The Role of Autophagy in Regulatory T Cell Function and Homoeostasis

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The Role of Autophagy in Regulatory T Cell Function and Homoeostasis

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A Thesis Submitted to the Faculty of

The Harvard Medical School

in Partial Fulfillment of the Requirements

for the Degree of Master of Medical Sciences in Immunology

Harvard University

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Abstract:

T regulatory cell are very crucial for the induction of tolerance. Autophagy is a dynamic process for which cells resort to in case of stress and starvation. Here, we elucidated some of phenotypic changes in autophagy among LRBA deficient subject suffering from regulatory T cell pathology. From there we decided to study role of Autophagy in physiological conditions and we deleted three key enzymes in the Autophagy pathway, namely Vps34, Stk3 and Stk4. This deletion was specific in T regulatory cell utilizing Foxp3\textsuperscript{YFP\textsuperscript{cre}} system and crossing them with flexed genes above. Our result shows that regulatory T cell in particular are more dependent on Autophagy and loss of Autophagy result in severe Autoimmunity.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>CHAPTER</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>TITLE PAGE</td>
<td>i</td>
</tr>
<tr>
<td>TABLE OF CONTENT</td>
<td>ii</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>iii</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>iv</td>
</tr>
<tr>
<td>ACKNOWLEDGMENT</td>
<td>v</td>
</tr>
<tr>
<td>CHAPTERS</td>
<td></td>
</tr>
<tr>
<td>CHAPTER ONE – Introduction</td>
<td>1</td>
</tr>
<tr>
<td>CHAPTER TWO – Method</td>
<td>13</td>
</tr>
<tr>
<td>CHAPTER THREE – Results</td>
<td>17</td>
</tr>
<tr>
<td>CHAPTER FOUR – Discussion</td>
<td>24</td>
</tr>
<tr>
<td>BIBLIOGRAPHY</td>
<td>28</td>
</tr>
<tr>
<td>APPENDICES</td>
<td></td>
</tr>
<tr>
<td>Appendix A – Supplementary Figures</td>
<td>37</td>
</tr>
<tr>
<td>Appendix B – Autophagy Dynamics</td>
<td>39</td>
</tr>
</tbody>
</table>
List of Figures

Chapter One: Illustrative Figures ........................................................................................................... 1

Figure 1: Defective Treg cell suppressive mechanisms in IPEX-like disorders .................. 5

Figure 2: Autophagy pathway and Regulation .................................................................................. 8

Chapter Three: Main Figures ......................................................................................................... 17

Figure 1: LRBA deficiency increases Autophagy ................................................................. 18

Figure 2: Vps34 KO in Tregs result in severe Autoimmunity ........................................... 19

Figure 3: Vps34 deficiency in Tregs results in Th1 and Th2 Disease ................................. 21

Figure 4: Delayed Autoimmunity in Stk3^Δ/Δ and Stk4^Δ/Δ .................................................. 22

Figure 5: 12 Weeks old Stk4^Δ/Δ mice have Th17 Type Inflammation ......................... 23

Appendix A: Supplementary Figures ......................................................................................... 37

Supplementary Figure 1: Method Validation for Autophagy Measurement ............... 37

Supplementary Figure 2: Canonical Markers of Regulatory T cells in Vps34^Δ/Δ mice ... 37

Supplementary Figure 3: Cellular Characterization of 4 weeks old Stk4^Δ/Δ mice .......... 38

Supplementary Figure 4: Cellular characterization of 12 weeks old Stk3^Δ/Δ mice .......... 38

Appendix B: Autophagy Dynamics ............................................................................................... 39

Appendix Figure 1: Autophagy Dynamics ............................................................................... 39
Acknowledgment:

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Chapter One: Introduction

The immune system functions to protect the host from pathogens and maintain homeostasis. These functions of the immune system involve coordinated action by its innate and adaptive arms, which provide for both early stereotypic responses to pathogens (innate immunity) as well as learned responses that are refined upon repeat exposures (adaptive immunity). The innate immune system is geared towards the recognition of pathogen-associated molecular patterns (PAMPs), such as bacterial cell-wall components (Lipopolysaccharides, LPS) and bacterial viral DNA and RNA species. It also recognizes damage-associated molecular patterns such as chromatin-associated protein high-mobility group box 1 (HMGB1), which are released upon tissue damage. The adaptive immune system recognizes specifically peptides antigens (via T cell receptors) or proteins (B cell receptors). A key conundrum of both the innate and adaptive immune responses is their potential to engage in injurious immune responses to “extend self”, including self-tissues, associated commensal microbiota, and harmless environmental antigens including foods. This conundrum suggested the presence of mechanisms that would have evolved to aid in the discrimination between extended self and non-self, leading to the introduction of the concept of immunological tolerance.

