GRADUATE PROGRAM IN
THE MOLECULAR BASIS OF HUMAN DISEASE

Master Thesis

The role of autophagy in T regulatory cell mediated suppression of immune responses

-Graduate Program: The Molecular Basis of Human Disease,
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Index

Abstract

Extensive research through the years has revealed that during an autoimmune disease, such as multiple sclerosis (MS), impaired T regulatory cell function could be responsible for the unbalanced tolerance against autoantigens, leading to disease susceptibility and affecting the course of autoimmunity. In parallel, studies have demonstrated that even though in the early stages of MS the pathology development is mainly controlled by inflammation, the progression of the disease is affected by mitochondrial impairment. In addition, It has been proved that disorders of mitochondrial dynamics can contribute to the pathogenesis of neurodegenerative diseases as well as in the pathogenesis of autoimmune diseases.

Even though impaired mitochondrial function is found to be implicated in autoimmunity and directly affects the cell growth and survival, the role of defective mitochondrial clearance by autophagy (mitophagy) in Treg cell function and survival, remains to be delineated.

In this context, aim of this study is to further assess the role of autophagy in Treg cells and shed light to the role of mitophagy in T regulatory mediated suppression during an autoimmune response, and more specifically in the context of multiple sclerosis. Understanding the implication of mitochondria clearance in autoimmunity could provide new evidence in MS pathogenesis, as well as in other autoimmune disorders, and lead to the discovery of new therapeutic targets.

Περίληψη

Έρευνες έχουν αποδείξει πως κατά τη διάρκεια ενός αυτοάνοσου νοσήματος, όπως η σκλήρυνση κατά πλάκας, διαταραγμένη λειτουργία των Τ ρυθμιστικών λεμφοκυττάρων μπορεί να ευθύνεται για την μη ισορροπημένη ανοχή έναντι αυτοαντιγόνων που οδηγεί σε ευπάθεια στη νόσο και επηρεάζει την αυτοανοσία. Ταυτόχρονα, μελέτες έχουν δείξει ότι διαταραχές στη λειτουργία των μιτοχονδρίων επηρεάζουν την εξέλιξη της νόσου στη σκλήρυνση κατά πλάκας. Επιπλέον, έχει αποδειχθεί ότι διαταραχές στο δυναμικό των μιτοχονδρίων συμβάλλει στην παθογένεση τόσο νευροεκφυλιστικών, όσο και αυτοάνοσων ασθενειών.

Παρά το γεγονός ότι η διαταραγμένη λειτουργία των μιτοχονδρίων εμπλέκεται στην αυτοανοσία και επηρεάζει άμεσα την επιβίωση των κυττάρων, ο ρόλος της απομάκρυνσης των μη λειτουργικών μιτοχονδρίων μέσω της αυτοφαγίας (μιτοφαγία) στην λειτουργία και επιβίωση των Τ ρυθμιστικών λεμφοκυττάρων δεν έχει περιγραφεί.

Ακολούθως, στόχος της συγκεκριμένης ερευνητικής εργασίας, είναι να διευκρινιστεί περεταίρω ο ρόλος της αυτοφαγίας των Τ ρυθμιστικών λεμφοκυττάρων και να αποσαφηνιστεί το πώς η μιτοφαγία των Τ ρυθμιστικών λεμφοκυττάρων επηρεάζει την καταστολή ανοσολογικών αποκρίσεων γενικότερα, και ειδικά στη νόσο της σκλήρυνσης κατά πλάκας. Η κατανόηση του ρόλου της απομάκρυνσης των διαταραγμένων μιτοχονδρίων από τα Τ ρυθμιστικά λεμφοκύτταρα σε κατάσταση αυτοανοσίας, μπορεί να δώσει νέα στοιχεία στην παθογένεση της σκλήρυνσης κατά πλάκας, καθώς και άλλων αυτοάνοσων νοσημάτων, και να οδηγήσει στην ανακάλυψη νέων φαρμακευτικών στόχων.

Introduction

A) Innate and adaptive immunity

Consisting of both humoral and cellular components, the immune system is a remarkably adaptive biological system having evolved in vertebrates to provide protection against evading pathogens. In order to be effective, it is divided into two major domains that act in a highly interactive and cooperative way, the innate immune system (innate immunity) and the adaptive immune system (adaptive immunity) (Hoebe, Janssen et al. 2004).

The innate immune system refers to the non-specific defense of vertebrates and consists of anatomic and physiologic barriers (skin, mucous membranes, temperature, pH and chemical mediators) as well as phagocytic cells (blood monocytes, neutrophils and tissue macrophages), which conduct the ingestion of pathogens. This process is of utmost importance and provides the first line of defense immediately after a pathogen enters the host's organism. Normally, most of the pathogens are cleared by this system before they activate the adaptive immune system. However, if the pathogen manages to escape from these disease-resistant mechanisms, the specific response of the adaptive immune system is mounted. In this case, lymphocytes (B and T cells) are activated by antigen-presenting cells, which display parts of the antigens on MHC class (Major Histocompatibility Complex) II molecules on their cell surface. After a crosstalk among activated lymphocytes, specific antibodies are produced by differentiated B cells (plasma cells) and specific cytotoxic responses from T lymphocytes begin with the ultimate aim to eliminate the foreign invader. Except for these responses, memory B cells are generated to sustain immunological memory for this pathogen (Hoebe, Janssen et al. 2004) (**Fig.A1**).

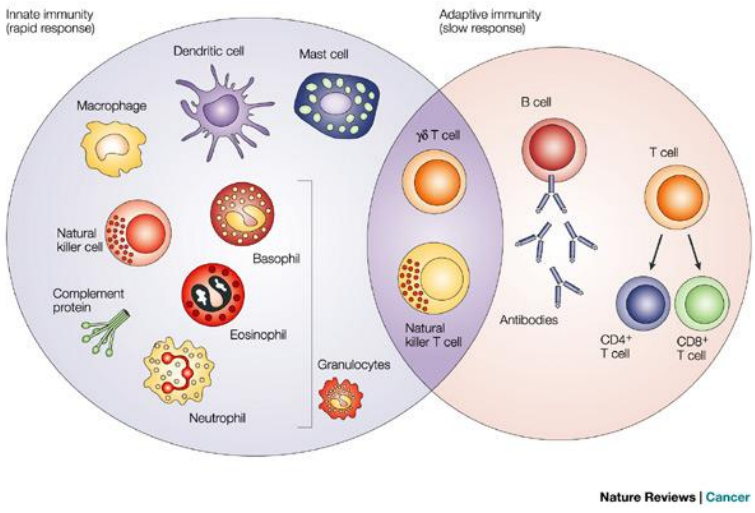

Figure A1: The innate and adaptive immune response. Dranoff et al., 2004

B) Autoimmune responses

Autoimmune response is the immune response in which antibodies or immune lymphoid cells are produced against the body's own tissues. The normal consequence of an adaptive immune response against a foreign antigen is the clearance of the antigen from the body. Virus-infected cells, for example, are destroyed by cytotoxic T cells, whereas soluble antigens are cleared by formation of immune complexes of antibody and antigen, which are taken up by cells of the mononuclear phagocytic system such as macrophages. When an adaptive immune response develops against self antigens, however, it is usually impossible for immune effector mechanisms to eliminate the antigen completely, and so a sustained response occurs. The consequence is that the effector pathways of immunity cause chronic inflammatory injury to tissues, which may prove lethal. The mechanisms of tissue damage in autoimmune diseases are essentially the same as those that operate in protective immunity and in hypersensitivity diseases.

Adaptive immune responses are initiated by the activation of antigen-specific T cells, and it is believed that autoimmunity is initiated in the same way. T-cell responses to self antigens can inflict tissue damage either directly or indirectly. Cytotoxic T-cell responses and inappropriate activation of macrophages by T_H1 cells can cause extensive tissue damage, whereas inappropriate T-cell help to self-reactive B cells can initiate harmful autoantibody responses. Autoimmune responses are a natural consequence of the open repertoires of both B-cell and T-cell receptors, which allow them to recognize any pathogen. Although these repertoires are purged of most receptors that bind with high affinity to self antigens encountered during development, they still include receptors of lower affinity reactive to some self antigens. It is not known what triggers autoimmunity, but both environmental and genetic factors, especially MHC genotype, are clearly important.

B1) Organ specific and systemic autoimmune diseases

It is useful to distinguish two major patterns of autoimmune disease, the diseases in which the expression of autoimmunity is restricted to specific organs of the body, known as 'organ-specific' autoimmune diseases, and those in which many tissues of the body are affected, the 'systemic' autoimmune diseases. Examples of organ-specific autoimmune diseases are Hashimoto's thyroiditis and Graves' disease, each predominantly affecting the thyroid gland, and type I insulin-dependent diabetes mellitus (IDDM), which affects the pancreatic islets. Examples of systemic autoimmune disease are systemic lupus erythematosus (SLE) and primary Sjögren's syndrome, in which tissues as diverse as the skin, kidneys, and brain may all be affected.

The autoantigens recognized in these two categories of disease are themselves respectively organ-specific and systemic. Thus, Graves' disease is characterized by the production of antibodies to the thyroid-stimulating hormone (TSH) receptor in the thyroid gland; Hashimoto's thyroiditis by antibodies to thyroid peroxidase; and type I diabetes by anti-insulin antibodies. By contrast, SLE is characterized by the presence of antibodies to antigens that are ubiquitous and abundant in every cell of the body, such as anti-chromatin antibodies and antibodies to proteins of the pre-mRNA splicing machinery—the spliceosome complex—within the cell.

It is likely that the organ-specific and systemic autoimmune diseases have somewhat different etiologies, which provides a biological basis for their division into two broad categories. Evidence for the validity of this classification also comes from observations that different autoimmune diseases cluster within individuals and within families. The organ-specific autoimmune diseases frequently occur together in many combinations; for example, autoimmune thyroid disease and the autoimmune depigmenting disease vitiligo are often found in the same person. Similarly, SLE and primary Sjögren's syndrome can coexist within a single individual or among different members of a family.

These clusters of autoimmune diseases provide the most useful classification into different subtypes, each of which may turn out to have a distinct mechanism. A working classification of autoimmune diseases based on clustering is given. It can be seen that the strict separation of diseases into 'organ-specific' and 'systemic' categories breaks down to some extent. Not all autoimmune diseases can be usefully classified in this manner. Autoimmune hemolytic anemia, for example, sometimes occurs as a solitary entity and could be classified as an organ-specific disease. In other circumstances it may occur in conjunction with SLE as part of a systemic autoimmune disease (Janeway, Travers et al. 1997) (**Fig.B1.1**).

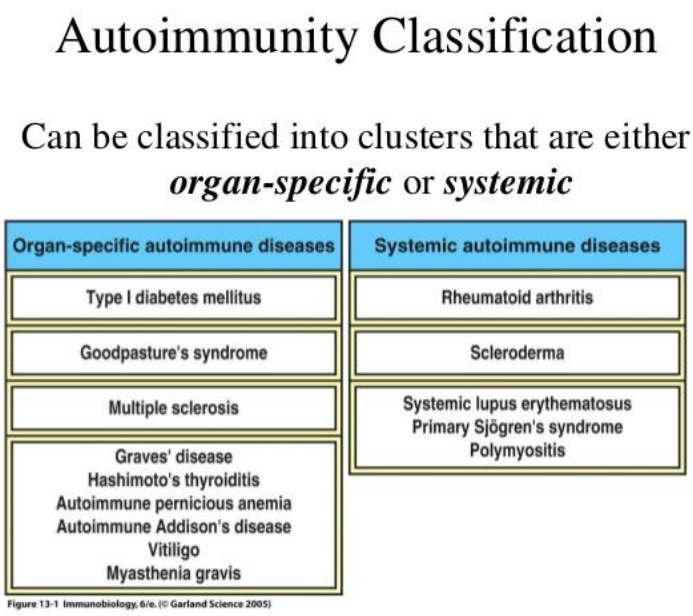

Fig.B1.1: Organ-specific and systemic diseases. Immunobiology, Garland Science, 2005

C) Multiple sclerosis

Multiple sclerosis (MS) is the most common cause of non-traumatic neurological disability affecting approximately 2.5 million people worldwide (Dendrou, Fugger et al. 2015). The onset of MS occurs in early to mid-adulthood and symptoms persist and typically worsen throughout life, resulting in significant health and socioeconomic problems (Aktas, Kieseier et al. 2010). Although the pathogenic pathways leading to MS are not well understood, it is believed to be an autoimmune disease in which self-reactive T cells specific for myelin proteins initiate an inflammatory cascade resulting in demyelination and axonal damage. The extensive heterogeneity in both disease course and pathological features seen in patients with MS suggests that multiple pathogenic pathways contribute to the disease development. The majority of MS patients exhibit a relapsing-remitting disease course (RRMS) in which recurring episodes of neurological symptoms are followed by periods of clinical stability. This stage of MS is characterized by the occurrence of focal inflammatory lesions within the central nervous system (CNS) that are detectable by MRI. Inflammation in the CNS leads to plaques of demyelination, which are a hallmark feature of RRMS. The majority of patients with RRMS have lesions disseminated in the brain and spinal cord; however, the focal lesion burden is typically heavier in the brain than in the spinal cord (Bot, Barkhof et al. 2004). Interestingly, a small subset of spinal cord-dominant patients (~2-3%) have lesions primarily localized in the spinal cord with comparatively less lesion burden in the brain (Bot, Barkhof et al. 2004). The mechanisms underlying these distinct neuroinflammatory patterns are not known. RRMS can persist for many years; however, approximately 80% of RRMS patients eventually convert to the secondary progressive stage of disease (SPMS) in which the extent of recovery after each episode of neurological deficit diminishes (Dendrou, Fugger et al. 2015). In contrast to RRMS, gadolinium-enhancing MRI lesions are less common during SPMS, despite the steady increase in brain atrophy and disability that occurs during this stage of disease (Segal 2014).

There are four key pathological features of MS: (a) inflammation, of complex pathogenesis, which is generally believed to be the main trigger of the events leading to CNS tissue damage in the majority of cases, although recent evidence suggests that initial damage to neuroglial elements can trigger secondary inflammation in some cases (Barnett and Prineas 2004); (b) demyelination, the hallmark of MS, where the myelin sheath or the oligodendrocyte cell body is destroyed by the inflammatory process; (c) axonal loss or damage; and (d) gliosis (astrocytic reaction to CNS damage). There is a certain degree of remyelination, which offers hope for therapies aimed at enhancing endogenous repair mechanisms in various experimental models (see below) but is partial and its efficiency is limited.

In addition to the clinical heterogeneity there is pathological heterogeneity, in terms of the relative proportion of the above key pathological features and the components of cellular and humoral immune response elements that mediate the inflammation. The pathological correlate of relapses is inflammation and disruption of the blood–brain barrier (BBB), clinical relapses being thought to correspond to fresh waves of inflammatory cell infiltration in the CNS.

The pathological correlate of long-term disability and progression is irreversible axonal loss. The acute MS lesion is characterized by inflammatory infiltrates with various immune cells and active demyelination (macrophages with myelin debris in their cytoplasm); when this lesion becomes chronic, there is significant loss of myelin with few if any inflammatory infiltrates and gliosis, which gives lesions their 'plaque' appearance (Constantinescu, Farooqi et al. 2011).

Axonal loss is most severe in the chronic plaques, but it is also present in what is known as the normal-appearing white matter (NAWM), or normal-appearing brain tissue (NABT), to take into account pathological changes in the normal appearing gray matter as well (Trapp, Peterson et al. 1998).

C1) Pathogenesis of MS

The interaction between multiple components of the immune system and all elements of the CNS determine the pathogenesis of MS.

During the course of MS, T cells in the periphery become activated by a viral or another infectious antigen or a superantigen. These show molecular similarity (mimicry) with some CNS antigen (Sospedra and Martin 2005). These T cells are capable of producing inflammatory cytokines and may be differentiated or have the potential to differentiate on activation into Th1 (producing IFN-gamma) or Th17 cells (IL-17, IL-22, IL-21) or cells producing both (McFarland and Martin 2007). Activated T cells up-regulate integrins such as VLA-4 and are capable of crossing the blood-brain barrier (BBB). Through the permeabilized BBB, attracted by chemokine release, other immune cells including B cells and monocytes/macrophages migrate into the CNS. There, they encounter the cognate antigen, probably derived from myelin antigen, presented by CNS resident or immigrant antigen-presenting cells (APC). These can be macrophages/microglia and in certain instances dendritic cells or astrocytes. On encountering the antigen, such autoreactive T cells are reactivated and differentiate, producing their signature cytokines, which activate the neighbouring immune or neural cells and attract further inflammatory cells into the CNS. Of these, it is especially activated macrophages that are thought to indirectly and directly damage the CNS. Myelin is phagocytosed by macrophages (Barnett, Henderson et al. 2006). Elements of the humoral immune response and soluble mediators also contribute to the pathology, via complement activation, direct cytokine cytotoxicity, nitric oxide, reactive oxygen and nitrogen species (Hemmer, Nessler et al. 2006). Plasma cells

produce antibodies, which can bind and activate complement or induce antibody-dependent cytotoxicity. Th2 cells (producing IL-4) may enhance antibody production. CD8 (cytotoxic) T cells may enhance the damage through further cytokine production as well as granzyme and perforin production and can directly transect axons (Fletcher, Lalor et al. 2010). The resolution of inflammation, which can be partial and subject to recrudescence, occurs when anti-inflammatory cytokines (e.g. IL-10) and other immunoregulatory mechanisms such as regulatory T cells (Treg) or NK cells come into play. The consequence is that the myelin is destroyed and typically lacks full regeneration potential, especially after repeated injury, and the axons degenerate, in part because they are devoid of myelin and more exposed and deprived of trophic support, in part through wallerian degeneration and metabolic injury (Piaton, Williams et al. 2009) (**Fig.C1.1**)

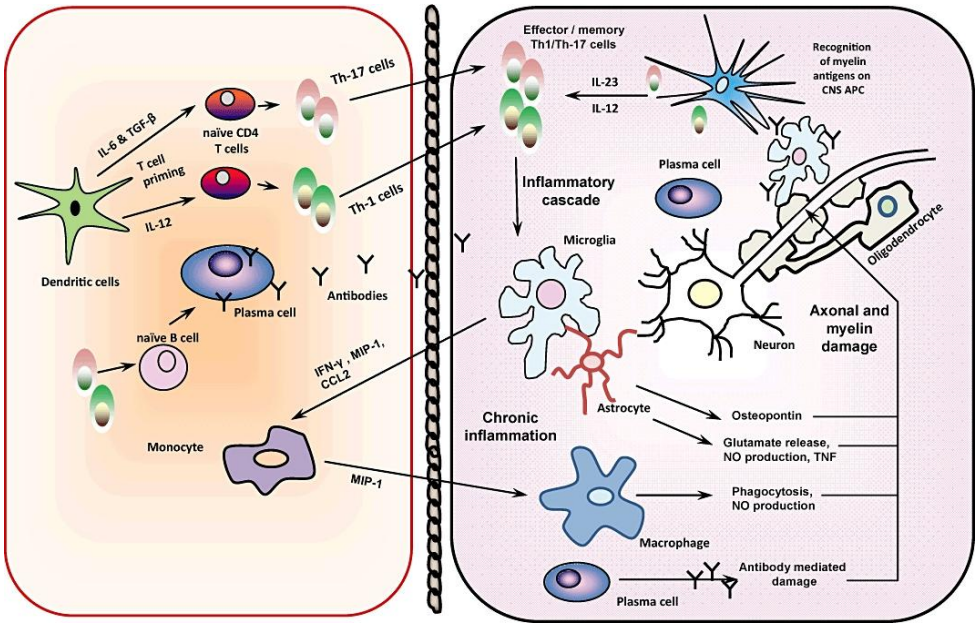

Fig.C1.1: Schematic diagram of some of the key pathological features of MS pathogenesis. Constantinescu et al., 2011

C2) Experimental autoimmune encephalomyelitis (EAE)

MS has been extensively studied using the animal model of experimental autoimmune encephalomyelitis (EAE). Experimental autoimmune encephalomyelitis is a T-helper (Th) cell-mediated autoimmune disease characterized by T-cell and monocyte infiltration in the central nervous system (CNS) associated with local inflammation. The autoimmune molecular target(s) identified and utilized have been proteins expressed by myelin-producing oligodendrocytes in the CNS. The result is primary demyelination of axonal tracks, impaired axonal conduction in the CNS, and progressive hind-limb paralysis. EAE is commonly employed as a model for multiple sclerosis (MS) and as such has been a powerful tool for studying disease pathogenesis as well as potential therapeutic interventions. There are currently many pathophysiologic forms of EAE with varying patterns of clinical presentation depending on the animal species and strain, priming protein/peptide, and route of immunization employed. Thus different models have been used to study disease development and specific histopathologic characteristics with relevance to MS, and to dissect mechanisms of potential therapeutic interventions.

