 2a**), they produced significantly lower levels of ATP compared to naïve animals (**Figure 2b**). This result forced us to the hypothesis that there might be a dysfunction in the mitochondria of these cells during an autoimmune response. Whereas it is not a quantitative method to export data, by using Transmission electron microscopy imaging, it was obvious that Treg cells sorted from immunized animals, had more damaged mitochondria than naïve (**Figure 3**). In addition, Treg mitochondria during EAE had disrupted membrane potential (**Figure 4 a,b,c**), and increased production of superoxide (**Figure 4 a,b**) in contrast to naïve. These data imply that Treg cells have damaged mitochondria unable to produce satisfying levels of ATP during an autoimmune response.

Next, we sought to determine whether the mechanism of autophagy is operating well in Treg cells during an autoimmune response in order to clear the dysfunctional mitochondria (**Figure 5a**). Indeed, LC3 levels in Mog Tregs were increased (**Figure 5b**) indicating a higher generation of autophagosomes in these cells. However, p62 was higher compared to naïve (**Figure 5c**), leading us to the conclusion that Mog Tregs cannot complete efficiently the mechanism of autophagy. Going one step further, we asked whether autophagy signaling was altered during EAE. PI3K-Akt-mTOR signaling (**Figure 6b**), which physiologically inhibits autophagy, was decreased in Mog-primed Tregs (**Figure 6a**), result that proves the increased numbers of autophagosomes we observe with LC3 staining.

Due to the defect we detected in the autophagy process of Treg cells, we examined the phenotype of naïve $Foxp3^{+}creAtg5^{fl/fl}$ animals. These mice present abnormal development (**Figure 7a**) in comparison to wild type animals. Also, the Treg frequencies under steady were altered (**Figure 7b**) probably due to high levels of death (**Figure 7c**). In addition, the autophagy-deficient Treg cells production significantly increased levels of mitochondrial ROS production (**Figure 7d**). Due to the developmental problems and the low survival rate of $Foxp3^{+}creAtg5^{fl/fl}$ animals, we occupied the $CD4^{+}creAtg5^{fl/fl}$ in order to move one step further and examine the mitochondrial status of activated autophagy-deficient Treg cells. MOG_{35-55}/CFA immunized $CD4^{+}creAtg5^{fl/fl}$ animals had lower numbers of $CD4^{+}$ T cells (**Figure 8a, b**) and the $CD4^{+}CD25^{+}GITR^{+}$ Tregs that were isolated presented higher levels of mitochondria ROS production (**Figure 8c**) and accumulation of mitochondria (**Figure 8c**) compared to immunized C57BL/6J mice. We

also examined under steady state the ATP production of Tregs in these mice. Autophagy-deficient Treg cells produced significantly lower levels of ATP compared to autophagy-sufficient cells (**Figure 8d**), which was also obvious in Treg cell during EAE (**Figure 2b**), implying a similarity between these two groups. These results indicate an impairment in the autophagic pathway of Treg cells during EAE, a process that seems to be critical for the function of the cell when is absent.

In addition to autophagy, in order to be more accurate for the removal of damaged mitochondria, we also studied mitophagy (**Figure 9**). Tregs isolated from immunized animals showed higher levels of mitochondria that were targeted for mitophagy (**Figure 9b**), but also increased accumulation of mitochondria in lysosomes (**Figure 9c**), indicating a partial inability of the cell to degrade the damaged mitochondria. In contrast, this is not observed in Teff cell population. We also performed an *in vitro* assay in order to question the proliferation and death of iTregs and nTregs after induction of mitophagy using CCCP. nTreg cells could not so well overcome this induction and demonstrated lower proliferation capacity (**Figure 10a**) and higher levels of apoptosis (**Figure 10b**).

Collectively, we focused in Tregs cells during EAE due to their critical role in MS pathogenesis. We characterized a Treg cell population that in an autoimmune environment accumulates damaged mitochondria that were unable to produce ATP for normal cell function, had decreased membrane potential and produced high amounts of superoxide. Due to this observation, we sought to determine whether these mitochondria could efficiently be cleared. Surprisingly, Treg cells from immunized animals showed a defect in the completion of the autophagic pathway. Interestingly, Tregs from cell specific autophagy deficient mice demonstrated the same mitochondrial characteristics as the ones from wild type immunized animals, which strengthens more the notion of autophagic dysfunction in Treg cell compartment during EAE. Moreover, we used further markers to assess mitophagy in these cells. Treg cells from immunized animals seemed to have higher number of mitochondria that were targeted for mitophagy, these mitochondria accumulated within the lysosomes, implying an impairment in the clearance. Finally, nTreg cells exhibited low proliferation capacity and increased apoptosis after in vitro mitophagy induction. To sum up, we lead to the conclusion that mitochondrial damage and impaired mitophagy of Treg cells contribute to the pathogenesis of autoimmune responses.