Tolerance towards the extend self is achieved by two general mechanisms. Central tolerance, in which the adaptive immune cells get deleted or edited if they mount a response against self. And peripheral tolerance in which the immune response is tuned down by means of anergy or actively enforced by dedicated regulatory immune cells, most notably regulatory T (Treg) cells. Treg cells are a dynamic modulator suppressing the proliferation, differentiation and effector function for CD4⁺, CD8⁺, Natural Killer T cells (NKT) and Antigen Presenting Cells (APC).
Treg cells were first invoked in 1970 by Gershon and Kondo. By giving large doses of sheep red blood cells to thymectomized, sub-lethally irradiated and bone marrow reconstituted mice, they found that tolerance is induced by thymic derived cells. Suppressor T cells along with its unique Major Histocompatibility Complex II (I-J) attracted the attention of immunologists for more than a decade. How, the concept of suppressor T cells suffered a setback when, in 1983, Kronenberg and his colleagues showed that the putative I-J gene doesn’t exist.

The resulting stigma that was associated the concept of suppressor T cells obscured accumulated evidence that thymic derived T cells harbored Treg cell subset(s) that dampened the immune response. In that era, the only acknowledged mechanisms of T cell tolerance were anergy and clonal deletion with several attempts to revisit the topic of suppressor CD4+ cells but under a different label such as Tr1 and Th3. It was through the studies of Sakagochi and colleagues that a subset of thymic derived Treg cells characterized by CD4+CD25 expression were identified as bona fide Treg cells that transferred tolerance from one host to another.

With this findings, Treg cells firmly emerged as enforcers of peripheral tolerance by means of modulating the immune response. Despite being extremely useful, CD25 (IL2 receptor alpha chain) is not very specific to (Tregs). Five years later, the Forkhead/winged helix transcription factor p3 (FOXP3) found to be the transcription factor that direct CD4+ cells into a Treg lineage. A mutation in this gene, was found to cause an X-linked autoimmunity, allergic and immune dysregulation, Hyper-IgE syndrome, Polyendocrinopathy and enteropathy (XLAAD/IPEX) (Illustrative Fig1). Concurrently, the murine orthologue Foxp3 was found to be responsible for the scurfy phenotype, also characterized by generalized Autoimmunity and inflammation.
Expression of Foxp3 is indispensable for Treg cells development as evident by the analysis of CD25⁺ CD4⁺ T cell population in the thymus and peripheral lymphoid organs of a mixed bone marrow chimeras. After transferring Foxp3 deficient and congenic wild type bone marrows into a T cell deficient mice, the CD25⁺ (Tregs) cell only developed from the Foxp3 sufficient progenitors. On top of that, retroviral expression of Foxp3 in CD25⁻ CD4⁺ or CD8⁺ T cell enhance the phenotypic feature of (Tregs) and induce suppressor function, while losing Foxp3 in Effector T Cells (Teffs) have no effect on their proliferation and cytokine production.

Collectively, these evidence show that Foxp3 is sufficient and necessary to induce (Tregs) and the lack of (Tregs) is the cause of disease in Foxp3 deficiency.

The transcription factor Foxp3 acts as both transcriptional activator and suppressor. It binds to more than 700 genes and its continuous high level of expression maintains the fitness of (Tregs). The exact transcriptional profile that Foxp3 potentiates was studied using a mouse model where Foxp3 is truncated (lacking the DNA binding domain) and fused with the reporter enhanced Green Fluorescence Protein eGFP Foxp3ΔeGFP. Unlike canonical (Tregs), eGFP⁺ Foxp3Δ produced IL-4 and IL-17 suggesting that Foxp3 modulate the Teff phenotype in cis. And it amplifies and stabilizes the expression of negative regulator genes such as CTLA4, IL-10 IL-10ra CD5 and FasL which maintains the (Treg) phenotype and suppress in trans. However, eGFP⁺ Foxp3Δ cells retain some of the phenotypic features of (Tregs) suggesting that there is a Foxp3-independent transcriptional program that arise in parallel or even proceeds Foxp3 transcriptome contributed by signaling from T cell receptor (TCR), IL-2R and TGF-βR.

To the expectation, mice with a loss of function in IL-2, IL-2Ra (CD25) or IL-2Rβ (CD122) or its signaling component such as Stat resulted in a scurfy phenotype. In similar
manner, patients with CD25 deficiency have an IPEX-like phenotype (Illustrative Fig1) with immune dysregulation, polyendocrinopathy and enteropathy. (Tregs) in CD25 deficient subjects losses its suppressive capacity, nonetheless patients do not have allergies and Hyper-IgE syndrome due to intact Foxp3 function. It’s very interesting that the loss of CD25 presented itself simplistically in similar fashion to Foxp3 deficiency indicating the complexity of Treg lineage specificity and function.