EAE in the mouse was first induced over 60 years ago, by active immunization with spinal cord homogenates (Olitsky and Yager 1949). Extensive research has led to the discovery of numerous encephalitogenic peptides, and mice remain the most commonly employed animal species, in part due to the wide availability of transgenic and knockout mice available for targeted mechanistic studies. In the SJL ($H\text{-}2^{s}$) mouse, EAE can be actively induced by immunization with CNS homogenate, proteolipid protein (PLP), myelin basic protein (MBP), or encephalitogenic epitopes of PLP ($PLP_{139–151}$, $PLP_{178–191}$), myelin oligodendrocyte protein ($MOG_{92–106}$), or MBP ($MBP_{84–104}$) in an emulsion with complete Freund's adjuvant (CFA). The disease follows a predictable clinical course, characterized by a prodromal period of 10–15 days followed by ascending paralysis beginning in the tail and hind limbs and progressing to the fore-limbs concurrent with weight loss. In SJL mice the disease is characterized by a relapsing-remitting course of paralysis, allowing for mechanistic studies or immunomodulatory strategies in a relapsing autoimmune disease setting. $MOG_{35–55}$ is a potent encephalitogen in C57BL/6 ($H\text{-}2^{b}$) mice, which presents clinically in the form of a chronic progressive disease course. EAE can be induced in other mouse strains, e.g., PL/J and B10.PL ($H\text{-}2^{u}$), but is normally acute and rectifying. Strain and immunizing antigen variations continue to be explored for atypical manifestations (inflammation, mononuclear cell (MNC) infiltration, and clinical presentation) pertinent to the heterogeneous forms of MS presentation and pathology. For example, a novel clinical form of disease was recently reported displaying a relapsing-remitting course that developed into a chronic progressive phenotype with lesions in the brain as well as the spinal cord (Levy, Assaf et al.

2010). This model may be particularly relevant to the most prevalent form of MS exhibiting relapsing-remitting followed by secondary progressive disease.

Method of immunization can be manipulated as well, allowing for more targeted studies of immunopathologic mechanisms. Whereas active EAE studies can be confounded by the robust immune response to the adjuvant itself, EAE can also be induced by adoptive transfer, whereby T cells are isolated from myelin peptide/protein-primed donors, stimulated *in vitro* with an encephalitogenic peptide, and resulting blast cells injected intravenously (i.v.) or intraperitoneally (i.p.) into naïve or immunodeficient recipient mice. This method allows for *in vitro* manipulation of the encephalitogenic T-cell population and disease induction with a fairly homogeneous population of antigen-specific T cells. Adoptive transfer of disease using T-cell receptor (TCR) transgenic mice allows for the study of myelin antigen-specific T cells (e.g., C57BL/6 2D2 $MOG_{35–55}$-specific or SJL/J 5B6 $PLP_{139–151}$-specific). "Humanized" mice expressing human TCRs specific for myelin epitopes presented by human major histocompatibility complex (MHC) class II molecules associated with genetic susceptibility to MS are also commercially available (e.g., a TCR specific for human $MBP_{84–102}$ bound to human leukocyte antigen (HLA)-DR2). Finally the adoptive transfer model is ideal for localizing T-cell populations *in vivo* throughout disease, as transferred cells can be labeled *in vitro* with fluorescent protein/dye or derived from congenic mice, allowing for *in vivo* tracking of encephalitogenic T-cell populations.

D) T regulatory cells (Tregs)

T regulatory cells, also known as suppressor T cells, are a subpopulation of T cells which modulate the immune system, maintain tolerance to self-antigens, and abrogate autoimmune disease. Regulatory T cells (Tregs) are critical to the maintenance of immune cell homeostasis as evidenced by the catastrophic consequences of genetic or physical ablation of the Treg population. Specifically, Treg cells maintain order in the immune system by enforcing a dominant negative regulation on other immune cells.

T regulatory cells are broadly classified into thymous-derived (natural) or adaptive (induced) Treg cells. Thymous-derived Treg cells are CD4+CD25+ T-cells which develop, and emigrate from the thymus to perform their key role in immune homeostasis. Induced Treg cells are non-regulatory CD4+ T-cells which acquire CD25 (IL-2R alpha) expression outside of the thymus, and are typically induced by inflammation and disease processes, such as autoimmunity and cancer (Sakaguchi, Yamaguchi et al. 2008).

T regulatory cells specifically express the transcription factor Foxp3 (forkhead box P3), a member of the forkhead/winged-helix family of transcription factors. Foxp3 is a master regulator of Treg development and function. The *Foxp3* gene was first

identified as the defective gene in the mouse strain Scurfy. Surfy is an X-linked recessive mutant that is lethal in hemizygous males within a month after birth, exhibiting hyperactivation of $CD4^+$ T cells and overproduction of proinflammatory cytokines. Mutations of the human gene *Foxp3* are the cause of the genetic disease IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome), which is the human counterpart of Scurfy. Experimental studies revealed that $CD25^+CD4^+$ peripheral T cells and $CD25^+CD4^+CD8^-$ thymocytes in normal mice express Foxp3, whereas other thymocytes/T cells, either in a resting or activated state, do not. Ectopic retroviral transduction of the *Foxp3*gene in $CD25^-CD4^+$ T cells can convert them to $CD25^+CD4^+$ Treg-like cells that are able to suppress proliferation of other T cells in vitro and inhibit the development of autoimmune disease and inflammatory bowel disease in vivo (Hori, Nomura et al. 2003). *Foxp3* transduction in naive T cells also upregulates the expression of CD25 and other Treg-associated cell-surface molecules, such as cytotoxic T cell-associated antigen-4 (CTLA-4) and glucocorticoid-induced TNF receptor family-related gene/protein (GITR), whereas it represses the production of IL-2, IFN-γ, and IL-4. Both Foxp3-deficient mice and Scurfy mice, whose Foxp3 protein lacks the forkhead domain, harbor few $CD25^+CD4^+$ Tregs, and inoculation of $CD25^+CD4^+$ T cells from normal mice prevents severe systemic inflammation in Scurfy mice (Fontenot, Gavin et al. 2003). In bone marrow chimera with a mixture of cells from wild-type and *Foxp3*-deficient mice, *Foxp3*-deficient bone marrow cells failed to give rise to $CD25^+CD4^+$ Tregs, whereas *Foxp3*-intact bone marrow cells generated Tregs that suppressed disease development. Conversely, in transgenic mice that overexpress Foxp3, the number of $CD25^+CD4^+$ T cells is enhanced; $CD25^-CD4^+$ T cells and $CD8^+$ T cells expressed high levels of *Foxp3* and exerted suppression in vitro (Khattri, Cox et al. 2003). Finally, analyses revealed that Foxp3-expressing T cells appear shortly after birth and show that the development of autoimmune/inflammatory disease follows their depletion (Lahl, Loddenkemper et al. 2007).

Thus, the transcription factor Foxp3 is critical for α/β TCR-positive T cells to differentiate to Tregs in the thymus. High-level expression of Foxp3 is sufficient to confer suppressive activity to normal non-Treg cells. Further, genetic disruption of Foxp3 in humans and mice provides unequivocal evidence that dominant self-tolerance is operating in both species. Foxp3 is currently the most reliable molecular marker for Tregs and provides clues with which to decipher the molecular and genetic basis of Treg development and function.

D1) Treg cell activation, proliferation and differentiation

Upon antigen exposure in the regional lymph nodes, $Foxp3^+$ Tregs become activated and exert suppression at a much lower concentration of antigen than naive T cells. In TCR transgenic mice, the concentration of peptide required to activate the peptide-specific Tregs to exert suppression in vitro has been estimated to be 10- to 100-fold lower than the concentration needed for activating naive T cells with the same antigen specificity (Takahashi, Kuniyasu et al. 1998). This finding suggests that natural Tregs can be activated even by immature dendritic cells whose expression levels of CD80/86 and self-peptide/MHC are too low to activate naive self-reactive T cells. This might enable natural Tregs to exert dominant and tonic suppression of self-reactive T cells. It could also contribute to the prevention of autoimmunity stemming from molecular mimicry (the antigenic crossreaction between a self-molecule and a microbial substance) because natural Tregs could be more easily activated than self-reactive T cells by such a substance (Stephens, Gray et al. 2005).

Notably, unlike their hypoproliferation upon antigenic stimulation in vitro, a sizable fraction of $Foxp3^+$ natural Tregs are continuously proliferating in vivo presumably through the recognition of self-antigen and commensal microbes (Setoguchi, Hori et al. 2005). Natural Tregs can also expand clonally in vivo and in vitro following antigenic stimulation and retain their suppressive function after expansion (Fehervari and Sakaguchi 2004) (Yamaguchi, Hirota et al. 2007).

Irrespective of active proliferation the number of $Foxp3^+$ natural Tregs is fairly constant in normal animals (10%–15% of $CD4^+$ T cells), indicating that cell death helps to maintain Treg homeostasis. Indeed, following antigenic stimulation Tregs downregulate Bcl-2, an antiapoptotic protein (Yamaguchi, Hirota et al. 2007). Further, among $Foxp3^+CD4^+$ T cells in humans, antigen-stimulated naive $Foxp3^{low}$ cells differentiate into effector/memory-like $Foxp3^{high}$ cells, which vigorously proliferate and then die.

Thus, $Foxp3^+$ Tregs that have migrated to lymphoid and nonlymphoid tissues become activated, proliferate, exert suppressive activity, and then die. However, it remains to be determined whether some Tregs further differentiate into long-lived memory-type cells.

D2) Treg cell mechanisms of suppression

Tregs can suppress a variety of immune cells including B cells, NK cells, NKT cells, $CD4^+$, and $CD8^+$ T cells, as well as monocytes and dendritic cells (DCs). In the following, I will describe the suppression of $CD4^+CD25^-$ conventional T cells (Tcons).

Upon cell–cell contact, Tregs inhibit TCR-induced proliferation and IL-2 transcription of Tcons, as shown for murine Tregs already in 1998 (Thornton and Shevach 1998). Suppression of murine or human Tcon proliferation by Tregs can occur directly, i.e.,

in the absence of antigen presenting cells (APCs). This direct suppression can involve immunosuppressive cytokines or other factors; however, contact-dependent direct suppression has also been described. In addition, Tregs can inhibit Tcons indirectly by influencing the activation status of APCs and therefore activation of Tcons. Based on these properties, a standard assay to assess Treg function is inhibition of responder Tcon proliferation upon stimulation via the TCR in the presence of APCs. A main function of Tregs is suppression of activation and expansion of naïve Tcons, but they can also inhibit activated effector T cells and memory $CD4^+$ (Levings, Sangregorio et al. 2001) and $CD8^+$ (Suvas, Kumaraguru et al. 2003) T cells. To be suppressive, Tregs themselves have to be TCR-activated in the presence of IL-2 (Takahashi, Kuniyasu et al. 1998) (de la Rosa, Rutz et al. 2004), while costimulation via CD28 is dispensable (Takahashi, Tagami et al. 2000). However, a more recent study questioned the requirement for Treg activation: Szymczak-Workman et al. showed that TCR-transgenic Tregs were able to suppress Tcons with different antigen specificity in the absence of the Treg-cognate antigen. Differences in the type of APCs used in the assays, the transgenic system, or “pre-activation” by the cell purification procedure might provide an explanation for the controversy (Szymczak-Workman, Workman et al. 2009). Yet, the study is in line with other reports that show that Tregs, once active, can suppress Tcons independently of antigen, leading to so-called bystander suppression (Karim, Feng et al. 2005).

T regulatory cells have been described to suppress Tcons by different mechanisms, depending on the experimental setup, site and type of immune response. Regulatory T cells can suppress by four basic mechanisms. The interaction between cytotoxic T lymphocyte antigen-4 (CTLA4) and CD80/CD86, expressed by antigen presenting cells (APCs), leads to CD80/CD86 down-regulation. Removal of these co-stimulatory molecules modulates APC function, limiting the initiation of an adaptive immune response. Tregs induce effector T cell (Teff) apoptosis by the interaction between Galectin-9 (Gal-9) and the T cell immunoglobulin and mucin domain-3 (TIM-3), and by the release of granzymes which enter Teffs via perforin pores. Tregs release the anti-inflammatory cytokines TGFβ, IL10 and IL35. Treg expression of the ecto-enzymes CD39 and CD73 enables the hydrolysis of pro-inflammatory adenosine triphosphate (ATP) into anti-inflammatory adenosine (ADO). All the possible mechanisms of Treg-mediated suppession are summarized in Figure D2.1 (**Fig.D2.1**).

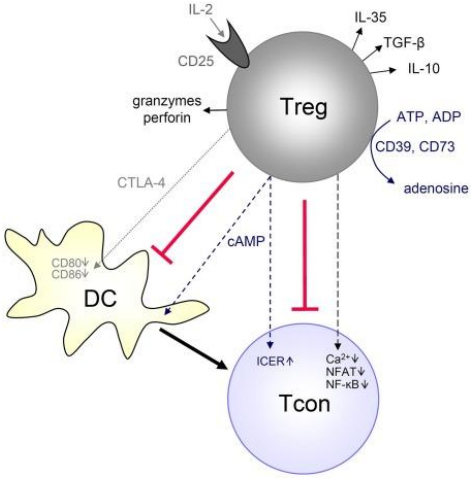

Fig.D2.1. Mechanisms of Treg-mediated Tcon suppression. Schmidt et al.,2012

D3. T regulatory cells in autoimmune diseases

Treg impairment has been reported in a number of human autoimmune conditions and includes Treg numerical and functional defects and conversion into effector cells in response to inflammation. In addition to intrinsic Treg impairment, resistance of effector T cells to Treg control has been described. Discrepancies in the literature are common, reflecting differences in the choice of study participants and the technical challenges associated with investigating this cell population.

The first suggestion that Tregs were defective in autoimmunity in humans was based on studies in MS patients where Baecher-Allan et al. showed that peripheral blood CD41CD25bright cells were defective in function and clonal expansion (Viglietta, Baecher-Allan et al. 2004). To date, studies have shown that in health, Tregs maintain tolerance by exerting suppression of effector T cells. In organ specific autoimmune disease, Tregs fail to suppress autoreactive effector T cells, therefore leading to target cell death. Reported reasons for this include inadequate numbers of Tregs, impaired suppressive ability, Treg conversion into effector cells and resistance of effector T cells to Treg-mediated suppression (Grant, Liberal et al. 2015), (**Fig.D3.1**).

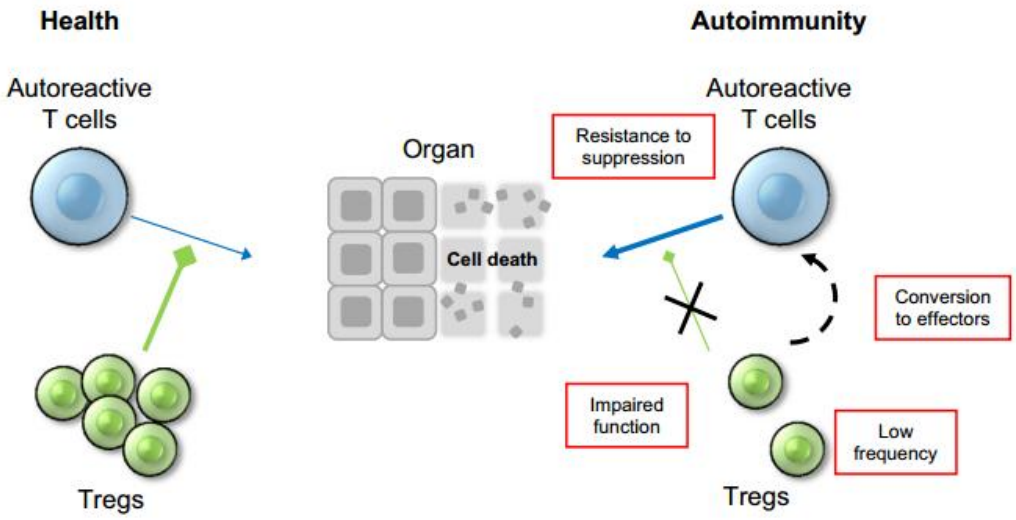

Fig.D3.1. Regulatory T cell defects in autoimmunity. Grant et al., 2015

E) Autophagy

The term autophagy refers to a collection of diverse processes — including macroautophagy, microautophagy, chaperone-mediated autophagy (Mizushima, Yoshimori et al. 2011) and non-canonical autophagy — that enable cells to digest their cytoplasmic contents in lysosomes. Macroautophagy (hereafter referred to as ‘‘autophagy’’) initiates with the sequestration of organelles or portions of the cytoplasm within double-membraned vesicles, called autophagosomes. Autophagosomes then fuse with lysosomes to generate autolysosomes which content is degraded (Codogno, Mehrpour et al. 2012).