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References

1. Smigiel, K.S., et al., *Regulatory T-cell homeostasis: steady-state maintenance and modulation during inflammation.* Immunol Rev, 2014. **259**(1): p. 40-59.
2. Boyman, O., et al., *Homeostatic maintenance of T cells and natural killer cells.* Cell Mol Life Sci, 2012. **69**(10): p. 1597-608.
3. Lio, C.W. and C.S. Hsieh, *Becoming self-aware: the thymic education of regulatory T cells.* Curr Opin Immunol, 2011. **23**(2): p. 213-9.
4. Vignali, D.A., L.W. Collison, and C.J. Workman, *How regulatory T cells work.* Nat Rev Immunol, 2008. **8**(7): p. 523-32.
5. Malek, T.R., *The biology of interleukin-2.* Annu Rev Immunol, 2008. **26**: p. 453-79.
6. Gu, H., et al., *New role for Shc in activation of the phosphatidylinositol 3-kinase/Akt pathway.* Mol Cell Biol, 2000. **20**(19): p. 7109-20.
7. Maloy, K.J. and F. Powrie, *Regulatory T cells in the control of immune pathology.* Nat Immunol, 2001. **2**(9): p. 816-22.
8. Sakaguchi, S., *Naturally arising Foxp3-expressing CD25+CD4+ regulatory T cells in immunological tolerance to self and non-self.* Nat Immunol, 2005. **6**(4): p. 345-52.
9. Gershon, R.K. and K. Kondo, *Cell interactions in the induction of tolerance: the role of thymic lymphocytes.* Immunology, 1970. **18**(5): p. 723-37.
10. Sakaguchi, S., et al., *Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases.* J Immunol, 1995. **155**(3): p. 1151-64.
11. Fontenot, J.D., M.A. Gavin, and A.Y. Rudensky, *Foxp3 programs the development and function of CD4+CD25+ regulatory T cells.* Nat Immunol, 2003. **4**(4): p. 330-6.
12. Kim, J.M., J.P. Rasmussen, and A.Y. Rudensky, *Regulatory T cells prevent catastrophic autoimmunity throughout the lifespan of mice.* Nat Immunol, 2007. **8**(2): p. 191-7.
13. Bennett, C.L., et al., *The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3.* Nat Genet, 2001. **27**(1): p. 20-1.
14. Newton, R., B. Priyadharshini, and L.A. Turka, *Immunometabolism of regulatory T cells.* Nat Immunol, 2016. **17**(6): p. 618-25.