The suppressive function of (Tregs) can be classified into contact-dependent mechanisms and contact independent ones (Illustrative Fig1). For the latter, inhibitory cytokine production such as IL-10, TGFβ, and IL-35 induces direct suppression on the effector T cells and the so-called licensed suppression on APC, which in turn reduces its capacity to activate naïve T cells. The high affinity IL-2 receptor (CD25) also acts as a vacuum depriving the inflammatory milieu from survival signals of IL-2. A less appreciated mechanism of suppression is mediated by the two ectoenzymes CD39 and CD73. CD39 is ectonucleotidase with triphospho-diphospho-hydrolase activity, which is the rate-limiting enzyme in converting the extracellular ATP and ADP to AMP. Then, the ecto-5’-nucleotidase CD73 releases the Adenosine from AMP. Adenosine signaling induces accumulation of intracellular cAMP in Effector T cells, which in turn suppress their proliferation.

The contact dependent mechanisms can be further classified into two. Firstly, the cytotoxic killing of target cells by means of Granzyme B (but not granzyme A). Secondly, through receptor-ligand interaction which is mediated by CTLA4, LAG3 and TIGIT. LAG3 is a CD4 homolog that binds with high affinity to MHC class II on APC. Upon engagement it leads to their inhibition and it reduces their co-stimulatory capacity. TIGIT and CTLA4 belong to the Ig super family and they are expresses with abundance on the surface of (Tregs). TIGIT appears
to be an inducer of immunosuppressive cytokines IL-10 and TGFβ. After the great success of anti-CTLA4 therapy, both LAG3 and TIGIT antagonizing antibodies are under investigation as potential therapies for cancer. Cytotoxic T lymphocyte antigen 4 (CTLA4) is well-documented suppressor molecule acting in cell intrinsic manner as well as cell extrinsic. Form the Tregs point of view, it acts by competing with CD28 to bind the co-stimulatory molecules CD80/86 on the APC and scavenging them away from (Teffs) via trans-endocytosis (Illustrative Fig1).

A total knock out of CTLA4 in mice give rise to a severe lymphoproliferative syndrome with mice dying at 2-3 weeks. While, the conditional knock-out of CTLA4 in (Tregs) resulted in a better survival with mice dying at two months due to severe Autoimmune disease. This

Illustrative Figure 1 Major pathways maintaining Treg cell homeostasis and function. Shown are highlights of human monogenic defects that lead to severe immune dysregulation due to altered Treg cell function. The engagement between IL-2 and IL-2R and the initiation of signal transduction through STAT5b phosphorylation are important for FOXP3 expression. While loss of FOXP3 result in IPEX, a loss in IL2Rα or STAT5b manifest with IPEX-like phenotype. Also, LRBA-CTLA4 pathway is indispensable for Treg cell suppressive activity. LRBA controls CTLA4 intra-cellular stores and expression. CTLA4 is a negative regulator both directly by competing with CD28 and indirectly by down-regulating the co-stimulatory molecules on APCs. Both LRBA and CTLA4 deficiency cause IPEX-like disorder.
suggest that CTLA4 deficiency exert its pathogenicity by means of defective (Treg) functionality and homeostasis. In humans, CTLA4 haploinsufficiency presents with autoimmune phenomena with colitis, cytopenia, hypogammaglobulinemia and respiratory infections. The interesting finding of a defective B cells homoeostasis on top the immune dysregulation syndrome adds on to the complexity of the disease. Adding more to the complexity, another cohort of patient suffered from severe lymphoproliferative syndrome, hypogammaglobulinemia and low surface expression of CTLA4 protein yet the CTLA4 gene was mutation free. A mutation was found in the gene coding for lipopolysaccharide responsive beige-like anchor protein LRBA. Those patients like many others were labeled with LRBA deficiency and treated accordingly as a form of the common variable Immunodeficiency given their hypo-gam status.