E1) Autophagic machinery- key players

Autophagy involves 3 morphological stages: initiation (formation of phagophores), elongation and closure (increase in the size of the phagophore and its closure into a completed autophagosome), and maturation (conversion of autophagosomes into degradative organelles, termed autophagolysosomes, by fusion with late endosomal and lysosomal organelles). The serine/ threonine kinase mammalian target of rapamycin (mTOR) plays a major role in the regulation of autophagy. The rapamycin-sensitive mTORC1 complex promotes mRNA translation and inhibits autophagy by integrating nutrient signals that are generated by amino acids, growth factors, energy and various stressors including hypoxia and DNA damage (Zoncu, Efeyan et al. 2011).

In mammals, the core autophagic pathway starts with the formation of an isolation membrane (also known as a phagophore), most often at contact sites between mitochondria and the endoplasmic reticulum (ER). The phagophore’s membrane then expands forming the so called autophagosome (Hamasaki, Furuta et al. 2013). The journey into the molecular realm of autophagy began with the identification of the AuTophaGy-related (ATG) genes. Among these ATG genes, one subset of genes is required for autophagosome formation. Autophagosome formation requires two ubiquitin-like conjugation systems, the Atg12 and Atg8 system, which are tightly associated with the expansion of the autophagosomal membrane (Xie and Klionsky 2007). Atg12 is conjugated to Atg5, forming the irreversible Atg12-Atg5 conjugate. During autophagosome formation the Atg12-Atg5 conjugate complexes with Atg16L; this complex dimerizes and associates with the exterior membrane of the phagophore. Upon completion of autophagosome formation, the Atg12–Atg5–Atg16 complex is released into the cytosol (Walczak and Martens 2013).

Another protein that decorates the autophagosome’s membrane is the microtubule-associated protein 1A/1B-light chain 3 (LC3). LC3 is a mammalian homolog of the yeast ATG8 protein, a ubiquitin-like protein that becomes lipidated and tightly associated with the autophagosomal membranes (Kabeya, Mizushima et al. 2000). A cytosolic form of LC3 (LC3-I) is conjugated to phosphatidylethanolamine

(PE) to form LC3-PE conjugate (LC3-II), which is recruited to autophagosomal membranes. In the autophagosome, LC3-II is distributed to both the exterior and the lumen of the vesicle. Superficial LC3-II is removed by cleavage of the PE by Atg4, while the luminal LC3-II is digested along with the cargo. LC3-II is essential for autophagosome biosynthesis and may be involved in membrane closure (Longatti and Tooze 2009). The final stages of the biogenesis of the autophagosome include the closure to form a double membrane vesicle.

LC3-II recruits the cargo adaptor protein p62. The p62 protein, also called sequestosome 1 (SQSTM1), is commonly found in inclusion bodies containing polyubiquitinated protein aggregates. In neurodegenerative diseases p62 is detected in ubiquitinated protein aggregates, including Lewy bodies in Parkinson disease, neurofibrillary tangles in Alzheimer disease, and Huntingtin aggregates in Huntington disease (Kuusisto, Salminen et al. 2001). p6 has been shown to bind directly to Atg8/LC3 to facilitate degradation of ubiquitinated protein aggregates by autophagy (Pankiv, Clausen et al. 2007).

Autophagosomes subsequently fuse with lysosomes, forming the mature autophagolysosome, exposing the inner compartment to lysosomal hydrolases which digest proteins, lipids, and nucleic acids in an acidic microenvironment (Kroemer and Jaattela 2005). Eventually the inner membrane of the autophagosome, together with the enclosed cargo, LC3-II and p62 proteins, is degraded and the resulting macromolecules are released into the cytosol through lysosomal membrane permeases for recycling (Kim and Lee 2014) (**Fig.E1.1)**.

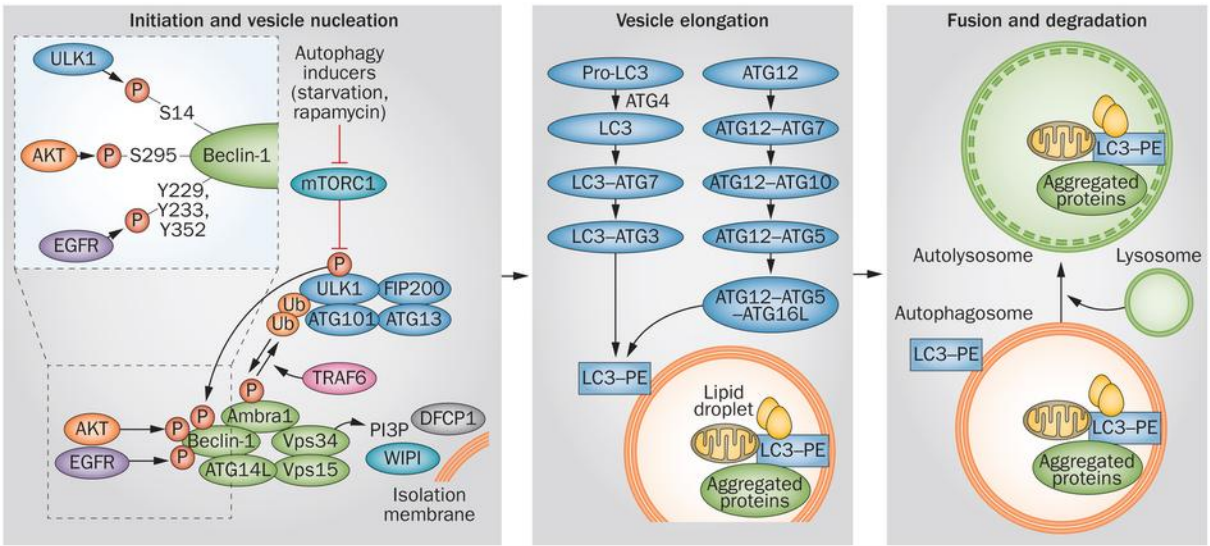

Fig.E1.1. Autophagy is regulated by multiple signaling pathways. Kim et al.,2014

Luminal LC3-II can be preserved by inhibiting its lysosomal degradation with protease inhibitors, such as the proton pump inhibitor, bafilomycin A1 or the lysosomotropic agent chloroquine (CQ) that prevents endosomal acidification. LC3-II/I ratio is often used as a marker for the index of autophagy (Kadowaki and Karim 2009).

Autophagy not only preserves cellular homeostasis in conditions of endogenous distress (Kroemer, Marino et al. 2010) but also plays a primordial role in controlling intracellular pathogens in evolutionarily distant species, ranging from unicellular organisms to humans (Levine and Kroemer 2008). Among the many functions of autophagy are cellular homeostasis (Levine and Klionsky 2004), anti-aging (Vellai, Takacs-Vellai et al. 2003) and development (Kuma, Hatano et al. 2004). Recent evidence indicates that autophagic responses in antigen-donor cells affect the release of several cytokines and ‘‘danger signals’’. Thus, especially when it precedes cell death, autophagy alerts innate immune effectors to elicit cognate immune responses. Autophagy is also important for the differentiation, survival, and activation of myeloid and lymphoid cells. Accordingly, inherited mutations in autophagy-relevant genes are associated with immune diseases, whereas oncogenesis-associated autophagic defects promote the escape of developing tumors from immunosurveillance. There are multiple mechanisms through which the cell-intrinsic regulation of autophagy is connected to cell-extrinsic stress response pathways. This is highlighted by the fact that although autophagy constitutes a cell-autonomous mechanism for the control of noninfectious stress and microbial pathogens is stimulated or inhibited by multiple soluble factors. In addition, autophagy can modulate the production of various cytokines (Ma, Galluzzi et al. 2013). In addition, it has been shown that autophagy is upregulated when cells are confronted with potentially dangerous environmental cues, like physical (thermal stress, irradiation), chemical (changes in pH, osmolarity), or metabolic (shortage in nutrients or oxygen) (Apel, Herr et al. 2008) (Xu, Su et al. 2011) (Boya, Reggiori et al. 2013).

E2) Autophagy in T regulatory cells

Owing to the cells fundamental necessity for autophagy, intense research on T cell implication of autophagy has been carried out. It has been proved that T cells indirectly depend on autophagy, maintaining HSC metabolism and differentiation, as well as regulating the presentation of peptides by APCs during positive and negative selection of thymocytes and during activation of mature T cells in the periphery (Bronietzki, Schuster et al. 2015).

Concerning Treg cells, there are only few studies indicating that autophagy directly regulates the development of Treg cells and supports their lineage stability and

survival fitness (Parekh, Wu et al. 2013) (Wei, Long et al. 2016). More specifically, Wei et al. demonstrated that Treg cell-specific deletion of Atg7 or Atg5 leads to loss of Treg cells, greater tumor resistance and development of inflammatory disorders. Atg7-deficient Treg cells showed increased apoptosis and readily lost expression of Foxp3, especially after activation. Finally, they proved that autophagy inhibition upregulated the metabolic regulators mTORC1, c-Myc and glycolysis, which contributed to defective Treg cell function (Wei, Long et al. 2016).

F) Mitochondria

It is well-known that mitochondria are maternally inherited double membrane-bound organellesfound in most eukaryotic cells. In 1890, Altman described them for the first time as ''bioblasts'' and later on, in 1898, Benda managed to observe their heterogeneous morphologic features. More specifically, the fact that they are sometimes ball-shaped and other times linear, led to the inspiration of the name mitochondrion, coming from the Greek words mitos (meaning thread) and chondrion (meaning granule) (O'Rourke 2010).

It was Merezhkovsky in 1905 and then Margulis in 1967 who proposed independently that mitochondria are derived from eubacterial endosymbionts and are capable of aerobic respiration. To date, we know that the aerobic alpha-proteobacteria are the precursors of mitochondria (Kroemer 1997). Moreover, the observations by Lewis in 1914 established the field of mitochondrial dynamics, since they form a dynamic interconnected intracellular network, moving through the use of cytoskeletal motors and undergoing continuously the process of mitochondrial fission and fusion (even when the cells are resting). As it was noted: ''Any one type of mitochondria such as a granule, rod or thread may at times change into any other type or may fuse with another mitochondrion, or it may divide into one or several mitochondria'' (Lewis and Lewis 1914).

Mitochondria range from 0.5 to 1.0 μm in diameter and obtain several characteristics that make them unique. First of all, the number of mitochondria inside a cell can vary widely depending on the organism, tissue and cell type. For instance, red blood cells lack mitochondria, whereas in liver cells more than 2000 mitochondria can be identified. Furthermore, mitochondria possess their own genome, transcriptome and proteome (Cloonan and Choi 2012). The human mitochondrial genome is a circular DNA molecule consisting of about 16 kilobases and by which 37 genes are encoded: 13 for subunits of complexes I, III, IV and V of the respiratory Electron Transfer Chain (ETC), 22 for mitochondrial tRNA (for the 20 standard amino acids, plus an extra gene for leucine and serine), and 2 for rRNA. One mitochondrion can contain two to ten copies of it's DNA (Wiesner, Ruegg et al. 1992).

Regarding their function, mitochondria are considered the main intracellular producers of energy (heat and ATP). Every single mitochondrion is able to carry out oxidative phosphorylation (OXPHOS) with the use of it's Electron Transfer Chain. During this process, the metabolic products generated from the Krebs cycle drive the generation of a proton gradient at the inner mitochondrial membrane (IMM), providing by this way the energy required for ATP generation. In addition to ATP 13 production, mitochondria have the ability to sense oxygen, calcium and fuel (such as carbohydrates and fatty acids), they are manufacturers of several metabolites as well as Reactive Oxygen Species (ROS) and also effective inducers of programmed cell death (apoptosis). Finally, it has been shown that they can sense danger signals and subsequently induce inflammation by activating and controlling the immune system (Cloonan and Choi 2013).

F1) Mitochondrial Dynamics- Mitochondrial Fission and Fusion

Undoubtedly, mitochondrial dynamics plays a pivotal role regarding cell growth and survival. It has been shown that disorders of mitochondrial dynamics can contribute to the pathogenesis of complex diseases that are not classically considered to involve mitochondria, such as cancer, cardiovascular disease as well as neurodegenerative diseases.

The balance between the rates of mitochondrial fission and fusion determines the length of mitochondria as well as mitochondrial redistribution inside the cell. It should be mentioned that mitochondria often exist as solitary units. However, they do occasionally fuse with other mitochondria so that they can form a healthy closed network that enhances communication with the Endoplasmic Reticulum (ER). Furthermore, fusion contributes to the diffusion of matrix content among mitochondria, diluting by this way the accumulated mitochondrial DNA mutations (Santel, Frank et al. 2003) and oxidized proteins. On the other hand, mitochondria can exit from this network by the process of fission, which gives rise to smaller, more discrete mitochondria with different membrane potential. Subsequently, the depolarized mitochondria (with persistently low membrane potential) are eliminated via mitophagy, whereas those ones that recover are capable of rejoining the mitochondrial network by fusion.

Both mitochondrial fission and fusion are mediated by a small number of highly conserved guanosine triphosphates (GTPases) that belong to the Dynamin family. Mitofusins (Mfn1, Mfn2) and Optic Atrophy 1 (OPA1) are proteins responsible for fusion; Dynamin-Related Protein 1 (DRP1) is involved in fission (van der Bliek, Shen et al. 2013). Mfn1 and Mfn2 are located in the outer mitochondrial membrane and tether adjacent mitochondria. They fulfill partially redundant function, since it has been observed that both of them are capable of supporting mitochondrial fusion by themselves. OPA1 is a GTPase located in the inner mitochondrial membrane and

mediates fusion of this membrane. Finally, DRP1 is a cytosolic GTPase that, once activated, translocates to the outer mitochondrial membrane. Following that, DRP1 multimerizes and creates a ringlike structure that constricts and divides the organelle. A number of non-GTPase receptor proteins activate DRP1 and target it to the outer mitochondrial membrane. These are the following ones: Mitochondrial fission 1 protein (Fis1), Mitochondrial fission factor (MFF), and Mitochondrial elongation factor 1. Interestingly, the fission apparatus is assebled with the assistance of Endoplasmic Reticulum (ER) which is in contact with the mitochondria (Hoppins and Nunnari 2012) (**Fig.F1.1**).

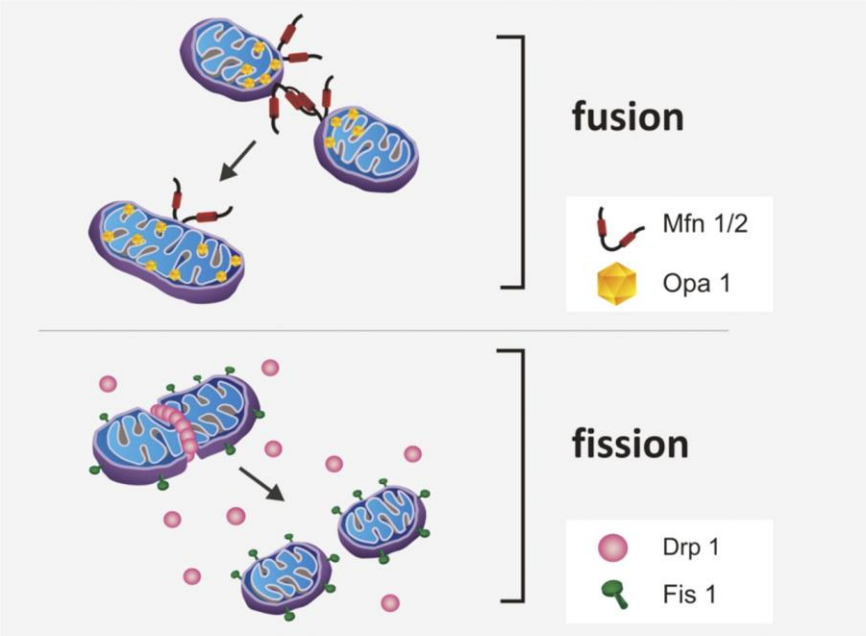

Fig. F1.1. Functions of the mitochondrial Dynamin family members. Hoppins et al.,2012

Moreover, mitochondrial outer membrane fusion occurs in most of the cases with concomitant inner membrane fusion. However, outer membrane fusion can take place without being coordinated with the inner membrane fusion when the latter one is blocked due to mutations or loss of membrane potential (Olichon, Baricault et al. 2003). Generally, mitochondrial fusion mediators are regulated by proteolysis and ubiquitination, whereas mitochondrial fission proteins are regulated by several protein modifications, such as phosphorylation, ubiquitination, sumoylation and nitrosylation (van der Bliek, Shen et al. 2013).

G) Mitophagy

It is well-known that the bulk degradation of cellular components occurs through a highly regulated process named autophagy. Additionally, mitophagy is considered as a selective form of autophagy that is chiefly responsible for the digestion and subsequent elimination of damaged or superfluous mitochondria. The term 'mitophagy' was coined by Lemasters in 2005 (Lemasters 2005). In this process, mitochondria are sequestered in double-membrane vesicles and delivered to lysosomes for degradation via the action of lysosomal hydrolytic enzymes (**Fig.G1**).