15. Putheti, P., et al., *Circulating CD4+CD25+ T regulatory cells are not altered in multiple sclerosis and unaffected by disease-modulating drugs.* J Clin Immunol, 2004. **24**(2): p. 155-61.
16. Brusko, T. and M. Atkinson, *Treg in type 1 diabetes.* Cell Biochem Biophys, 2007. **48**(2-3): p. 165-75.
17. Lee, J.H., et al., *Inverse correlation between CD4+ regulatory T-cell population and autoantibody levels in paediatric patients with systemic lupus erythematosus.* Immunology, 2006. **117**(2): p. 280-6.
18. Miyara, M., et al., *Global natural regulatory T cell depletion in active systemic lupus erythematosus.* J Immunol, 2005. **175**(12): p. 8392-400.
19. van Amelsfort, J.M., et al., *CD4(+)CD25(+) regulatory T cells in rheumatoid arthritis: differences in the presence, phenotype, and function between peripheral blood and synovial fluid.* Arthritis Rheum, 2004. **50**(9): p. 2775-85.
20. Lord, J.D., et al., *Paradoxically increased FOXP3+ T cells in IBD do not preferentially express the isoform of FOXP3 lacking exon 2.* Dig Dis Sci, 2012. **57**(11): p. 2846-55.
21. Pollizzi, K.N. and J.D. Powell, *Integrating canonical and metabolic signalling programmes in the regulation of T cell responses.* Nat Rev Immunol, 2014. **14**(7): p. 435-46.
22. Wang, R., et al., *The transcription factor Myc controls metabolic reprogramming upon T lymphocyte activation.* Immunity, 2011. **35**(6): p. 871-82.
23. Pearce, E.L., et al., *Fueling immunity: insights into metabolism and lymphocyte function.* Science, 2013. **342**(6155): p. 1242454.
24. van der Windt, G.J., et al., *Mitochondrial respiratory capacity is a critical regulator of CD8+ T cell memory development.* Immunity, 2012. **36**(1): p. 68-78.
25. Chang, C.H., et al., *Posttranscriptional control of T cell effector function by aerobic glycolysis.* Cell, 2013. **153**(6): p. 1239-51.
26. Gerriets, V.A., et al., *Foxp3 and Toll-like receptor signaling balance Treg cell anabolic metabolism for suppression.* Nat Immunol, 2016. **17**(12): p. 1459-1466.
27. Mizushima, N., T. Yoshimori, and B. Levine, *Methods in mammalian autophagy research.* Cell, 2010. **140**(3): p. 313-26.
28. Ma, Y., et al., *Autophagy and cellular immune responses.* Immunity, 2013. **39**(2): p. 211-27.
29. Kroemer, G., G. Marino, and B. Levine, *Autophagy and the integrated stress response.* Mol Cell, 2010. **40**(2): p. 280-93.
30. Levine, B., N. Mizushima, and H.W. Virgin, *Autophagy in immunity and inflammation.* Nature, 2011. **469**(7330): p. 323-35.
31. Fleming, A., et al., *Chemical modulators of autophagy as biological probes and potential therapeutics.* Nat Chem Biol, 2011. **7**(1): p. 9-17.
32. He, C. and D.J. Klionsky, *Regulation mechanisms and signaling pathways of autophagy.* Annu Rev Genet, 2009. **43**: p. 67-93.
33. Klionsky, D.J. and S.D. Emr, *Autophagy as a regulated pathway of cellular degradation.* Science, 2000. **290**(5497): p. 1717-21.
34. Kuma, A., et al., *The role of autophagy during the early neonatal starvation period.* Nature, 2004. **432**(7020): p. 1032-6.
35. Komatsu, M., et al., *Impairment of starvation-induced and constitutive autophagy in Atg7-deficient mice.* J Cell Biol, 2005. **169**(3): p. 425-34.
36. Schmid, D. and C. Munz, *Innate and adaptive immunity through autophagy.* Immunity, 2007. **27**(1): p. 11-21.

37. Schmid, D., M. Pypaert, and C. Munz, *Antigen-loading compartments for major histocompatibility complex class II molecules continuously receive input from autophagosomes.* Immunity, 2007. **26**(1): p. 79-92.
38. Pua, H.H., et al., *A critical role for the autophagy gene Atg5 in T cell survival and proliferation.* J Exp Med, 2007. **204**(1): p. 25-31.
39. Pua, H.H., et al., *Autophagy is essential for mitochondrial clearance in mature T lymphocytes.* J Immunol, 2009. **182**(7): p. 4046-55.
40. Wei, J., et al., *Autophagy enforces functional integrity of regulatory T cells by coupling environmental cues and metabolic homeostasis.* Nat Immunol, 2016. **17**(3): p. 277-85.
41. Mizushima, N., et al., *In vivo analysis of autophagy in response to nutrient starvation using transgenic mice expressing a fluorescent autophagosome marker.* Mol Biol Cell, 2004. **15**(3): p. 1101-11.
42. Starr, T.K., S.C. Jameson, and K.A. Hogquist, *Positive and negative selection of T cells.* Annu Rev Immunol, 2003. **21**: p. 139-76.
43. Steinman, R.M., D. Hawiger, and M.C. Nussenzweig, *Tolerogenic dendritic cells.* Annu Rev Immunol, 2003. **21**: p. 685-711.
44. Sarkar, S., *Regulation of autophagy by mTOR-dependent and mTOR-independent pathways: autophagy dysfunction in neurodegenerative diseases and therapeutic application of autophagy enhancers.* Biochem Soc Trans, 2013. **41**(5): p. 1103-30.
45. Chi, H., *Regulation and function of mTOR signalling in T cell fate decisions.* Nat Rev Immunol, 2012. **12**(5): p. 325-38.
46. Zeng, H., et al., *mTORC1 couples immune signals and metabolic programming to establish T(reg)-cell function.* Nature, 2013. **499**(7459): p. 485-90.
47. Nguyen, T.N., B.S. Padman, and M. Lazarou, *Deciphering the Molecular Signals of PINK1/Parkin Mitophagy.* Trends Cell Biol, 2016. **26**(10): p. 733-44.
48. Yamano, K., N. Matsuda, and K. Tanaka, *The ubiquitin signal and autophagy: an orchestrated dance leading to mitochondrial degradation.* EMBO Rep, 2016. **17**(3): p. 300-16.
49. Wang, K. and D.J. Klionsky, *Mitochondria removal by autophagy.* Autophagy, 2011. **7**(3): p. 297-300.
50. Yen, W.L. and D.J. Klionsky, *How to live long and prosper: autophagy, mitochondria, and aging.* Physiology (Bethesda), 2008. **23**: p. 248-62.
51. Kundu, M. and C.B. Thompson, *Macroautophagy versus mitochondrial autophagy: a question of fate?* Cell Death Differ, 2005. **12 Suppl 2**: p. 1484-9.
52. Koyano, F., et al., *Ubiquitin is phosphorylated by PINK1 to activate parkin.* Nature, 2014. **510**(7503): p. 162-6.
53. Shiba-Fukushima, K., et al., *PINK1-mediated phosphorylation of the Parkin ubiquitin-like domain primes mitochondrial translocation of Parkin and regulates mitophagy.* Sci Rep, 2012. **2**: p. 1002.
54. Narendra, D., et al., *Parkin is recruited selectively to impaired mitochondria and promotes their autophagy.* J Cell Biol, 2008. **183**(5): p. 795-803.
55. Compston, A. and A. Coles, *Multiple sclerosis.* Lancet, 2008. **372**(9648): p. 1502-17.
56. Dendrou, C.A., L. Fugger, and M.A. Friese, *Immunopathology of multiple sclerosis.* Nat Rev Immunol, 2015. **15**(9): p. 545-58.
57. Haider, L., et al., *The topograpy of demyelination and neurodegeneration in the multiple sclerosis brain.* Brain, 2016. **139**(Pt 3): p. 807-15.
58. Kearney, H., et al., *Cervical cord lesion load is associated with disability independently from atrophy in MS.* Neurology, 2015. **84**(4): p. 367-73.