Later on, (Tregs) in LRBA deficient subject was found to be highly apoptotic with a defect in its suppressive capacity. Last year, Bernice Lo and colleagues showed elegantly that LRBA protein is responsible for maintaining CTLA4 intracellular store and surface translocation. This discovery has shifted the paradigm of homestay therapy in CTLA4 and LRBA deficiency from bone marrow transplantation to CTLA4 fusion protein. Although some of the main features of both disorders where explained, a huge part of the pathogenesis is remained to be elucidated. Indeed, LRBA is responsible for CTLA4 trafficking yet loss of function in LRBA present differently than CTLA4 deficiency. LRBA loss of function result in more severe autoimmunity that is less responsive to the conventional immunosuppressive medication. On top of that CTLA4 haploinsufficiency presents later in life compared to LRBA deficiency.
The Pi3k-Akt-mTOR pathway is relatively well characterized in (Tregs) physiology. This pathway is involved in regulating metabolism and survival. Activation of this pathway by growth signals such as cytokines or co-stimulation (CD28) results in anabolism and increased growth and proliferation. In (Tregs), CD28 or TCR signal result in activation of Class I Phosphatidylinositol-3-kinase (PI3K) which phosphorylate membrane phosphatidylinositol at three locations giving Phosphatidylinositol (3,4,5)-trisphosphate (PI(3,4,5)P3). These phospholipids act as docking site for another kinase called Phosphoinositide-dependent kinase1 (PDK1), which in turn phosphorylate and activate (AKT). AKT kinase is an activator of the mammalian Target Of Rapamycin complex 1 (mTORc1). Then, mTORc1 diverges to many pathways involved in promoting survival, anabolism and inhibiting autophagy and apoptosis. Mitogen-activated protein kinase (ERK) and mTORc2 are non-canonical activator of AKT-mTORc1 axis. Lack of survival signal or nutrition results in accumulation of intracellular AMP which inhibits mTORc1 through AMP activated-Kinase (AMPK) (Illustrative Fig2).

A conditional deletion of Raptor, the core protein of mTORc1, in (Tregs) results in a loss of their suppressive capacity leading to a scurfy phenotype. On the other hand, constitutively active PI3K or AKT result in the same phenotype. This dichotomy can be explained in concordance with the thymic selection model. That is, mTORc1 signaling is crucial for (Tregs) suppressive capacity. However, over signaling of mTORc1 result in changing the metabolic landscape of (Tregs) which also render them inactive. The fine tuning of mTORc1 activity in Tregs is essential in their development and suppressive capacity.

A more mechanistic explanation of Akt-mTOR axis is that pulsar mTORc1 activity is required to maintain the Lipogeneic-lipolytic program of Treg, which is required for their longevity and
suppressive capacity\textsuperscript{69}. Other than changing the metabolic program of (Treg), the non-physiological constitutive activity of PI3K or AKT results in phosphorylation and cytosolic translocation of transcription factors Foxo1 and Foxo3\textsuperscript{74}. The Foxo transcription factors are essential for Foxp3 expression as they bind to Foxp3 promotor region as well as downstream to the Conserved Non-coding Sequence (CNS) to enhance and maintain the transcription\textsuperscript{75}. It

Illustrative Figure 2 Autophagy pathway and Regulation. Shown are the initiation, elongation, sequestration and degradation steps of autophagy with the main proteins involved in the machinery. The regulatory mechanisms lie at the beginning where mTORc1 phosphorylate and inhibits ULK1 at Serine 757 and inhibit and Ambra1 by phosphorylating Serine 52. AMP Kinase (AMPK) inhibit mTORCc1 and reverse its action on ULK1 by phosphorylating Serine 317, which in turn phosphorylate and release Ambra1 on unknown site. Once Ambra1 is release a second regulatory element is maintained by Serine-Threonine Kinases 3 and 4 (STK3/4). STK3/4 phosphorylate the BH3 homology domain on Threonine 108 of Beclin1 increasing it interaction with Bcl2 and inhibiting autophagy. Death Associated Protein Kinase (DAPK) phosphorylate Beclin1 at Threonine119 of the BH3 domain releasing it from Bcl2. Also, STK3/4 phosphorylate LC3 at Threonine 50 to enhance its binding with lysosome. STK3/4 inhibit YAP-TAZ transcription factor to program the cell into quiescence and activate FOXO3 to inhibit glycolysis and decrease the oxidative stress.
was reported that (Tregs) in *LRBA* deficient subjects have lower mTORc1 activity as compared to a healthy (Tregs)\(^{56}\). Lack of TCR stimulation or survival signaling result in mTORc1 suppression\(^{76},\) \(^{77}\). The cell then turns into a catabolic programing for survival and sustenance. However, the dynamic migratory nature of (Tregs) may result in status where nutrients might not be readily available. Without stimulating signals and lacking external nutrition, (Tregs) taps into an internal source for metabolites where proteolysis and amino-acids are the primary source of ATPs\(^ {70},\) \(^{78},\) \(^{79}\). In process involving self-eating (Autophagy), (Tregs) degrades its non-essential macro-molecules and organelles providing ATPs during starvation\(^{73}\).