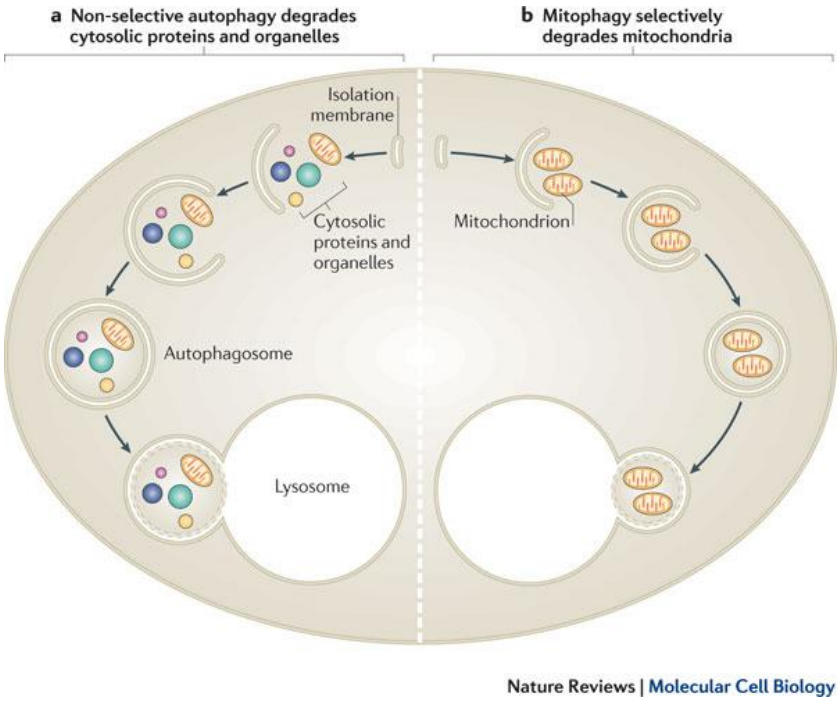

Fig.G1. Non-selective autophagy and mitophagy. Youle et al., 2011

Although the turnover and clearance of dysfunctional/depolarized (low Δψm) mitochondria and/or mitochondria producing the highest levels of ROS may represent the primary functions of mitophagy, some other specialized cases of mitophagy for the regulation of organelle number in response to developmental or physiological cues have been identified as well. One of these cases is the complete removal of mitochondria during erythrocyte maturation that occurs through the action of a specific mitochondrial protein named Nix (Saraste 1999). Moreover, the selective destruction of sperm-derived mitochondria after oocyte fertilization takes place also via the process of mitophagy (Al Rawi, Louvet-Vallee et al. 2011).

G1) The PINK1/Parkin pathway of Mitophagy

A recently identified pathway that has emerged as a paradigm for mammalian mitophagy is mediated by the effectors PINK1 and Parkin. The genes encoding these proteins have been found mutated in certain forms of autosomal recessive Parkinson's disease (PD), also termed Parkinsonism (Valente, Abou-Sleiman et al. 2004). Accumulation of dysfunctional mitochondria in the brains of PD patients in combination with the clinical similarity between patients with PINK1 or Parkin mutations suggest that these molecules might function in a common pathway regarding the regulation of mitochondrial quality control. Genetic studies in Drosophila have further proposed a role for PINK1 and Parkin in the regulation of mitochondrial integrity (Greene, Whitworth et al. 2003). However, Parkin and PINK1 may exert independent functions in some other cellular pathways.

PINK1 is a serine/threonine protein kinase that is targeted to mitochondria due to the expression of a mitochondrial targeting sequence (MTS) (Youle and Narendra 2011). Like most other mitochondrial proteins, PINK1 is encoded by the nuclear genome and subsequently synthesized in the cytoplasm before it is imported in mitochondria via the TOM (translocase of outer membrane)/TIM23 (translocase of inner membrane 23) complexes (Greene, Grenier et al. 2012). Moreover, PINK1 requires the electrical component of the inner mitochondrial membrane potential ($\Delta\psi$) for it's import (Ashrafi and Schwarz 2013).

Interestingly, imported PINK1 is constitutively cleaved into an unstable product by an inner mitochondrial membrane rhomboid protease called PARL as well as by other mitochondrial proteases (Meissner, Lorenz et al. 2011); thus PINK1 is present at very low levels in those mitochondria that are healthy enough to maintain the $\Delta\psi$-dependent import pathway. This is the explanation for the fact that PINK1 cannot be detected in proteomics studies very often, is spite of it's relatively large size. In addition, PINK1 contains a second weaker signal sequence that targets it to the outer mitochondrial membrane (OMM) in the case that $\Delta\psi$ has collapsed and consequently the TOM/TIM import pathway is blocked (Narendra, Walker et al. 2012).

So, PINK1 is responsible for the assessment of the internal state of all mitochondria inside the cell due to the aforementioned features. Therefore, the healthy mitochondria acquiring a strong inner membrane potential (high $\Delta\psi$) quickly dispose of PINK1 via degradation, whereas failing mitochondria (low $\Delta\psi$) are unable to import and degrade the kinase, accumulating it on their surface and thus displaying their inner dysfunction on their outer surface. This mechanism is very elegant and fine-tuned, since PINK1 constitutively associates with the TOM complex (once it is on the outer mitochondrial membrane), so that it can be re-imported and degraded if mitochondrial membrane potential is restored (Lazarou, Jin et al. 2012). This is a quick on/off signal of mitochondrial dysfunction.

Mammalian Parkin is an E3 ubiquitin ligase that under basal conditions resides in the cytoplasm (likely inert). This E3 ligase is the most recent candidate for ATG5-dependant mitophagy in mammals (Narendra, Tanaka et al. 2008). Upon mitochondrial damage (low $\Delta\psi$), PINK1 is exposed to the cytosolic surface and thereby activates and directs Parkin specifically to the failing mitochondrion. Afterwards, Parkin ubiquitinates several outer mitochondrial membrane proteins, including VDAC1, that serve as a recognition mark for the autophagic machinery. Ubiquitinated mitochondrial proteins are bound by p62 which is a multi-functional protein that also interacts with LC3 and thereby promotes association with autophagosomal membranes, leading to clearance of the damaged mitochondrion by selective autophagy (mitophagy) (**Fig.G1.1**).

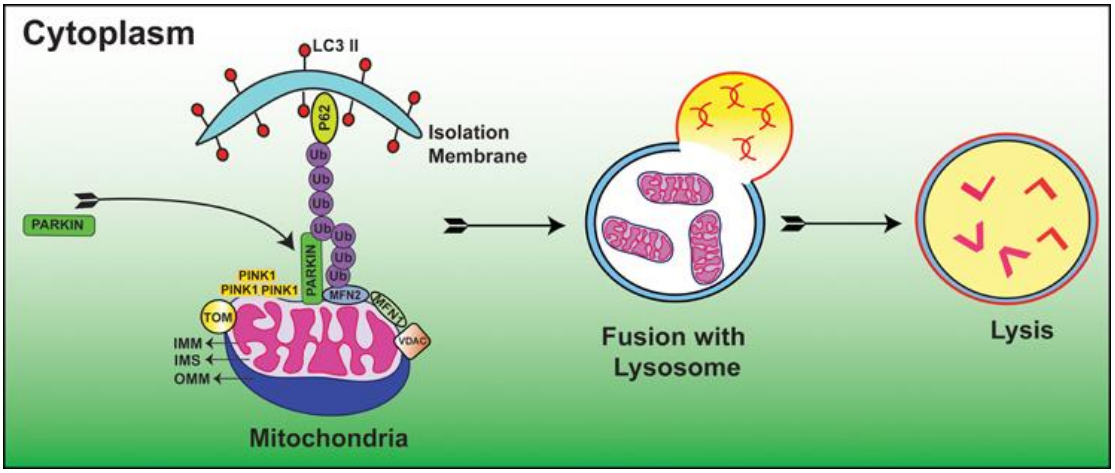

Fig.G1.1. Parkin/PINK1 mediated mitophagy process. Sureshbabu et al., 2013

However, the exact mechanism for the recruitment of Parkin selectively to mitochondria, on the surface of which PINK1 accumulates, has not been deciphered so far. There are three major hypotheses. Firstly, it is suggested that PINK1 and Parkin can directly bind each other, since PINK1-Parkin complexes of different sizes have been observed by size-exclusion chromatography and native PAGE electrophoresis (Narendra, Walker et al. 2012). Secondly, there is mixed evidence that PINK1 directly phosphorylates Parkin on residues Thr175 and Thr217 at the linker region of Parkin (Narendra, Tanaka et al. 2008). Finally, an alternative but not mutually exclusive hypothesis includes the priming through phosphorylation of particular substrates of Parkin on the outer mitochondrial membrane (OMM) by PINK1. The latter hypothesis is known as the 'shared-substrate model', supporting the existence of a motif that is recognized by PINK1 in it's unphosphorylated form and by Parkin in it's phosphorylated one (Narendra, Walker et al. 2012).

To date, it is known that Parkin mediates directly or indirectly the ubiquitination of mitochondrial outer membrane proteins with various ubiquitin linkages. The target proteins with predominantly K48-linked ubiquitin chains are removed by proteasomal degradation, whereas those with K63-linked ubiquitin chains are able to recruit adaptor proteins from the cytoplasm (Lim, Dawson et al. 2006). There are many substrates that have been identified to be ubiquitinated by Parkin, but which one of them is ubiquitinated more efficiently in vivo and which is of greatest physiological importance is still less clear. Remarkably, these substrates differ also in the number of transmembrane domains they possess, their size and their association with other proteins (Narendra, Walker et al. 2012). Conclusively, the substrates towards which Parkin has high activity display significant diversity. Representative examples of these are Mitofusins (Mfn1/2), Miro1, VDAC and TOM70.

Finally, besides ubiquitination, Parkin can interact with other proteins such as Ambra1 (Autophagy/Beclin1 regulator 1). This interaction leads to the activation of PI3K complex around damaged mitochondria. Since this complex is essential for the formation of new phagophores, it can subsequently facilitate selective mitophagy. So, the interaction of Parkin with Ambra1 can be considered as a key mechanism for the induction of the final clearance step of Parkin-mediated mitophagy (Strappazzon, Nazio et al. 2015).

G2) Parkin-independent pathway of Mitophagy

A second pathway of mitophagy that is mostly induced under hypoxic conditions and that is independent of the action of Parkin has been proposed too (Ding and Yin 2012). During this process, dysfunctional mitochondria exhibit elevated expression of the mitochondrial proteins BNIP3, NIX and FUNDC1, which in turn are able to recruit autophagosomes to mitochondria by direct interaction with LC3 (through their LIR domains). Upon mitochondrial depolarization after CCCP treatment of cells (mitochondrial uncoupler), the mitochondrial protein Smurf1 has been also found to promote mitophagy, most likely via ubiquitination of a number of mitochondrial proteins. Furthermore, the complex Hsp90-Cdc37 leads to the stabilization and activation of Ulk1, which is responsible for the phosphorylation of ATG13. Once phosphorylated, ATG13 is targeted to damaged mitochondria and induces the mitophagic flux. Both the Parkin-dependent and the Parkin-independent pathways are considered as ATG-dependent mechanisms due to the participation of ATG proteins in these processes. It should be mentioned that in the ATG-dependent mechanisms ROS and ATP depletion are considered as significant inducers of mitophagy by repressing mTOR complex. However, an ATG-independent mechanism has been described as well but is less understood so far (Ding and Yin 2012). In this particular mechanism, 15-lipoxygenase promotes mitochondrial degradation. In

addition, direct lysosomal invagination or interaction with damaged mitochondria (microautophagy) might also play a pivotal role in the clearance of the defective organelles (**Fig.G2.1**).

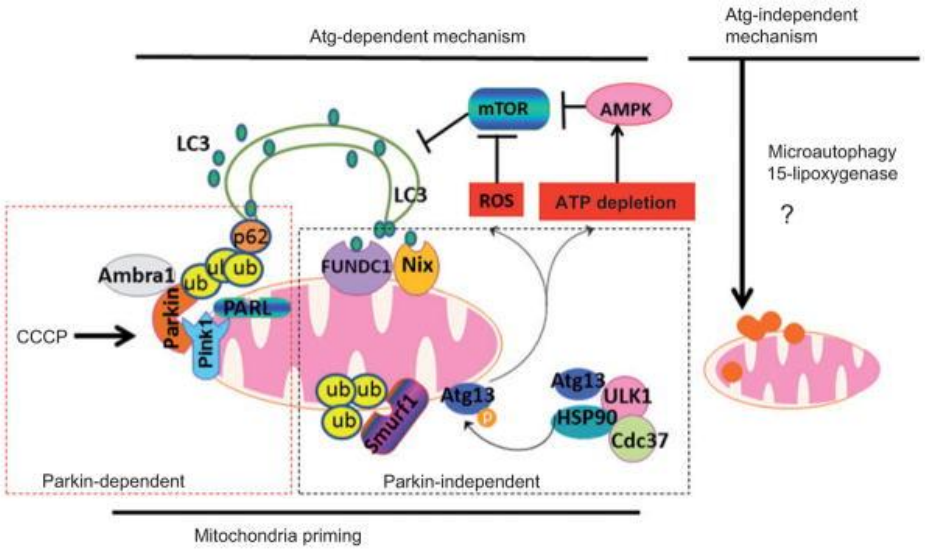

Fig.G2.1. Proposed models for mitophagy in mammalian cells. Ding and Yin, 2012

G3) Mitophagy in T regulatory cells

Given the central role of mitophagy in maintaining mitochondrial function, it is reasonable to expect that immune cell viability and differentiation as described above may be disrupted in its absence. Despite the discovery that T-cells depend on autophagy to maintain their homeostasis and survival, only few evidence exist concerning the role of mitophagy in Treg cells. Recently, Gavin et al., were the first to correlate Pink1, and thus mitophagy, with Treg cell differentiation and function. More specifically, they showed that under polarization conditions favoring the development of induced regulatory T cells, *PINK1*$^{-/-}$ T cells exhibited a reduced ability to suppress bystander T cell proliferation despite normal FoxP3 expression kinetics (Ellis, Zhi et al. 2013). These results indicated a critical role for PINK1 in T regulatory cell function and thus, more intensive research needs to be done in order to further understand the role of mitophagy in Treg cell function and survival.

Aim of the project

The role of T regulatory cells in autoimmunity has been characterized thoroughly. Treg impairment has been reported in a number of human autoimmune conditions and includes Treg numerical and functional defects. In addition Treg cells are found to directly depend on autophagy for their development and survival fitness. Even though mitochonrial dysfanction is strongly correlated to cell death, but also correlated to autoimmune diseases such as multiple sclerosis, the role of defective mitochondria clearence (mitophagy) in T regulatory cells until now remains obscure.

Consequently, aim of this study is to further assess the role of autophagy in Treg cells and shed light to the role of mitophagy in T regulatory mediated suppression in autoimmunity, and more specificaly in the context of multiple sclerosis. At the same time, differences between T effector and T regulatory concerning their autophagy/mitophagy regulation mechanisms will be also noticed. This will be achieved by firstly characterizing the role of autophagy and mitophagy in T effector and T regulatory cell survival and function and then, by investigating its association with the progress of Multiple Sclerosis.

Materials and methods

A. Mice

Foxp3^{YFPcre} mice kindly provided by Dr. Alexander Rudensky (Department of Immunology, Memorial Sloan-Kettering Cancer Center, New York, USA) (Rubtsov, Rasmussen et al. 2008) and *Atg5*$^{fl/fl}$ mice were kindly brovided by Dr. Noboru Mizushima (Department of Biochemistry and Molecular Biology, Graduate School and Faculty of Medicine, University of Tokyo, Tokyo, Japan) (Hara, Nakamura et al. 2006). *CD4cre* mice were a generous gift from Dr. Werner Müller (Department of Immunology and Molecular Microbiology, University of Manchester, Manchester, UK). In order to generate the Foxp3creAtg5fl/fl mice, mice expressing the YFP-Cre fusion from the Foxp3 locus (Foxp3Cre) were crossed to Atg5 flox/flox mice, carrying loxP-flanked Atg5 (Atg5fl/fl) and to generate the CD4creAtg5fl/fl mice, we crossed mice carrying loxP-flanked Atg5 (Atg5fl/fl) with mice expressing YFP-Cre fusion from the CD4 locus (CD4Cre). 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 injections at the base of the tail, with 100 μg MOG_{35-55} emulsified (1:1) in complete Freund's adjuvant (CFA, Sigma Aldrich). 9 days after immunization, mice were sacrificed and their inguinal lymph nodes (LNs) were isolated. For the experimental autoimmune encephalomyelitis (EAE) induction: mice were immunized with 200 μg Mog_{35-55} emulsified (1:1) in CFA, supplemented with extra 4 μg/ml Mycobacterium (Difco). Emulsion was injected subcutaneously over three sites (i.e. 50 μl per site): one along the midline of the back between the shoulders, and two on each hind flank. Additionally mice received intraperitonially (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 (**Fig.B**):

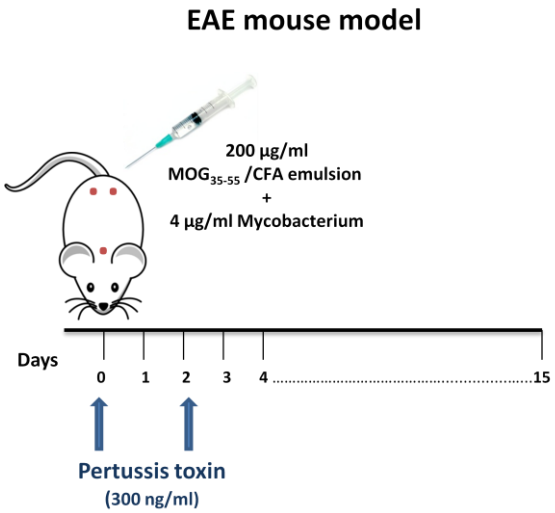

Figure B: Protocol of EAE induction in mice. Red dots indicate the 3 sites of the emulsion subqutaneous injections. Pertussis toxin is injected intraperitonially.