59. Frischer, J.M., et al., *The relation between inflammation and neurodegeneration in multiple sclerosis brains.* Brain, 2009. **132**(Pt 5): p. 1175-89.
60. Mastorodemos, V., M. Ioannou, and P. Verginis, *Cell-based modulation of autoimmune responses in multiple sclerosis and experimental autoimmmune encephalomyelitis: therapeutic implications.* Neuroimmunomodulation, 2015. **22**(3): p. 181-95.
61. Fletcher, J.M., et al., *T cells in multiple sclerosis and experimental autoimmune encephalomyelitis.* Clin Exp Immunol, 2010. **162**(1): p. 1-11.
62. Steinman, L., *Assessment of animal models for MS and demyelinating disease in the design of rational therapy.* Neuron, 1999. **24**(3): p. 511-4.
63. Olsson, T., *Cytokines in neuroinflammatory disease: role of myelin autoreactive T cell production of interferon-gamma.* J Neuroimmunol, 1992. **40**(2-3): p. 211-8.
64. Danikowski, K.M., S. Jayaraman, and B.S. Prabhakar, *Regulatory T cells in multiple sclerosis and myasthenia gravis.* J Neuroinflammation, 2017. **14**(1): p. 117.
65. Feger, U., et al., *Increased frequency of CD4+ CD25+ regulatory T cells in the cerebrospinal fluid but not in the blood of multiple sclerosis patients.* Clin Exp Immunol, 2007. **147**(3): p. 412-8.
66. Haas, J., et al., *Reduced suppressive effect of CD4+CD25high regulatory T cells on the T cell immune response against myelin oligodendrocyte glycoprotein in patients with multiple sclerosis.* Eur J Immunol, 2005. **35**(11): p. 3343-52.
67. International Multiple Sclerosis Genetics, C., et al., *Analysis of immune-related loci identifies 48 new susceptibility variants for multiple sclerosis.* Nat Genet, 2013. **45**(11): p. 1353-60.
68. Huan, J., et al., *Decreased FOXP3 levels in multiple sclerosis patients.* J Neurosci Res, 2005. **81**(1): p. 45-52.
69. Fritzsching, B., et al., *Intracerebral human regulatory T cells: analysis of CD4+ CD25+ FOXP3+ T cells in brain lesions and cerebrospinal fluid of multiple sclerosis patients.* PLoS One, 2011. **6**(3): p. e17988.
70. Kohm, A.P., et al., *Cutting edge: CD4+CD25+ regulatory T cells suppress antigen-specific autoreactive immune responses and central nervous system inflammation during active experimental autoimmune encephalomyelitis.* J Immunol, 2002. **169**(9): p. 4712-6.
71. Zhang, X., et al., *IL-10 is involved in the suppression of experimental autoimmune encephalomyelitis by CD25+CD4+ regulatory T cells.* Int Immunol, 2004. **16**(2): p. 249-56.
72. Stephens, L.A., D. Gray, and S.M. Anderton, *CD4+CD25+ regulatory T cells limit the risk of autoimmune disease arising from T cell receptor crossreactivity.* Proc Natl Acad Sci U S A, 2005. **102**(48): p. 17418-23.
73. Korn, T., et al., *Myelin-specific regulatory T cells accumulate in the CNS but fail to control autoimmune inflammation.* Nat Med, 2007. **13**(4): p. 423-31.
74. Miller, S.D., W.J. Karpus, and T.S. Davidson, *Experimental Autoimmune Encephalomyelitis in the Mouse.* Current protocols in immunology / edited by John E. Coligan ... [et al.], 2007. **CHAPTER**: p. Unit-15.1.
75. Costes, S.V., et al., *Automatic and quantitative measurement of protein-protein colocalization in live cells.* Biophys J, 2004. **86**(6): p. 3993-4003.
76. Long, S.A. and J.H. Buckner, *CD4+FOXP3+ T regulatory cells in human autoimmunity: more than a numbers game.* J Immunol, 2011. **187**(5): p. 2061-6.
77. Bjorkoy, G., et al., *p62/SQSTM1 forms protein aggregates degraded by autophagy and has a protective effect on huntingtin-induced cell death.* J Cell Biol, 2005. **171**(4): p. 603-14.