Autophagy\(^ {80}\) can be looked at as recycling process in which intracellular proteins are degraded in the lysosome. This degradation can extend to entire organelles like mitophagy, pexophagy and ERphagy. Depending on the cargo’s size it can be classified into Microautophagy and Macroautophagy. A chaperone mediated autophagy were soluble small protein are transferred across the lysosomal membrane or a direct invagination of lysosomal membrane to engulf larger proteins, are both collectively fall under Microautophagy\(^ {81}\).

Macroautophagy is different in which a newly formed double membrane structure engulfs, in a selective or non-selective manner\(^ {82}\), intracellular proteins and organelles. Other than providing an alternative source of nutrition during stress, Macroautophagy can be in itself a mechanism of apoptotic cell death (type II apoptosis)\(^ {83},\) \(^ {84}\). Although in the canonical apoptotic cell death (type I apoptosis) there can be varying amounts autophagosome, type II apoptosis differs in the cell membrane and nuclear structures. There are minor changes in the nucleus as compared to nuclear condensation in type I apoptosis or nuclear swelling in necrosis. On the other hand, the plasma membrane can go either way, blebbing as in type I apoptosis or
rupture as necrosis. These morphological and biochemical distinctions between type I and type II apoptosis needs further exploration\(^84,\)\(^85\).

The molecular mechanism of autophagy can be classified into four stages, membrane isolation (phagophore formation), elongation, sequestration and degradation (Illustrative Fig2). Lack of mTORc1 activity or AMPK activation result in in activation of more than 30 proteins that orchestrate autophagy, collectively termed Autophagy-related Genes (Atg). It begins with an activation of the kinase ULK1 (Atg1) downstream of AMPK and mTORc1\(^76,\)\(^86\). This kinase mediates the escape of Autophagy initiating enzyme PIK3c3 complex from microtubules by phosphorylating the regulatory protein Ambra1 that tethers Pik3c3 complex inactive. The released Pik3c3, a Class III (PI3K) that phosphorylate membrane phosphatidylinositol at one site only (PI(3)P), is activated\(^86,\)\(^87\) (Illustrative Fig2). It is composed of multiple subunits Vps34, Atg14, p150 and Beclin1 where Vps34 is the catalytic subunit that phosphorylate phosphatidylinositol at the rER membrane and Beclin1 which act as major regulatory element\(^88,\)\(^89\). These negatively charged PI3Ps buds out escaping the hydrophobicity of the membrane forming an omega shaped structure, omegasome, the autophagosome nidus. Also, these phosphorylated lipids act as niche for other Autophagy proteins such as Atg18, Atg21 and Atg24 (Illustrative Fig2).

After nucleation of the double membrane, elongation begins. The aforementioned Atgs and Pik3c3 recruit a series of two Ubiquitin like systems (Ubl). Atg12 is conjugated to Atg5 through the action of Atg7 and Atg10 (E1 and E2 like enzymes, respectively). Atg5-Atg12 is further interacts with Atg16L that oligomerizes the Atg5-Atg12-Atg16L into a tetramer and binds to the membrane. The second Ubl system, LC3b (Microtubules associated protein 1 light Chain 3) is first process by Atg4 exposing a c-terminus glycine forming LC3I. Then, Atg7 and Atg3 (E1
and E2 like enzymes, respectively) conjugate LC3I to phosphatidylethanolamine (PE) giving a mature protein LC3II. This protein can serve as a specific marker for the Autophagosome. Other than being a key regulator of phagophore elongation LC3II can act as receptor for the prospective cargo\textsuperscript{81}. \textsuperscript{90}. \textsuperscript{91}. Through p62/sequestosome1 (SQSTM1) that binds both ubiquitinated proteins and LC3II\textsuperscript{92}. Then, Autophagosome fuses with the lysosome in process requiring LAMP2 and the small GTPase Rab7.

Being a two-edge sword, Autophagy is lying under a tight regulation\textsuperscript{93}. Other than the upstream regulation of initiation by mTORc1-ULK1-AMPK loop, Bcl2 (B Cell Lymphoma 2) plays an off-switch role halfway on the Autophagy cascade\textsuperscript{94}. Bcl2 is pro-survival anti-apoptotic protein, which binds the Beclin-1 at the rER membrane rendering the Pik3c3 complex inactive (Illustrative Fig2). When Bcl2 is phosphorylated by tumor suppressor kinase JNK1 (c-Jun N-terminal protein kinase 1) it dissociate from Beclin-1 and only then Autophagy starts. Continuous JNK1 signaling will result in apoptosis through Bax activation\textsuperscript{95}.