Progression of the disease was evaluated daily according to the grading system for clinical assessment of EAE (Miller, Karpus et al. 2007):

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

In order to obtain Teff ($CD4^+Foxp3^-$ or $CD4^+CD25^-$) and Treg ($CD4^+Foxp3^+$ or $CD4^+CD25^+$) cells, mice inguinal (after Mog_{35-55}/CFA immunization) or cervical (after EAE induction) LNs were isolated. Cells were stained with conjugated antibodies to mouse: CD4 (GK15) and CD25 (3C7) (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

For characterization of mitophagy *in vivo,* 500.000 cells from total LNs isolated from either naive or Mog_{35-55}/CFA immunized mice, were stained with conjugated antibodies to mouse CD4 and CD25 (if needed) for 20 minutes at 4 ^oC. Next, cells were stained with 100 nM TMRE, 5 nM Mitosox, 100 nM Mitotracker or 100 nM Lysotracker (ENZ-52309, M36008, M22425 and L7528 respectively). Dyes were added to the cells in a 96-flat bottom well plate, and after required incubation according to manufacturer's protocol, cells were centrifuged at 1.800 rpm, for 7 minutes, at 4 ^oC. After centrifugation, cells were resuspended in 5% PBS-FBS (Fetal Bovine Serum) and tranferred in flow cytometry tubes. Then, the cells were subjected to flow cytometry in FACS ARIA III (BD Biosciences). Analysis was performed using FlowJo software.

E. In vitro co-culture experiments for mitophagy assessment

For proliferation 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 100 nM Wortmannin (Sigma Aldrich) or with its control Dimethyl Sulfoxide (DMSO) (SIGMA life science). After 96 h cells were stained (TMRE, Mitosox, Mitotracker, Lysotracker) and subjected to FACS ARIA III (BD Biosciences).

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 100 nM Wortmannin (Sigma Aldrich) or with DMSO. After 96 h cells were stained (TMRE, Mitosox, Mitotracker, Lysotracker) and subjected to FACS.

F. 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^oC, 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), 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.

For Mitotracker Fluorescence, $1x10^6$ sorted Teff and Treg cells were seeded in coverslips pretreated with poly lysine, stained with 100nM Mitotracker in cell medium for 30 minutes in 37 ^oC, fixed with 4% PFA for 15 min in room temperature and washed twice with PBS. As above, 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 Mitotracker/cell were calculated using a macro developed in Fiji software.

G. RNA isolation

Total RNA from Teff and Treg cells was purified using the Macherey-Nagel RNA isolation kit, according to manufacturer's protocol.

H. Real-Time PCR

cDNA was prepared from isolated RNA, using Superscript II reverse transcriptase cDNA Synthesis Kit (Invitrogen) according to manufacturer's protocol. 60 ng of RNA were used as a template for every reaction and were mixed initially with appropriate volume of Oligo dT primers and mixture of dNTPs. After incubation for 5 minutes at 65^{o}C, the samples were cooled immediately on ice. Next, appropriate volumes of 5x First-Strand buffer, RNase inhibitor, and DTT were added and samples were incubated at 42^{o}C for 2 minutes. Finally, Superscript II Reverse Transcriptase was added and the samples were incubated at 25^{o} C for 10 minutes, 42^{o}C for 50 minutes and at 70^{o}C for 15 minutes, followed by cooling on ice. cDNA was stored at -20^{o} C. PCR amplification of the resulting cDNA samples was performed using appropriate volumes of KAPA SYBR® FAST Universal 2x qPCR Master Mix (KAPA BIOSYSTEMS) at a Sacace Biotechnologies, Real-Time System. Total volume of each PCR reaction was 20μl. The following PCR conditions were used for *Pink1* and *Hprt*: 95°C for 3 minutes at 95°C, for 3 seconds at 95°C, for 20 seconds at 63^{o}C and annealing temperature at 90°C for 6 seconds. *Pink1* expression was calculated by the change-in-threshold method ($\Delta\Delta C_T$) and *Hprt* was used as a reference gene for normalization. The primers used for *Pink1* and Hprt genes were the following:

Pink1- Forward primer: 5'- GAG GGC GTG GAC CAT CTG- 3'

Reverse primer: 5' – AGG ATG TTG TCG GAC TTG AGA TC- 3'

Hprt - Forward primer: 5'-GTGAAACTGGAAAAGCCAAA-3'

Reverse primer: 5'-GGACGCAGCAACTGACAT-3'

I. 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

A) Characterization of autophagy in T effector and T regulatory cells during an autoimmune response.

To directly examine the role of autophagy in activated cells *ex vivo*, we measured the expression of two indispensable genes for the formation of autophagosomes, *Beclin-1* and *Atg5*, in immunized mice. Foxp3^{YFPcre} female mice (transgenic mice expressing YFP fused to Foxp3) (Rubtsov, Rasmussen et al. 2008) were immunized after subcutaneous injections with 100 µg Mog_{35-55}/CFA emulsion (Myelin Oligodendrocyte Glycoprotein /Freund's Complete Adjuvan). 3 or 9 days after immunization, draining lymph nodes (dLNs) (inguinal LNs) were isolated and cells were sorted for two distinct populations: CD4$^+$Foxp3$^-$ cells (Teff cells) and CD4$^+$Foxp3$^+$ cells (Treg cells) (**Fig.A.1**).

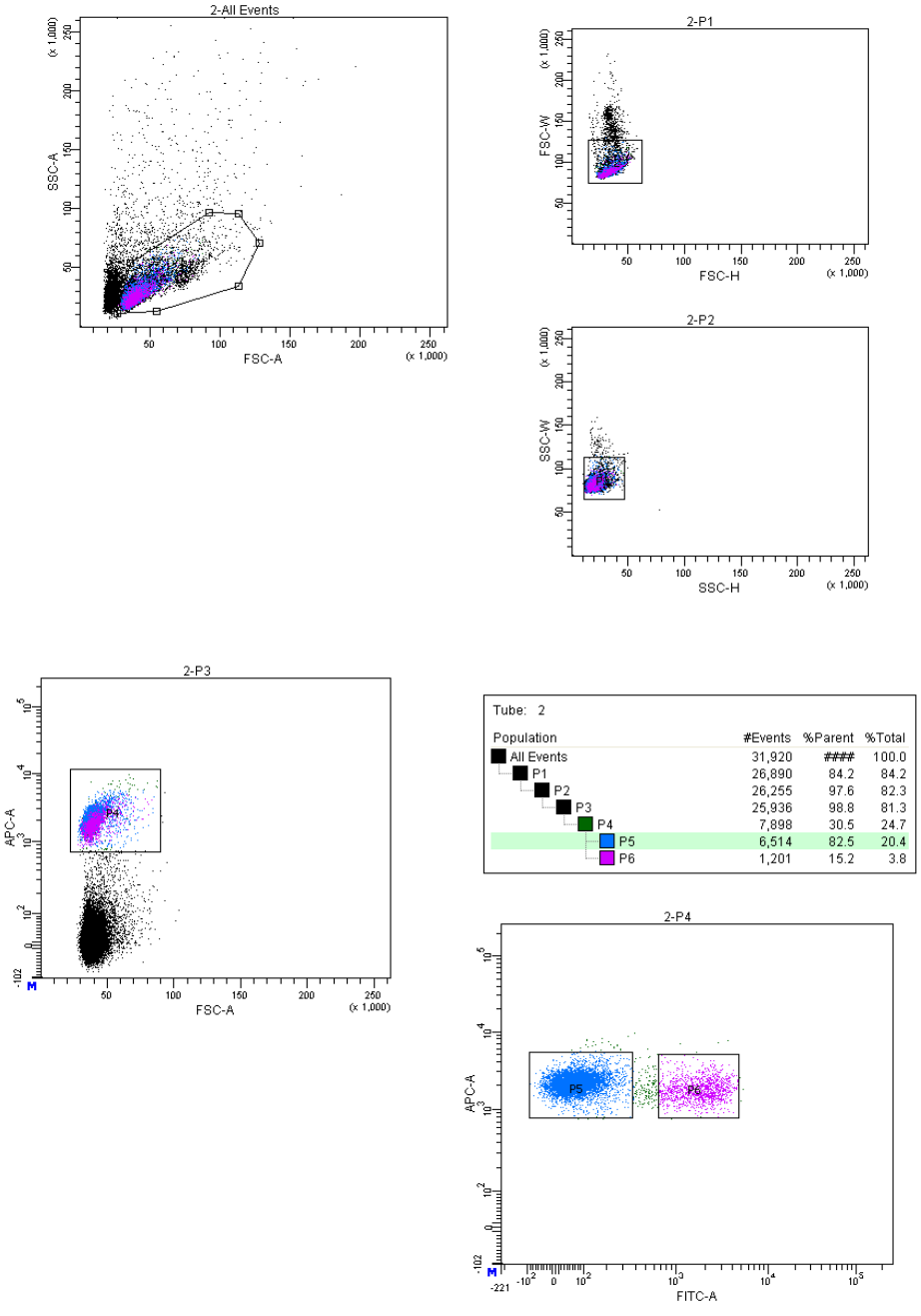

Figure A.1: FACS gating strategy for sorting $CD4^+Foxp3^-$(Teff) and $CD4^+Foxp3^+$ (Treg) cells from total LN cells. Discrimination between live and damaged or dying cells, side scatter (SSC-A, log) and forward scatter (FSC-A, log) are plotted. Higher SSC-A signals indicate increased granularity and therefore sorting gate is on the population with the lower SSC-A signal (P1). In order to exclude double-cells from P1 and obtain clear populations, FSC-W and SSC-W are plotted and P2 and P3 populations are chosen. P4 population resembles all APC-CD4 positive cells (T cells). Plotting the APC signal against FITC-A (the same excitation of cells with YFP), reveals two distinct populations, P5 ($CD4^+Foxp3^-$ Teff cells) and P6 ($CD4^+Foxp3^+$ Treg cells).

The expression of *Beclin-1* and *Atg5* was measured by Real-time PCR in both populations. In Teff cells the levels of Beclin-1 which is implicated in the initiation of the autophagosome are increasing after 9 days of immunization, while the expression of Atg5 which is indispensable for the elongation of the autophagosome is significantly reducing upon immunization. On the other hand, Treg cells from immunized mice exhibited significantly decreased Beclin-1 expression compared to Treg cells from naive mice, followed by a small reduction in Atg5 expression, suggesting that Treg cell autophagy is impeded/reduced upon an immune response (**Fig.A.2**).

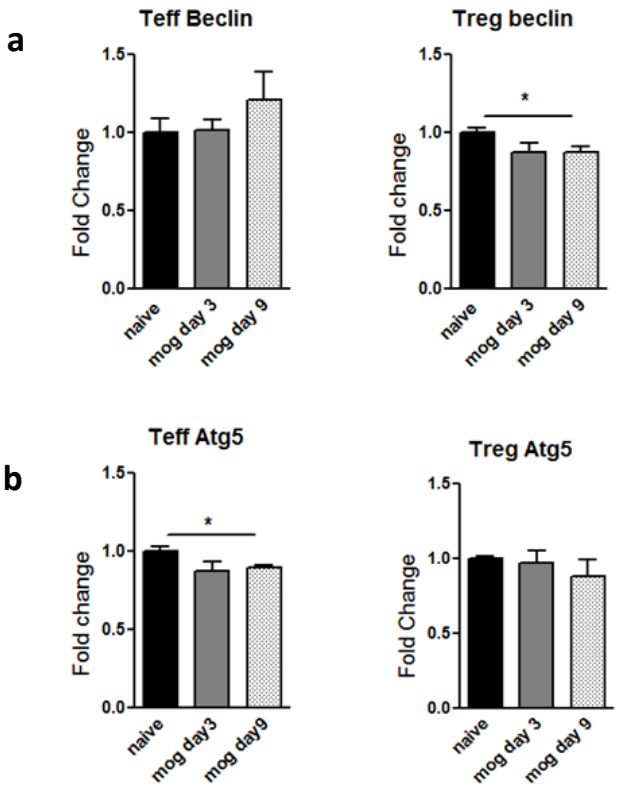

Figure A.2: Reduced Beclin-1 expression in Treg cells upon Mog_{35-55}/CFA immunization. Real-time PCR for (a) Beclin1 and (b) ATG5 mRNA levels of $CD4^+Foxp3^-$(Teff) and $CD4^+Foxp3^+$ (Treg) cells, 3 and 9 days after immunization (n=7 mice per group) . Data are mean ± SD values, *p<0.05, **p<0.01, and ***p<0.001 (unpaired Student's t test).

Another way to evaluate the levels of autophagy upon stimulation in Teff and Treg cells *ex vivo,* is to quantify their autophagosomes; for this reason LC3 and p62 puncta were visualized by confocal microscopy in T effector and T regulatory cells, in naive mice and mice after 9 days of Mog_{35-55}/CFA immunization. LC3 labels autophagic membranes while p62, an autophagosome cargo protein, recognizes toxic cellular waste, which is then scavenged by autophagy, thus, low levels of p62 in cells imply enhanced autophagic activity. Our results demonstrate that in Teff cells p62 accumulation is increased in Mog_{35-55}/CFA treated mice, while LC3 puncta present an even higher increase compared to naive Teff cells. This result could be explained by the fact that even though there is a higher number of autophagosomes in activated T effectors, these autophagosomes are not able to sequestrate totally the dysfunctional cellular components. Additionally, Tregs maintain their p62 expression at the same levels after immunization, but significantly increase their LC3 expression. Finally, by comparing active Teffs with active Tregs, we can see that Tregs from immunized mice exhibit more effective autophagic activity since their p62 is lesser and their LC3 expression is importantly higher than in Teff cells (**Fig.A.3**)

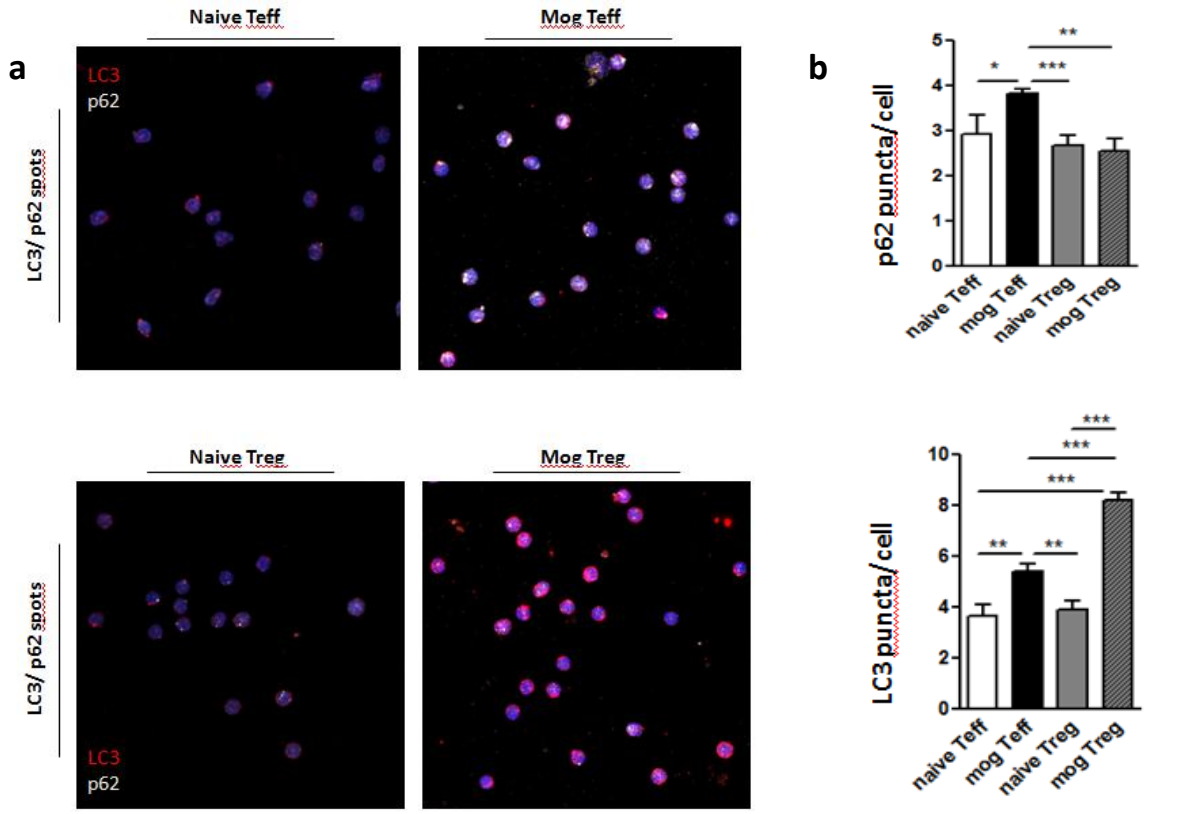

Figure A.3: Upon immunization Treg cells exhibit reduced p62 and increased LC3 expression compared to Teff cells. (a) Confocal images and (b) quantification of cells with LC3 and p62 puncta in Teff and Treg cells purified from YFP-cre mice, naive or 9 days after MOG/CFA immunization (n = 9 mice).

B1. *In vivo* autophagy deletion in Treg cells

Aiming to investigate whether autophagy is implicated in T reg mechanism of action, we elaborated Foxp3creAtg5fl/fl mice lacking autophagy in their Foxp3^{+} Treg compartment. In order to generate these mice, mice expressing a YFP-Cre fusion from the Foxp3 locus (Foxp3^{YFPCre}) were crossed to Atg5 flox/flox mice, carrying loxP-flanked Atg5 (Atg5fl/fl). Following immunization with Mog_{35-55}/CFA, draining lymph nodes (dLNs) were isolated and the frequency of Treg cells was evaluated by flow cytometry. Our results demonstrated a robust decrease of Treg cell frequency in Foxp3creAtg5fl/fl mice compared to their Foxp3gfp controls, indicating that abscence of autophagy in Foxp3^{+} cells affects activated T reg cell survival (**Fig.B.1**).