78. Fontenot, J.D. and A.Y. Rudensky, *A well adapted regulatory contrivance: regulatory T cell development and the forkhead family transcription factor Foxp3.* Nat Immunol, 2005. **6**(4): p. 331-7.
79. Graca, L., et al., *Dominant tolerance: activation thresholds for peripheral generation of regulatory T cells.* Trends Immunol, 2005. **26**(3): p. 130-5.
80. Sakaguchi, S., et al., *Foxp3+ CD25+ CD4+ natural regulatory T cells in dominant self-tolerance and autoimmune disease.* Immunol Rev, 2006. **212**: p. 8-27.
81. Ochs, H.D., E. Gambineri, and T.R. Torgerson, *IPEX, FOXP3 and regulatory T-cells: a model for autoimmunity.* Immunol Res, 2007. **38**(1-3): p. 112-21.
82. Baecher-Allan, C., et al., *CD4+CD25high regulatory cells in human peripheral blood.* J Immunol, 2001. **167**(3): p. 1245-53.
83. Zozulya, A.L. and H. Wiendl, *The role of regulatory T cells in multiple sclerosis.* Nat Clin Pract Neurol, 2008. **4**(7): p. 384-98.
84. Detmer, S.A. and D.C. Chan, *Functions and dysfunctions of mitochondrial dynamics.* Nat Rev Mol Cell Biol, 2007. **8**(11): p. 870-9.
85. Lood, C., et al., *Neutrophil extracellular traps enriched in oxidized mitochondrial DNA are interferogenic and contribute to lupus-like disease.* Nat Med, 2016. **22**(2): p. 146-53.
86. Blokhin, A., et al., *Variations in mitochondrial DNA copy numbers in MS brains.* J Mol Neurosci, 2008. **35**(3): p. 283-7.
87. Campbell, G.R. and D.J. Mahad, *Clonal expansion of mitochondrial DNA deletions and the progression of multiple sclerosis.* CNS Neurol Disord Drug Targets, 2012. **11**(5): p. 589-97.
88. Fischer, M.T., et al., *NADPH oxidase expression in active multiple sclerosis lesions in relation to oxidative tissue damage and mitochondrial injury.* Brain, 2012. **135**(Pt 3): p. 886-99.
89. Prinz, J., et al., *Time-Dependent Progression of Demyelination and Axonal Pathology in MP4-Induced Experimental Autoimmune Encephalomyelitis.* PLoS One, 2015. **10**(12): p. e0144847.
90. Singhal, N.K., et al., *Changes in Methionine Metabolism and Histone H3 Trimethylation Are Linked to Mitochondrial Defects in Multiple Sclerosis.* J Neurosci, 2015. **35**(45): p. 15170-86.
91. Lu, F., et al., *Oxidative damage to mitochondrial DNA and activity of mitochondrial enzymes in chronic active lesions of multiple sclerosis.* J Neurol Sci, 2000. **177**(2): p. 95-103.
92. Reinke, S.N., et al., *Metabolomic profiling in multiple sclerosis: insights into biomarkers and pathogenesis.* Mult Scler, 2014. **20**(10): p. 1396-400.
93. Mao, P., et al., *MitoQ, a mitochondria-targeted antioxidant, delays disease progression and alleviates pathogenesis in an experimental autoimmune encephalomyelitis mouse model of multiple sclerosis.* Biochim Biophys Acta, 2013. **1832**(12): p. 2322-31.