There are different enzymes that regulate Bcl2-Beclin-1 interaction. For example, Death Associated Protein Kinase (DAPK) phosphorylates Beclin-1 and dissociates it from Bcl2\textsuperscript{87}. \textsuperscript{96}. Another negative regulator that maintains the stability of Bcl2-Beclin-1 complex is the Serine-Threonine Kinase 3 and 4, Hippo Kinase in yeast, which phosphorylate Beclin-1 increasing its affinity to Bcl2 and inhibiting autophagy\textsuperscript{97}. Nonetheless, the Hippo kinases also play another integral role in advancing autophagy machinery by phosphorylating LC3. Only phosphorylated LC3 on the Autophagosome can induce lysosomal clustering and fusion while non-phosphorylated LC3 can’t\textsuperscript{98}.

The Hippo Kinases (STK3/4) also phosphorylates two transcription factors Foxo3 and YAP- TAZ (Yorkie in yeast). The oncogene YAP-TAZ is phosphorylated, inhibited and translocated
out of the nucleus by STK3/4\textsuperscript{99}. That decreases the capacity of the cell to proliferate and go into mitosis. On the other hand, STK3/4 phosphorylation to Foxo3 maintains its nuclear localization regardless and overriding the metabolic signals\textsuperscript{100}. The Foxo transcriptional program is equipped to minimize the damage of stress by up-regulating anti-oxidant machinery and turning the cell into non-glycolytic metabolism. It seems that Hippo signaling pathway can decide the fate of autophagy spectrum from apoptotic cell death (type II) to pro-survival mechanism. It inhibits Pik3c3 activity (forming new phagosomes), enhances lysosomal fusion and degradation (providing nutrients), and changes the transcriptional landscape to a quiescent long-lived program.

With above information we decided to study the role of Autophagy in (Tregs). It was reported that \textit{LRBA} deficient subjects have a dysregulated autophagy machinery in B cell\textsuperscript{55}. Also, \textit{LRBA} loss of function results in decreased mTORc1 activity. With that in mind we deleted two Autophagy genes specifically in (Tregs). A core machinery gene and regulatory gene, namely \textit{Vps34} and \textit{STK3/4} respectively.
Chapter Two: Method

Patients. Affected individuals with LRBA deficiency previously reported\textsuperscript{54}. With a 2-bp deletion, NM_006726:c.6657_6658del resulted in a frameshift and premature truncation of the protein p.(Glu2219Aspfs*3). Control subjects were age group-matched. All study participants were recruited using written informed consent approved by the local Institutional Review Boards. Studies at the Boston Children’s Hospital were conducted under approved protocol #04-09-113R.

Mice. Foxp3\textsuperscript{YFP\textsuperscript{Cre}}, Vps34\textsuperscript{fl/fl}, Stk3\textsuperscript{fl/fl} and Stk4\textsuperscript{fl/fl} mice were purchased form Jackson Laboratory on C57BL/6 background. 4 or 12 week-old mice were used in this study (Age is specified in each experiment). The mice were housed under specific pathogen-free conditions and used according to the guidelines of the institutional Animal Research Committees at the Boston Children’s Hospital.

Autophagy Detection. PBMCs were isolated from whole blood by centrifugation over Ficoll-Hypaque gradients. Autophagy was measured with the Cyto-ID autophagy detection kit (Enzo, Cat. No. ENZ-51031-K200) according to the manufacturer’s instructions. Cells were starved for 4 hours in nutrient free medium EBSS (Thermofisher, Cat. No. 14155063) or treated with 10 μM Hydroxychloroquine and 1μM Rapamycin in RPMI-1640 media supplemented with 5% FBS, 100 U/mL penicillin G, and 0.1 mg/mL streptomycin for 18 hours in a humidified atmosphere of 5% CO2 at 37 °C. This test measures autophagic vacuoles and monitors autophagic flux in live cells using a fluorescent cationic amphiphilic dye that selectively labels autophagic vacuoles. Briefly, cells (5×10\textsuperscript{5}) were washed in PBS (pH 7.2), and resuspended in 250 μl of freshly diluted Cyto-ID\textsuperscript{®} Green Detection Reagent to a final volume of 500 μl with PBS, and incubated for 30 minutes with Anti-CD4 mAb (RPA-T4, BioLegend). The
fluorescence of 10,000 CD4+ cells was analyzed using Fortessa cytometer with DIVA software (BD Biosystems) and were analyzed using FlowJo (Tree Star).