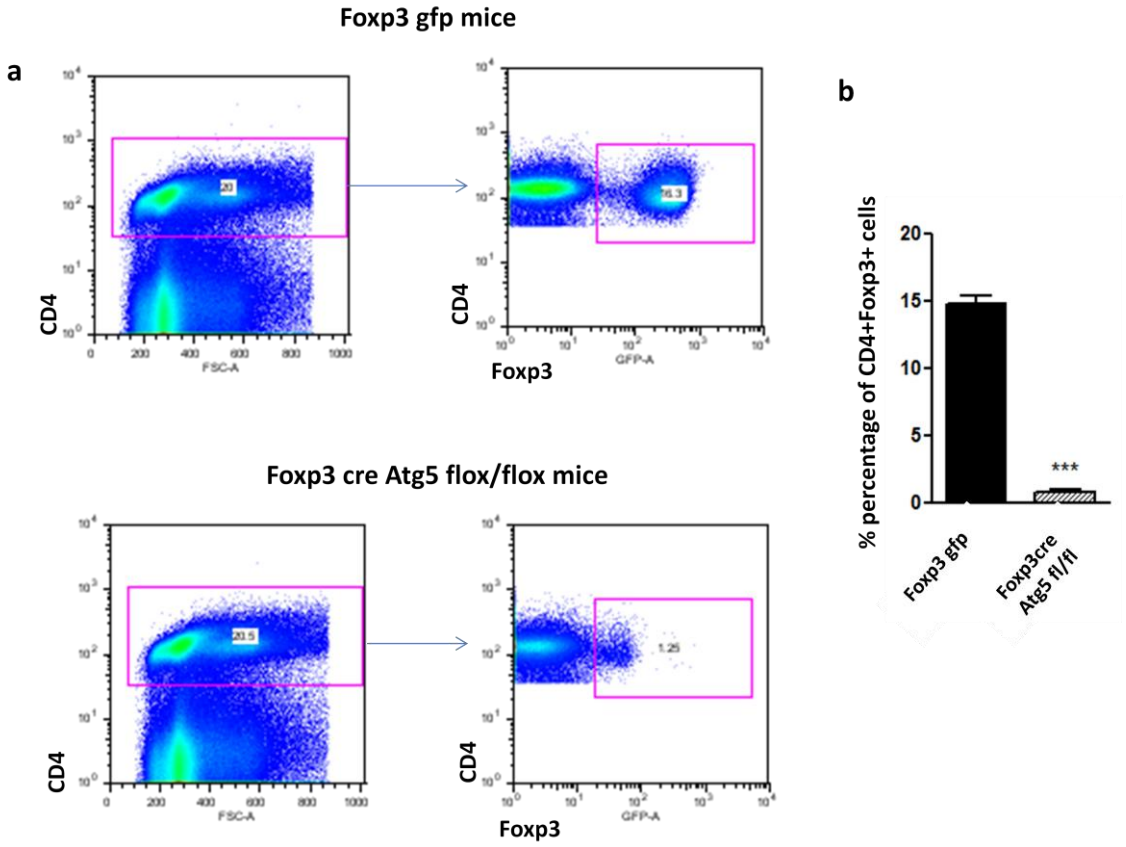

Figure B.1: Autophagy affects Treg cell survival. (a) Flow cytometry analyzing the expression of CD4^{+}Foxp3^{+} cells (Tregs) of Foxp3 gfp mice (up) and Foxp3 Cre Atg5 flox/flox mice (down), 9 days after Mog/CFA immunization. Numbers in plots indicate percent of the cells. (b) % percentage of Foxp3^{+} Treg cells analyzed by FACS cytometry. Representative data from one experiment is shown, n=3 mice per group. Data are mean ± SD values, *p<0.05, **p<0.01, and ***p<0.001 (unpaired Student's t test).

B2. *In vitro* autophagy inhibition in T cells

In order to further assess the role of autophagy in Treg cells, we treated thymus-derived Treg cells and induced Treg cells (iTreg cells, produced by the conversion of Teff cells in the presence of TGF-b) in vitro with an autophagy inhibitor, Wortmannin (WM). Wortmannin is a selective and irreversible inhibitor of phosphatidylinositol 3-kinase (PI3K), thus prevents the initial formation of autophagosome-like structures and inhibits the autophagic sequestration (Blommaart, Krause et al. 1997). To study the response of T cells when their autophagy is blocked, we isolated total lymph nodes (LNs) from naive Foxp3^{YFPcre} mice (transgenic mice expressing YFP fused to Foxp3) and sorted cells in order to obtain 2 distinct populations as described in section A: CD4^{+}Foxp3^{-} cells (Teff cells) and CD4^{+}Foxp3^{+} cells (Treg cells). Labeled with the division-tracking dye CellTrace, T effectors and T regulatory cells were activated for 4 days *in vitro* with beads coated with monoclonal antibody (mAb) to the invariant signaling protein CD3 plus mAb to CD28 in the presence of IL-2. Four days after activation we evaluated by flow cytometry the proliferative capacity of originally derived from thymus Tregs (Proliferation) and induced Tregs (Induction).

B2.1 Evaluation of the proliferating capacity of thymus derived Tregs

Comparing cells treated with WM or its control Dimethyl Sulfoxide(DMSO), we found that inhibition of autophagy slightly increased the proliferation of Tregs (**Fig.B2.1.1**).

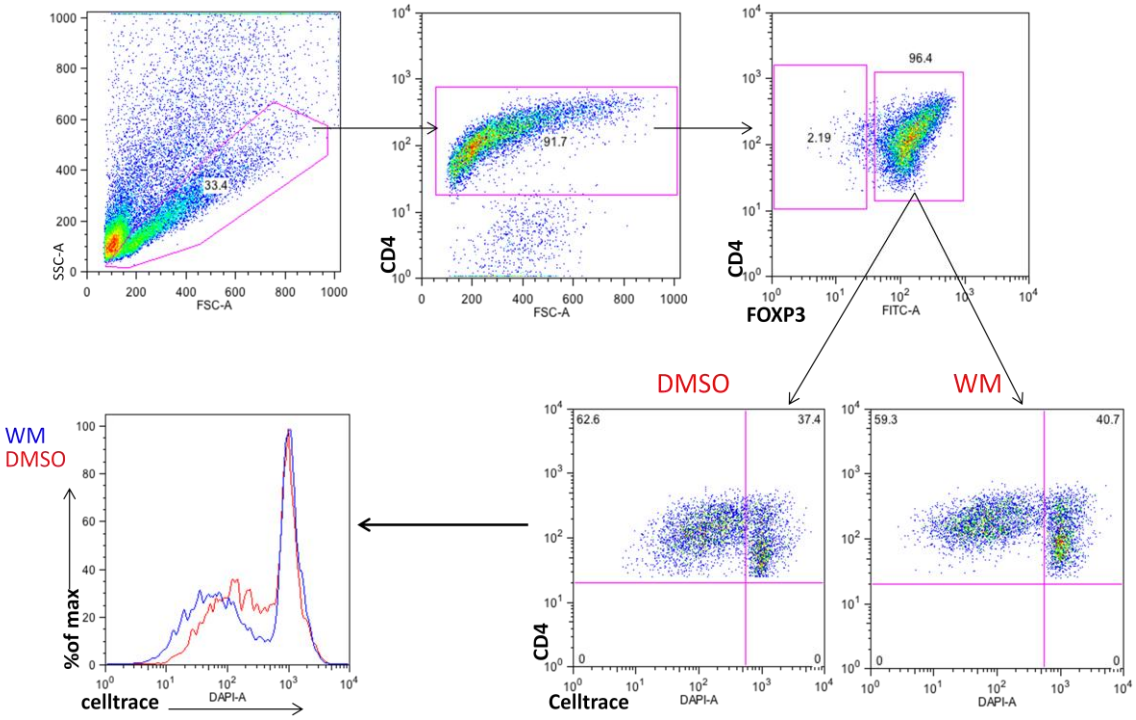

Figure B2.1.1: Inhibition of autophagy with WM slightly increases naturally-occurring Treg cell proliferation. (a) Flow cytometry analyzing the expression of activated CD4^{+}Foxp3^{+} cells treaded with WM or DMSO. Numbers in plots indicate percent of the cells. (b) Proliferation of CellTrace-labeled CD4^{+}Foxp3^{+} cells stimulated for 4 days *in vitro* with mAb to CD3 plus mAb to CD28 and cultured with IL2 and WM or DMSO. Data are from one experiment representative of 4 experiments with 8 mice in total.

Autophagy is a catabolic process aimed at recycling cellular components and damaged organelles in response to diverse conditions of stress such as nutrient deprivation, viral infection and genotoxic stress. Reactive oxygen species (ROS) are one of the main intracellular signal transducers sustaining autophagy, thus we assumed that the inhibition of autophagy in the activated T regulatory cells could possibly affect the ROS production of the cell. Surprisingly, activated T regulatory cells treated with Wortmannin had no significant difference in their total ROS production compared to Treg cells with normal autophagy, suggesting that other metabolic networks must also be implicated in total ROS regulation of the cell (**Fig.B2.1.2**).

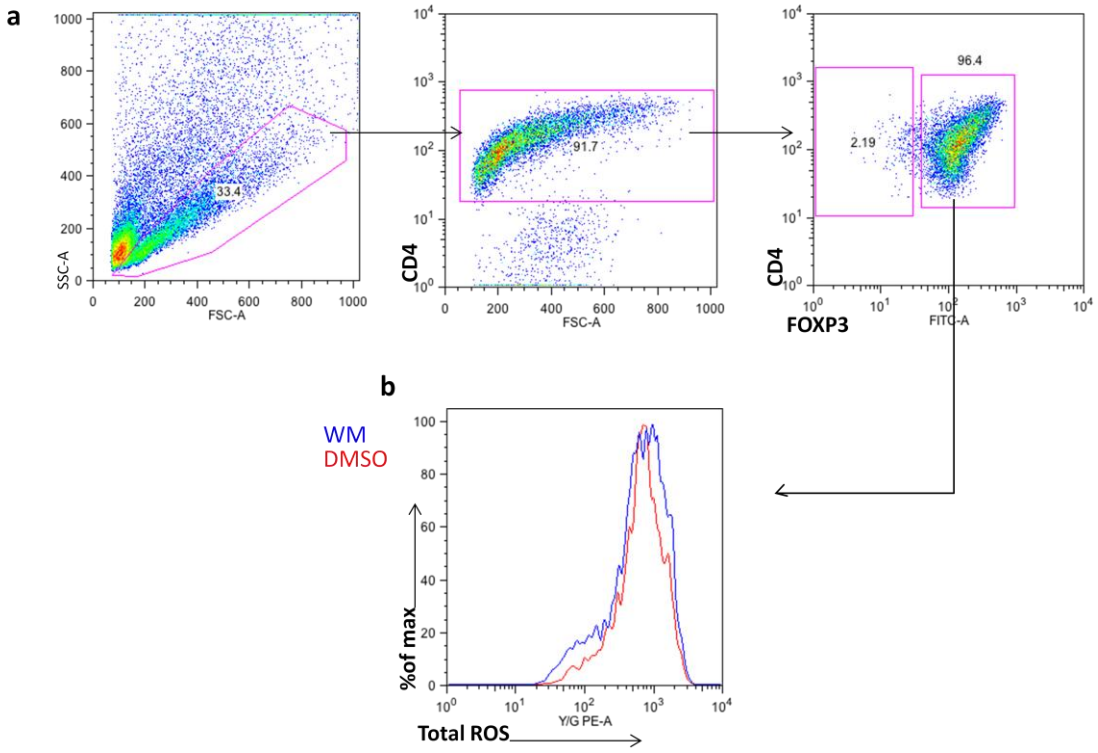

Figure B2.1.2: Total ROS levels of activated naturally-occurring Treg cells do not alter after inhibition of their autophagy with WM. (a) Flow cytometry analyzing the expression of activated $CD4^+Foxp3^+$ cells treaded with WM or DMSO and (b) real time ROS production of $CD4^+Foxp3^+$ cells measured by flow cytometry, stimulated for 4 days *in vitro* with mAb to CD3 plus mAb to CD28 and cultured with IL2 and WM or DMSO. Numbers in plots indicate percent of the cells. Data are from one experiment representative of 3 experiments.

B2.2. Study of the induction of Tregs

Isolated Teff cells from naive Foxp3^{YFPcre} mice were activated *in vitro* with mAb to CD3 plus mAb to CD28 in the presence of TGFb plus Interleukin 2 (IL2) and cultured under the proper conditions for 4 days, in order to induce the development of T regulatory cells (iTreg cells). Next, Teffs were treated either with Wortmannin or DMSO, in order to investigate the proliferative capacity of induced Tregs (iTregs) under the inhibition of their autophagy and to elucidate possible differences between thymus derived Tregs and iTregs when their autophagy mechanism is blocked. T effector cells flow cytometry analysis revealed that CD4^{+}Foxp3^{-} (Teff) as long as CD4^{+}Foxp3^{+} (iTreg) cells, labeled with the division-tracking dye CellTrace, presented significantly higher proliferation upon their autophagy inhibition, (approximately a 2-fold increase) (**Fig.B2.2.1 and Fig.B2.2.2**), while total ROS production levels were elevated in both populations (**Fig.B2.2.3**). An important observation is the fact that upon autophagy inhibition with wortmannin, T effector cell population was significantly decreased while T regulatory cells exhibited a substantial increase in their frequency (**Fig.B2.2.4**).

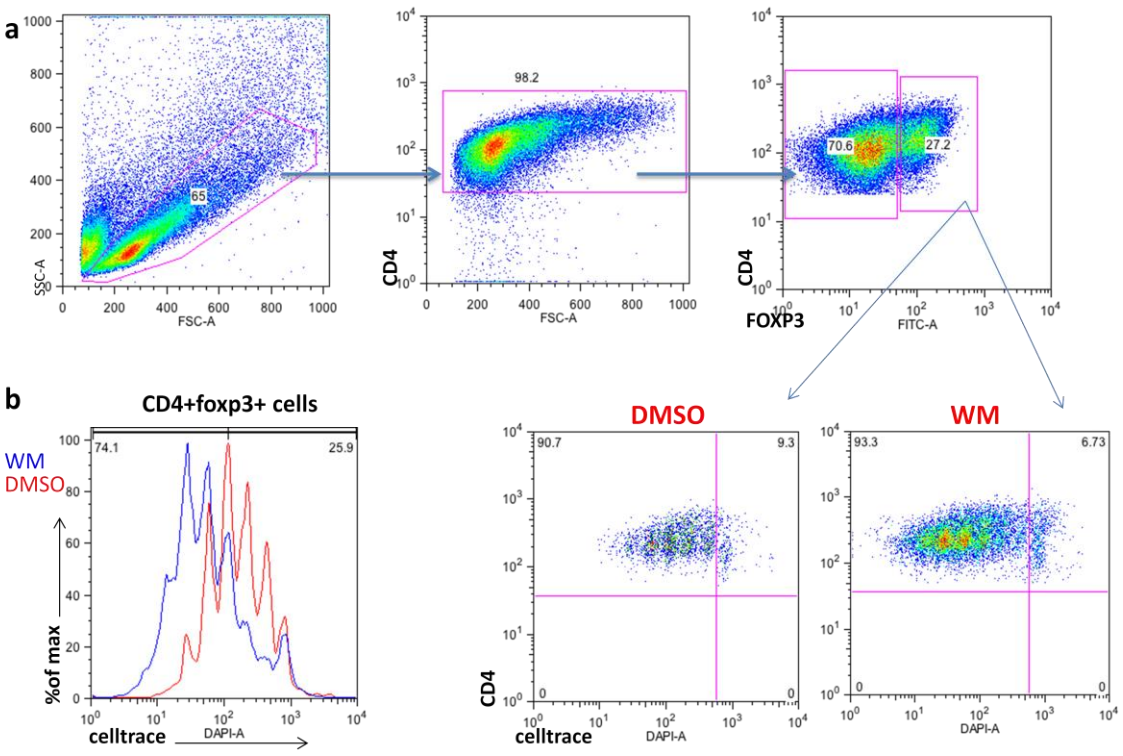

Figure B2.2.1: Increase of proliferation of induced T regulatory cells after inhibition of their autophagy with WM. (a) Flow cytometry analyzing the expression of iTreg cells treaded with WM or DMSO. Numbers in plots indicate percent of the cells. (b) Proliferation of iTreg cells as developed after the stimulation of T conventional cells for 4 days *in vitro* with mAb to CD3 plus mAb to CD28, TGFb and IL2, treated with WM or DMSO. Data are from one experiment representative of 4 experiments with 8 mice in total.

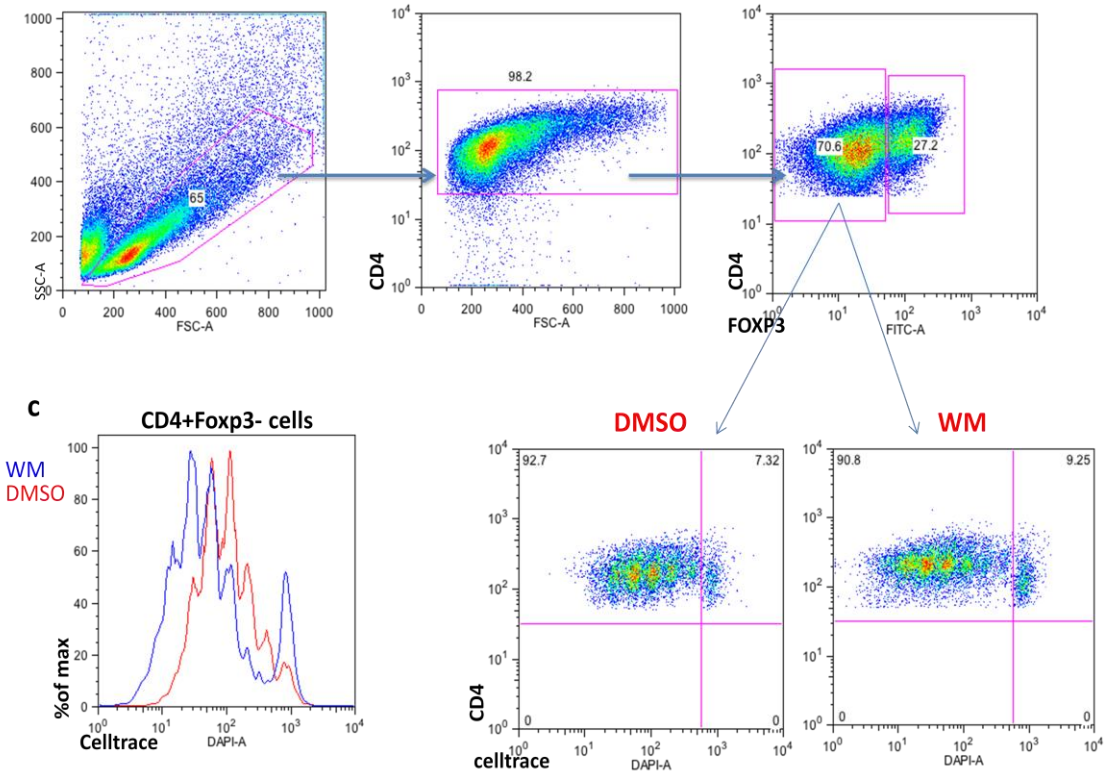

Figure B2.2.2: Increase of proliferation of T effector cells after inhibition of their autophagy with WM. Flow cytometry analyzing the expression of Teff cells treated with WM or DMSO. Numbers in plots indicate percent of the cells (c) Proliferation of T effector cells after their stimulation *in vitro* with mAb to CD3 plus mAb to CD28, TGFb and IL2, and treatment with WM or DMSO. Data are from one experiment representative of 4 experiments with 8 mice in total.