**Flow Cytometry.** For surface staining, lymphocytes were harvested from spleen and suspended in PBS with 0.5% Fetal bovine serum (FBS). Stained with Anti-CD4 (RM4-5), Anti-CD8 (53-6.7), Anti-CD90.2(30-H12), Anti- CD25 (PC61), Anti- Nrp1 (3 E12), Anti- GARP (F011-5), Anti- LAP (TW7-20B9) all from (BioLegend). And Anti-CD44 (IM7) Anti-CD62L (MEL-14) Anti- ICOS (7E.17G9) (from eBioscience). Intra-cellular staining for Foxp3 was done according to manufacturer recommendation (FJK-16S, eBioscience). Anti-CTLA4 (UC10-4B9), Anti- Ki67 (SolA15), Anti-Helios (22F6) and all was performed by using eBioscience Fixation/Permeabilization kit. For cytokine detection, cell suspensions were pre-incubated for 4h with 50 ng/ml PMA (phorbol 12-myristate 13-acetate), 500 ng/ml ionomycin and 10 μg/ml brefeldin A in complete medium before blockade of CD16/32, followed by surface staining, permeabilization and intracellular overnight staining of IFN-γ(XMG1.2), IL4 (11B11), and IL-17 (TC11-18H10.1) using BD Bioscience Fixation/Permeabilization kit. All acquisitions for flow cytometry were performed on a Fortessa cytometer with DIVA software (BD Biosystems) and were analyzed using FlowJo (Tree Star).
Histology. Lung, Liver and ear sections were stained by H&E Histopathological scoring of tissue was done by a blinded observer, and the final scores reflected averages of scores from 3 different mice. Lung inflammation was scored separately for cellular infiltration around blood vessels and airways, as follows: 0, no infiltrates; 1, few inflammatory cells; 2, a ring of inflammatory cells 1 cell layer deep; 3, a ring of inflammatory cells 2–4 cells deep; 4, a ring of inflammatory cells >4 cells deep. A composite score was determined by adding the inflammatory scores for both vessels and airways. Large Intestine inflammation was scored as follows: 0, no inflammatory cells; 1, small, isolated infiltrates; 2, moderately sized infiltrates; 3, extensive, multifocal infiltrates; 4, severe inflammatory cell infiltration with loss of epithelial barrier integrity. Liver inflammation was scored at portal areas, as follows: 0, no inflammatory cells; 1, mild, scattered infiltrates; 2, moderate infiltrates occupying less than 50% of the portal areas; 3, extensive infiltrates in the portal areas; 4, severe, with infiltrates completely packing the portal area and spilling over into the parenchyma. Ear inflammation was scored as followed: 0, no inflammation, no infiltration; Mild inflammation associated with few cells infiltration; 2, moderately severe inflammation associated with mild infiltration; 3, severe inflammation associated with large infiltration of cells and mild skin dryness; 4, very severe inflammation associated with skin dryness and cartilage erosion.

Suppression Assay. As published by our group, CD4\(^+\) T cells were isolated with a CD4 negative isolation kit (Miltenyi), then were labeled with CellTrace Violet Cell Proliferation dye according to the manufacturer's instructions (Life Technologies) and were used as responder cells. T\(_{reg}\) cells (CD4\(^+\)EGFP\(^+\)) were isolated on a FACSARia and were used as suppressor
cells. Responder cells were used at a fixed concentration of $1 \times 10^5$ cells per well and were stimulated for 3 d with 2 μg/ml of soluble anti-CD3 and 5 μg/ml of soluble anti-CD28 in the presence of $4 \times 10^5$ feeder Rag1$^{-/-}$ spleen cells in 96-well, round-bottomed plates in triplicate$^{101}$. 
Chapter Three: Result

LRBA deficient subjects have an increase in their Autophagic flux.

The Autophagic flux is crucial for cell sustenance during the time of stress or starvation by providing an internal source of nutrition. The first report on LRBA deficiency described that patient B cells have a decrease in their Autophagosome formation and degradation\textsuperscript{54, 55}. Since LRBA deficient subjects suffer mainly from a regulatory T cells (Tregs) pathology we decided to study the role of Autophagy in (Tregs)\textsuperscript{56}. Several groups reported that Autophagy increases upon T cell activation. However, using a murine model where Foxp3 gene is fused with Red Fluorescent Protein (RFP) we stimulated solenocytes using a different stimulatory conditions and we measured autophagy (method validation for murine cells (Supplementary Fig.1a and 1b). We didn’t observe any increase in autophagic flux (Fig.1a). Then we sought to observe autophagy at shorter time scale to see the immediate effects of T cells stimulation (Fig.1b). Despite what have been published\textsuperscript{91, 102}, a significant decrease in autophagy was observed. Other groups had the same finding in CD8\textsuperscript{+} cells, these contradicting results can be explained by the timing of measurement\textsuperscript{103}. We also, observed a general trend of higher Autophagy among Tregs compared to the T effector cells (Teff) in various stimulatory conditions and time points (Fig.1a,b). This observation reached significance with starvation and Hydroxychloroquine (HCQ) owning this to blocking the autophagic dye degradation in the lysosome by means of HCQ (Fig.1c). This finding was in agreement with data published from other groups.