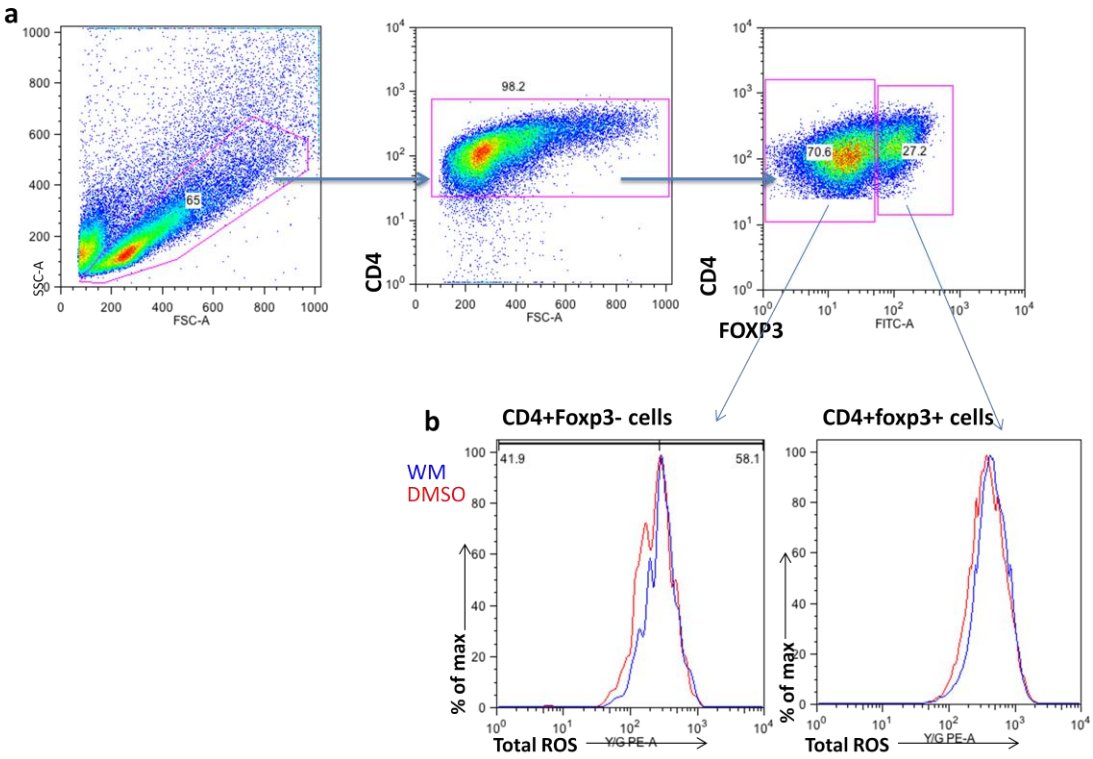

Figure B2.2.3: Inhibiting autophagy of Teff and induced Treg cells with WM slightly increases their total ROS levels. (a) Flow cytometry analyzing the expression of Teff ($CD4^+Foxp3^-$) and iTeg ($CD4^+Foxp3^+$) cells treaded with WM or DMSO and (b) real time ROS production of Teff and iTreg cells measured by flow cytometry, stimulated for 4 days *in vitro* with aCD3/aCD28 and cultured with TGFb, IL2 and WM or DMSO. Numbers in plots indicate percent of the cells. Data are from one experiment representative of 3 experiments.

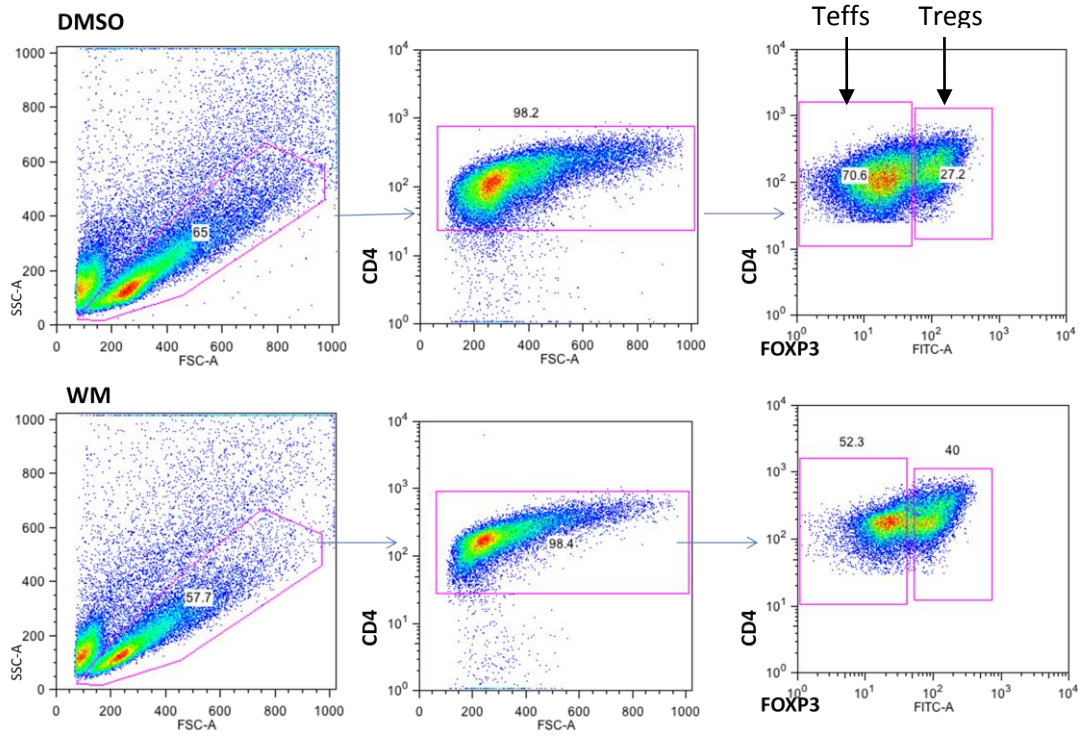

Figure B2.2.4: *In vitro* inhibition of autophagy with WM leads to alterations in the ratio of Teff and iTreg cells. Teff and iTreg cells treaded with DMSO (up) or WM (down). Numbers in plots indicate percent of the cells. Data are from 1 experiment representative of 4 experiments with n=2 per exp.

B3. Autophagy depletion in T cells CD4creATG5$^{fl\backslash fl}$ mice

In order to confirm our *in vitro* results, we crossed mice carrying loxP-flanked Atg5 (Atg5fl/fl) with mice expressing YFP-Cre fusion from the CD4 locus (CD4Cre) to generate CD4CreAtg5fl/fl mice, in which Atg5 is deleted from all T helper lymphocytes. We isolated total lymph nodes from CD4CreAtg5fl/fl mice and their control, CD4cre mice, and sorted cells in order to obtain $CD4^+CD25^-$ Teff and $CD4^+CD25^+$ Treg cells. As described in section B1, T effectors and T regulatory cells were activated for 4 days *in vitro* with beads coated with monoclonal antibody (mAb) to the invariant signaling protein CD3 plus mAb to CD28. On day four results were evaluated by flow cytometry analysis which revealed that the ratio between the two cell populations shifted towards Tregs after inhibition of autophagy. This fact further validated our previous results, highlighting the importance of autophagy on Treg cell proliferation and/or survival (**Fig.B2**).

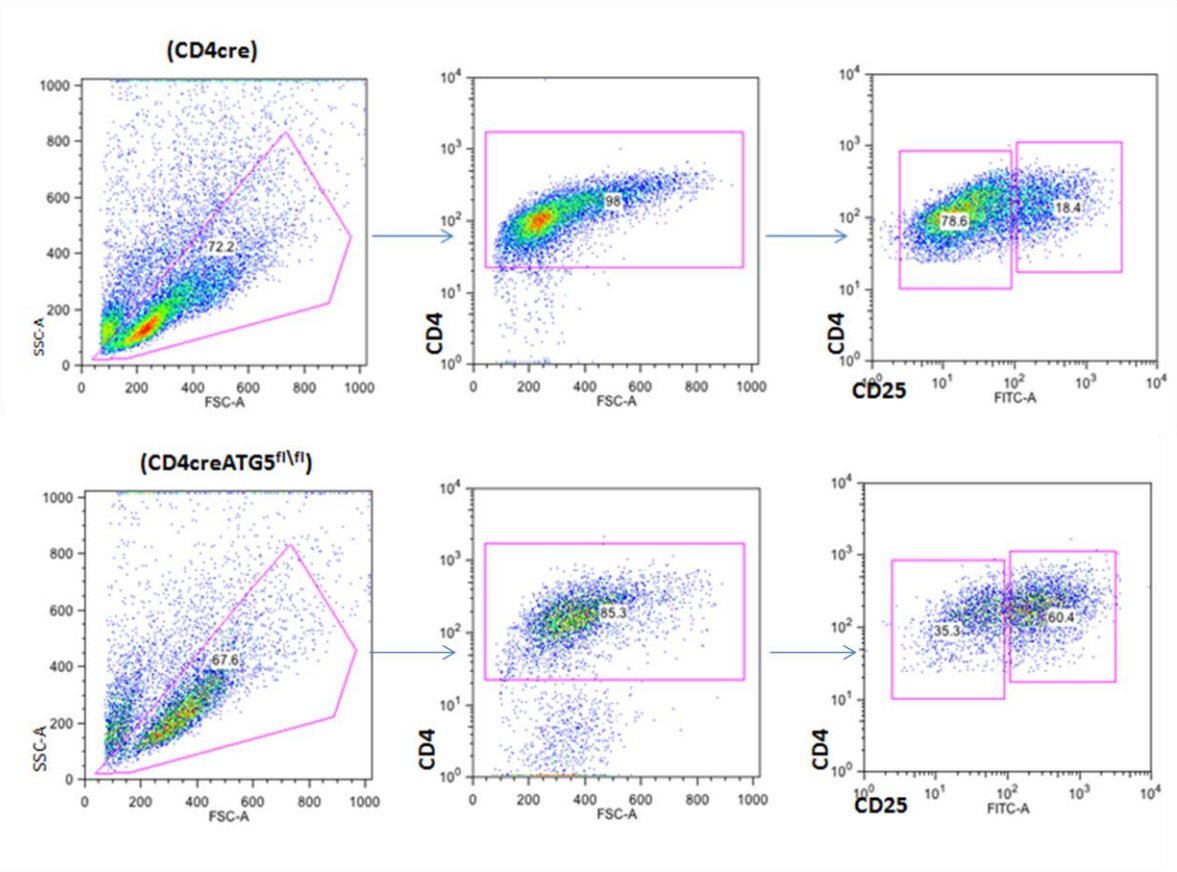

Fig.B2: *In vivo* inhibition of autophagy in CD4CreAtg5fl/fl mice leads to alterations in the ratio of Teff and iTreg cells. Flow cytometry analysis of Teff and iTreg cells from CD4cre (up) or CD4CreAtg5fl/fl mice (down). Numbers in plots indicate percent of the cells. Data are from 1 experiment representative of 2 experiments with n=2 per exp.

C) Autophagy of mitochondria in Teff and Treg cells- Mitophagy

C.1) Characterization of mitophagy after an autoimmune response

As mentioned above, mitophagy is the selective degradation of defective mitochondria by autophagy. Given the significance of autophagy for T cells we wanted to study the efficacy of mitochondrial clearance by autophagy in activated Teff and Treg cells *in vivo.* We therefore immunized Foxp3^{YFPcre} mice using Mog_{35-55}/CFA emulsion and after 9 days of immunization, cells from their draining lymph nodes were sorted for $CD4^+Foxp3^-$ and $CD4^+Foxp3^+$ cell populations. In both populations the levels of Pink1 expression, the mitochondrial protein implicated in the induction of mitophagy, were measured by Real-time PCR. Results demonstrated that there is no significant difference in Pink1 expression between activated T regulatory and T effector cells (**Fig.C.1.1**).

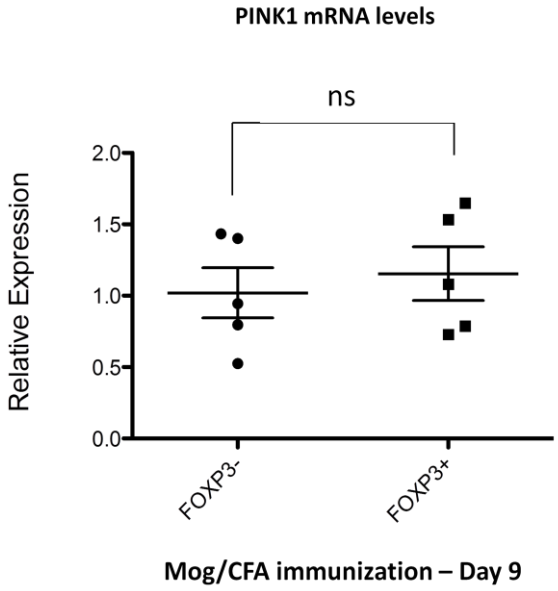

Figure C.1.1: Pink1 mRNA levels of Teff and Treg cells are similar upon MOG/CFA immunization. Real-time PCR for Pink1 mRNA levels of $CD4^+Foxp3^-$(Teff) and $CD4^+Foxp3^+$ (Treg) cells, 9 days after immunization (n=5 mice per group). Data are mean ± SD values, *p<0.05, **p<0.01, and ***p<0.001 (Student's t test).

In contrast, dynamic changes were observed in the mitochondrial quality between Treg and Teff cells in response to Mog_{35-55}/CFA immunization in vivo. Treg cells exhibited decreased mitochondrial cell membrane potential (TMRE) and a concomitant increase in mitochondrial production of superoxide (Mitosox) compared to Teff cells (**Fig.C.1.2**), whereas their overall mitochondrial content was decreased (**Fig.C.1.3.**)

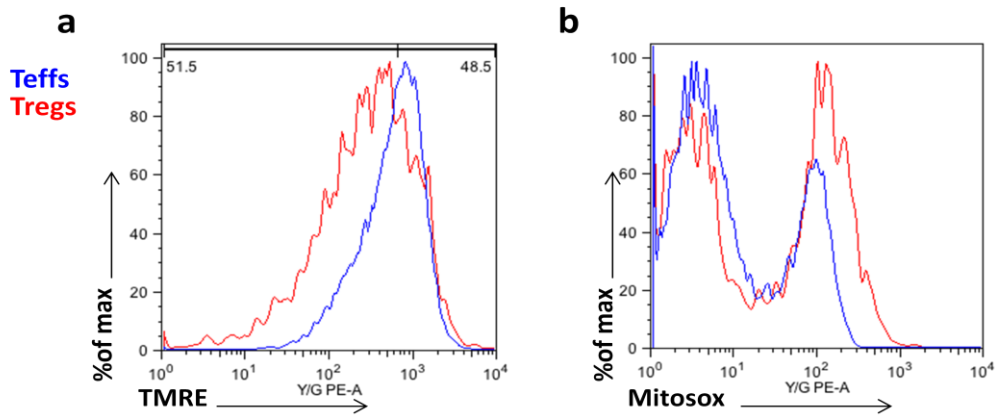

Figure C.1.2: TMRE reduction and Mitosox increase in Treg cells compared to Teff cells. **(a) mitochondrial membrane potential and (b) mitochondrial superoxide production of Teff and Treg cells measured by flow cytometry after 9 days of Mog/CFA immunization. Numbers in plots indicate percent of the cells. Data are from one experiment representative of 7 experiments.**

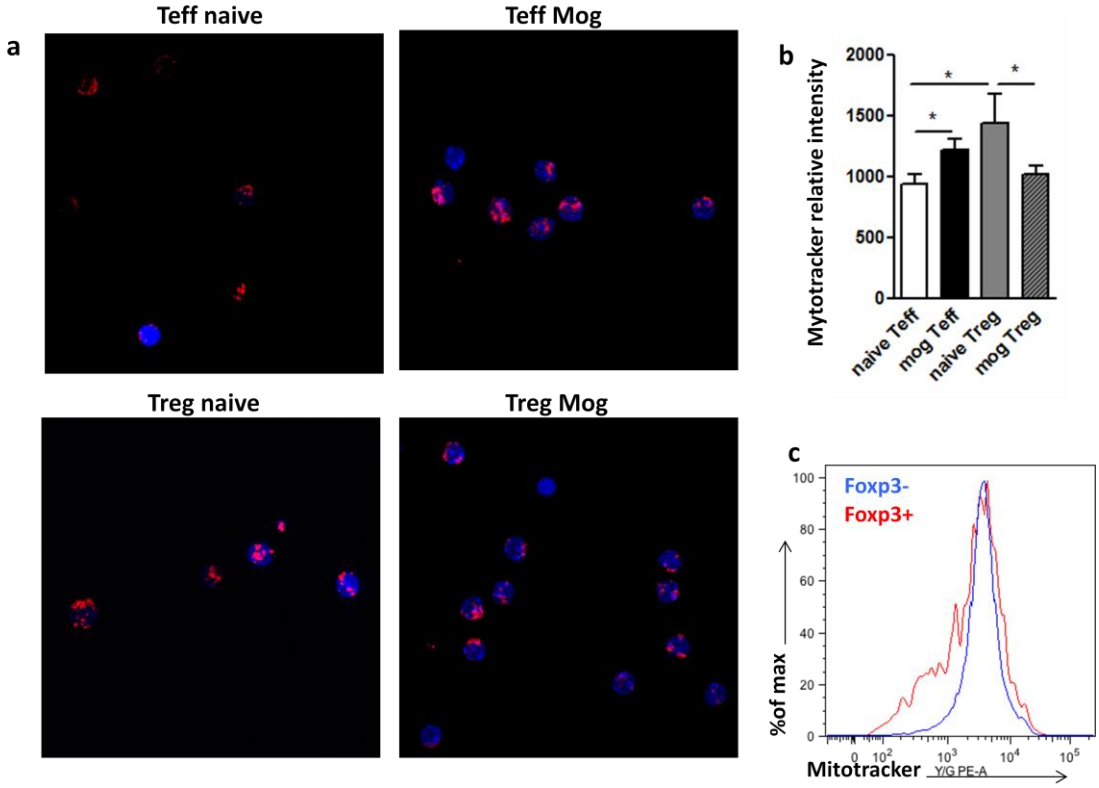

Figure C.1.3: Overall mitochondrial content diminished in Treg cells compared to Teff cells. **(a) Confocal images and (b) quantification of Teff and Treg cells from YFP-Foxp3 mice immunized for 9 days, labeled with mitotracker. (c) Flow cytometry analysis results. (a,b) Data are from one experiment with n=3, mean ± SD values, *p<0.05, **p<0.01, and ***p<0.001 (Student's t test). (c) Data are from one experiment representative of 3 experiments with n=2 per exp.**

Mitochondrial clearance by mitophagy depends on lysosomes. We therefore examined the frequency of acidic organelles- lysosomes in Treg and Teff cells. The frequency of lysosomes in Treg cells found decreased compared to Teff cells, in naive state as well as upon immunization (**Fig.C.1.4**)

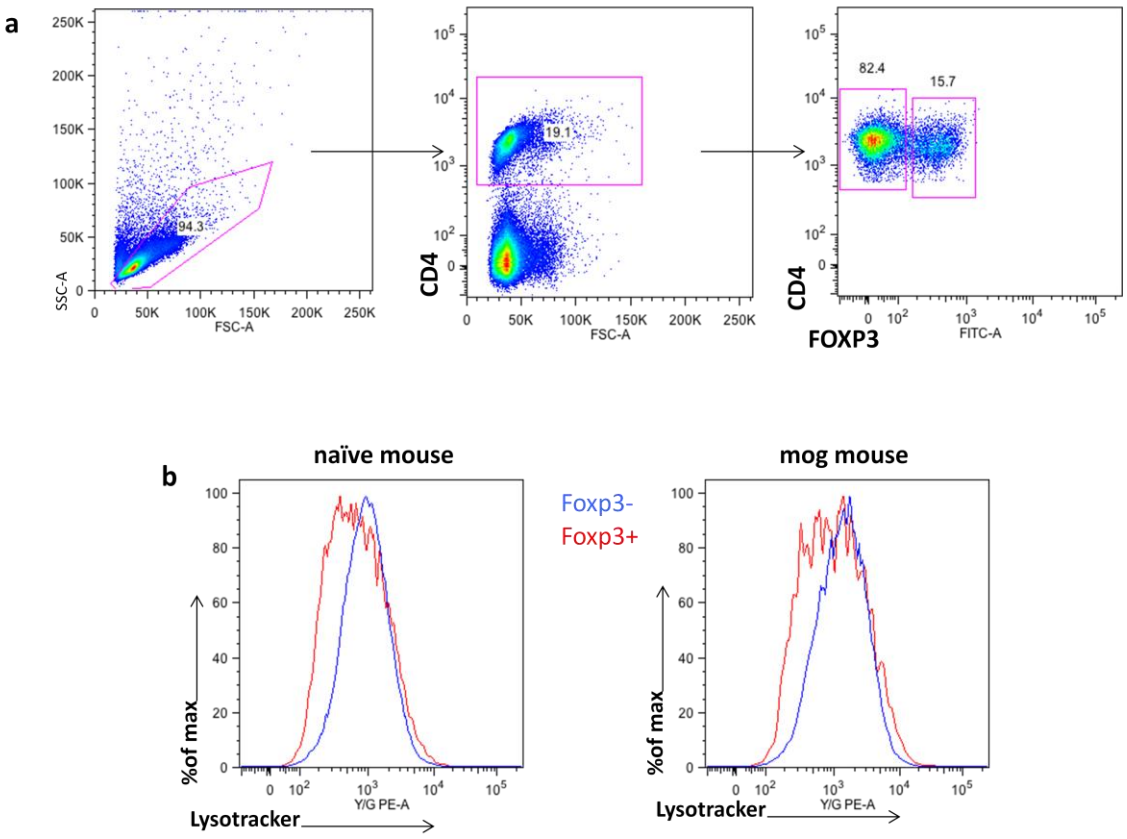

Figure C.1.4: Treg cells exhibit reduced amounts of lysosomes copmared to Teff cells. (a) Flow cytometry analyzing the expression of Teff ($CD4^+Foxp3^-$) and Teg ($CD4^+Foxp3^+$) cells of dLNs from YFP-Foxp3 mog/cfa immunized mouse and (b) representative histograms of mean fluorescent intensity (MFI) of Lysotracker staining in Teff and Treg cells from naive (left) and immunized (right) mice. Numbers in plots indicate percent of the cells. Data are representative of 2 independed experiments with n=2-3 per exp.

C.2) Characterization of mitophagy in the context of EAE

EAE is the experimental autoimmune encephalomyelitis mouse model, resembling human central nervous system (CNS) demyelinating diseases such as multiple sclerosis (MS). In order to induce EAE in Foxp3^{YFPcre} mice, apart from Mog_{35-55}/CFA injections, mice were co-injected with pertussis toxin in order to break down the blood-brain barrier and allow immune cells to enter the CNS tissue. At the time point that the manifestations of the disease were obvious (almost 14 days after the induction), cervical LNs were isolated and cells were stained with conjugated antibodies to CD4, additionaly to TMRE, Mitosox and Mitotracker stainings. Flow cytometry analysis revieled that the levels of TMRE and Mitosox underwent the same changes as in Mog_{35-55}/CFA immunization alone, while Mitotracker expression did not demonstrate any particular differences between Teff and Treg cells (**Fig.C.2.1**)

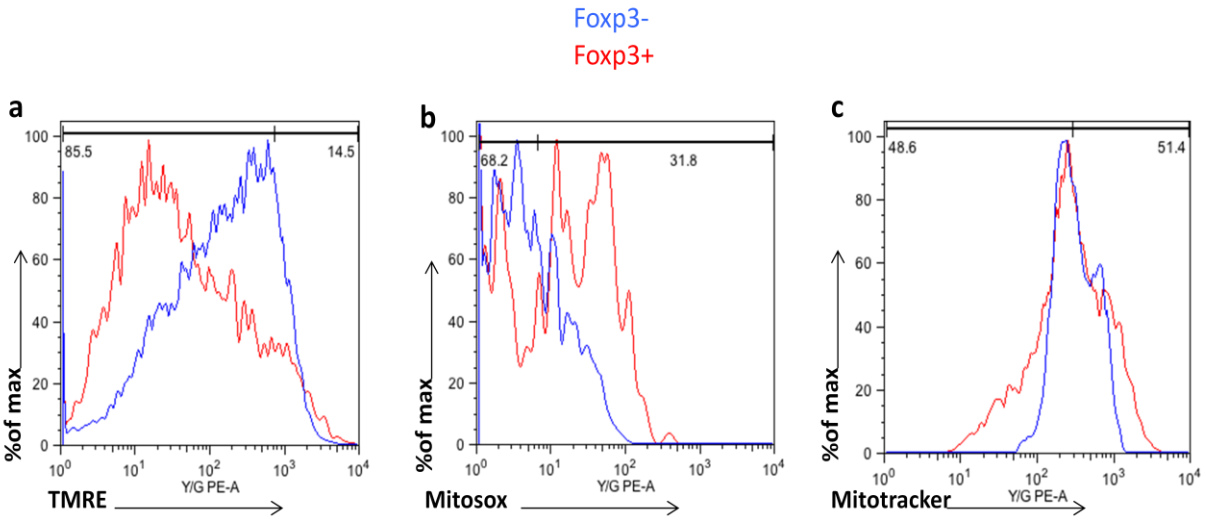

Figure C.2.1: Dynamic changes in mitochondrial quality of Treg cells compared to Teff cells. Representative histograms of MFI for (a) TMRE, (b) Mitosox and (c) Mitotracker staining in Teff and Treg cells from EAE mice. Data are representative of 2 independed experiments with n=2 per exp.

Finally, the Pink1 mRNA expression levels did not alter in the context of EAE, neither in Treg nor in Teff cells, but significantly reduced in Treg cells upon Mog_{35-55}/CFA immunization (**Fig.C.2.2**)

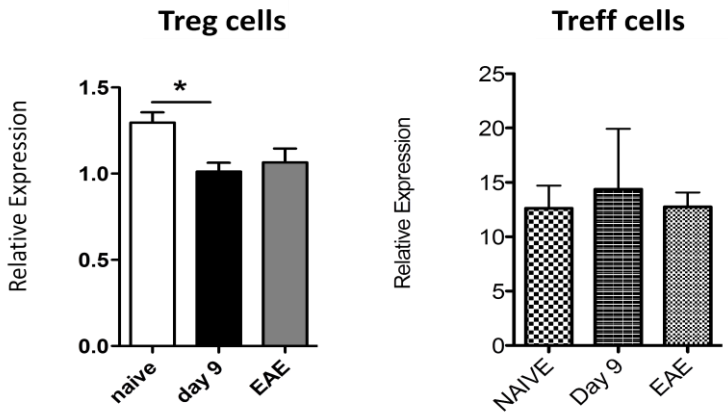

Figure C.1.2: Pink1 mRNA levels are reduced upon MOG/CFA immunization in Treg cells, while they remain the same in Treg and Teff cells of EAE mice. **Real-time PCR for Pink1 mRNA levels of $CD4^{+}Foxp3^{-}$(Teff) and $CD4^{+}Foxp3^{+}$ (Treg) cells, 9 days after immunization or in the context of EAE (n=5 mice per group). Data are mean ± SD values, *p<0.05, **p<0.01, and ***p<0.001 (Student's t test).**

Discussion

Understanding of autoimmune diseases, including multiple sclerosis, has expanded considerably during the last years. Accordingly, the role of regulatory T cells in autoimmunity has been described thoroughly; in mice depletion of Tregs is found to increase autoimmunity while adoptive transfer of Tregs can prevent and reverse it (Sakaguchi, Ono et al. 2006). In humans, deficient Treg cells can also lead to aggressive autoimmune diseases (Ochs, Gambineri et al. 2007). Studies on Multiple Sclerosis (MS) revealed that patients and healthy controls exhibit the same frequency of $CD4^{+}CD25^{high}$ Treg cells (Putheti, Pettersson et al. 2004) but MS patient's T regulatory cells were functionally impaired or unable to maturate properly (Zozulya and Wiendl 2008). Thus impaired Treg cell function could be responsible for the unbalanced tolerance against autoantigens, leading to disease susceptibility and affecting the course of autoimmunity in MS.

Even though studies have demonstrated that in the early stages of MS the pathology development is mainly controlled by inflammation (Centonze, Muzio et al. 2009), mitochondrial impairment is found to play a substantial role in the progression of the disease (Centonze, Muzio et al. 2010, Campbell and Mahad 2012). Undoubtedly, mitochondrial dynamics play a pivotal role regarding cell growth and survival. It has been proved that disorders of mitochondrial dynamics can contribute to the pathogenesis of neurodegenerative diseases and as recently discovered, in the pathogenesis of autoimmune diseases, (Detmer and Chan 2007, Lood, Blanco et al. 2016).

Extensive research have revealed that during MS development, mitochondria impairment is related to altered structure and distribution along with many molecular and biochemical defects (Blokhin, Vyshkina et al. 2008, Campbell and Mahad 2012, Fischer, Sharma et al. 2012, Prinz, Karacivi et al. 2015, Singhal, Li et al. 2015). In active MS lesions, oxidative damage to mitochondrial DNA has been observed (Lu, Selak et al. 2000), while studies have demonstrated globally impaired bioenergetics and overproduction of lactate in the cerebrospinal fluid of MS patients (Lutz, Viola et al. 2007, Reinke, Broadhurst et al. 2014). In addition, a mitochondria-targeted antioxidant found to have neuroprotective role and thus delayed the progression of the disease in EAE mice (Mao, Manczak et al. 2013). Finally, Vergara and collaborators discovered that mitochondrial lipid metabolism is altered in $CD4^{+}$ T cells from MS patients, indicating a possible correlation between mitochondria and regulation of T cell lipid metabolism in multiple sclerosis (Vergara, D'Alessandro et al. 2015).

Autophagy is found to be crucial for T-cell development at the precursor stage, as self-renewability and quiescence of hematopoietic stem cells depend on autophagy of the mitochondria and the endoplasmic reticulum. Later, during development in the thymus, autophagy regulates peptide presentation in stromal cells and

professional antigen-presenting cells, which mediate thymocyte selection. Furthermore, the metabolic changes when mature T cells enter the periphery and when they are activated are both dependent on autophagy (Bronietzki, Schuster et al. 2015).

Even though the implication of mitochondria in MS progression has been extensively examined, the role of mitophagy in Treg cell function and survival, as well as its efficacy in damaged mitochondrial clearance in the course of the disease, until know remain obscure.

Our data demonstrate that 9 days after Mog_{35-55}/CFA immunization, Tregs exhibited significantly decreased *Beclin-1* expression followed by a small reduction in *Atg5* expression, while their p62 expression levels remained the same. On the contrary, LC3 expression levels substantially increased, indicating that even though LC3 is present, the initiation and elongation of the autophagosome cannot be performed properly and thus p62 levels are not diminishing. In the context of mitochondrial clearance, the fact that Pink1 expression is reducing in Treg cells after immunization could also play a role in this result.

On the other hand, activated Teff cells exhibited increased *Beclin-1* expression as well as LC3 and p62 expression, with a concomitant significant *Atg5* reduction. The fact that the toxic cellular waste (recognized by p62) are being increased despite the elevated *Beclin-1* and LC3 expression levels, indicates that autophagy in Teff cells is not carried out properly and highlights the importance of *Atg5* in the process of autophagy. In Teff cells, the Pink1 expression did not alter upon immunization. Finally, Treg cells found to exhibit reduced p62 and increased LC3 expression compared to Teff cells indicating that autophagy is more active in Treg cells upon immunization.

The above results, made obvious that immune responses affect the autophagic procedure in Teff and Treg cells, as well as that the Teff and Treg cells exhibit different mechanisms of autophagy regulation upon activation.

In vivo deletion of *Atg5* from activated Tregs led to significant decrease of Treg cell population, indicating that autophagy strongly affects Treg cell survival upon an immune response. Additionally, *in vitro* inhibition of autophagy with Wortmannin slightly increased the proliferation of thymus derived Treg cells, while their total cell ROS levels were not affected. On the other hand, *in vitro* autophagy inhibition of induced Treg (iTreg) cells and Teff cells, led to a profound increase of their proliferation rates, followed by a small increase in their total ROS levels. This observation suggests that thymus derived Treg cells are governed by different mechanisms concerning their autophagy compared to iTreg and Teff cells. However, another study demonstrated that *in vitro* inhibition of autophagy with 3-MA, an autophagy inhibitor which also inhibits autophagosome formation, led to reduced activation-induced Teff cell proliferation (Hubbard, Valdor et al. 2010). These controversial results could be explained by off-target effects of using these

inhibitors. To this end, more research needs to be done in order to elucidate the above differences.

Another important finding upon *in vitro* autophagy inhibition with Wortmannin, was the observed alterations in the ratio of Teff and Treg cell frequencies. More specifically, treatment with WM augmented Treg cell frequency, while simultaneously decreased Teff frequency, indicating that upon inhibition of autophagy Teff cells are intensely converted to iTreg cells. This effect further confirmed by *in vivo* depletion of autophagy in T cells (CD4creATG5$^{fl\backslash fl}$ mice). However, these decreased Teff cells upon autophagy inhibition could be due to their defective survival, possibly caused by mitochondria volume accumulation (Jia and He 2011).

Studying the efficacy of mitochondrial clearance through autophagy, we observed that while Teff and Treg cells exhibited the same levels of Pink1 expression upon activation, their mitochondrial dynamics presented significant differences. Treg cells exhibited substantially decreased membrane potential and increased mitochondrial superoxide production compared to Teff cells upon immunization, followed by diminished mitochondrial cell content. This result suggests that under an immune response Treg cell mitophagy should be more active, as they exhibit features of unhealthy/damaged mitochondria. The paradox of this observation is that even though Pink1 is normally accumulated on mitochondria outer membrane when their membrane potential is low, in Treg cells that their TMRE is importantly decreased compared to Teff cells, the Pink1 expression is at the same levels with Teff cells.

Although the mitochondrial dynamics of activated Tregs suggested an increased need for mitochondrial clearance, our results demonstrated that lysosomes in Treg cells were decreased compared to Teff cells.

Finally, examining the mitochondrial features of Tregs in the context of EAE, we observed that the mitochondrial dynamics follow the same changes as in the activated stage after Mog_{35-55}/CFA immunization, concerning their membrane potential and the ROS production, but the total mitochondrial content between Treg and Teff cells did not present any differences in EAE mice. In addition, upon EAE induction, the expression of Pink1 did not alter neither in Treg nor in Teff cells.

The above findings are providing a new insight in the field of autophagy/mitophagy of Treg cells upon immunization in general, and more specificaly in the context of MS. Of course more research needs to be done in order to further assess the role of mitophagy in T regulatory cell survival and function, in the context of multiple sclerosis.

Until today, treatment for autoimmune diseases mostly involves regulation of the autoimmune reaction by suppressing the immune system. However, these drugs suppress not only the autoimmune reaction but also the body's ability to defend itself against foreign substances, this way increasing the risk of additional

pathological conditions. Consequently, discovering alternative therapeutic methods that are targeted specifically to autoimmunity is of vital importance. In this context, delineating the influence of defective mitochondria clearance in the role of T regulatory cells in autoimmunity and more specifically in multiple sclerosis could provide new evidence in the pathogenesis of the disease, indicating novel therapeutic targets towards the design of immunotherapeutic protocols.

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