Then we looked at Autophagy in human PBMCs from healthy subjects and LRBA deficient deficient ones; method validation for human cells (Supplementary Fig.1c). We found that Autophagy is similar in (Tregs) between the two groups in non-stressful situations that is un-
stimulated un-starved conditions (Fig.1d). However, we observed an increase in Autophagy in LRBA deficient subjects compared to Healthy donors when autophagy was induced (Fig1.e,f). This observation was more significant in starvation condition compared to Rapamycin. This finding in part, could be owed to the already compromised mTORc1 function in LRBA deficient subjects. Causing more significant increase in Autophagy by starvation and stress, but decreasing that significance by nullifying mTORc1 activity through Rapamycin. With these finding in mind we decided to take a closer look into the role of autophagy in (Treg) homeostasis and function.

Figure 1. LRBA deficiency increases Autophagy. a, splenocytes stimulated in RMPI complete medium for 4H with activation beads with or without IL2. b, splenocytes stimulated with 8μL activation beads and autophagy was measured at different time points. c, splenocytes incubated in EBBS medium with 10 μM Hydroxychloroquine for 4h. d, CD4+ cells from LRBA patient and aged match healthy donors incubated in complete medium for 4h or in EBSS and 10 μM Hydroxychloroquine as in. e, f, CD4+ cells from LRBA patient and aged match healthy donors incubated in complete medium for 4h with 1 Mm Rapamycin and 10 μM Hydroxychloroquine. Plots are expressed as mean ± s.e.m and representative of two independent experiments.
Compromising Autophagy in Regulatory T cells result in severe Autoimmune phenomenon.

Knocking out Vps34 in regulatory T cells resulted in severe lymphoproliferative proliferative syndrome. Mice that are homozygous for Vps34^{fl/fl}, failed to thrive, with splenomegaly and lymphadenopathy (Fig.2a). At 4 weeks of age mice had severe Treg lymphopenia compared to their wild type (wt) letter mate (Fig.2b). At three weeks of age mice present with severe and global dermatitis that is similar to graft versus host disease (GVHD) in the caliber of infiltration and severity. Also they present with pericholangitis, alveolar and interstitial, pneumonitis with vasculitis (Fig.2c).

Figure 2 Vps34 KO in Tregs result in severe Autoimmunity. a, 4 weeks old mouse with failure to thrive, splenomegaly and lymphadenopathy. b, a comparison of Foxp3^{+} cells in Foxp3^{YFPCre} and Foxp3^{YFPCre Vps34^{Δ/Δ}}. c, Hematoxylin and Eosin staining for skin(X20), liver (X10) lung (X10). d, gated on CD4^{+} cells and plotted as naive (CD44^{+}, CD62L^{+}), central memory (CD44^{+}, CD62L^{+}) and effector memory (CD44^{+}, CD62L^{-}). Plots are expressed as mean ± s.e.m and representative of two independent experiments.
The CD4⁺ compartment is shifted to the activated phenotype (CD44⁺ CD62L⁻) from the naive (CD44⁻ CD62L⁺) as compared to wt letter mates (Fig.2d). CTLA4 is increased in Vps34 deficient Tregs (Supplementary Fig.2a) but not other markers of Tregs (Supplementary Fig.2b,2c,2d) such as LAP, ICOS and CD25 respectively.

**Defective Autophagy in Tregs results in Th1 and Th2 but not Th17 Disease.**

Measurement of Ki67 in Tregs, a marker of proliferation, in Vp34 deficient Tergs show higher proliferative index as compared to wt letter mates (Fig.3a). This result in part could be a compensatory mechanism due to the massive inflammation. The phenotype of the inflammation in the CD4⁺ Foxp3⁻ (Teff) compartment is driven by INFg and IL4 but not IL17 (Fig.3b). The CD8⁺ compartment has two-fold increase in cytokines production (Fig.3c). The finding of increased INFg and IL4 also extend to the Treg compartment (Fig.3d). The decreased (Treg) percentages (Fig.2b) among the the CD4⁺ cell is in part responsible for the generalized inflammation, however it doesn't explain the Th1 and Th2 but not Th17 skewing. For that, we then decided to characterize the Treg compartment in Vps34 deficient cells. There was a significant lose in the antigen inexperienced Tregs (Helios⁺), with lose of Nuriopilin-1 (Nrp1⁺) Tregs which is a marker for Thymic drove Treg (Fig.3e). Indeed, the thymus in Foxp3⁺ is atrophic (data not shown) but this atrophy could be a consequence of generalized inflammation rather than a primary cause since the thymus is atrophic in mice with loss of function mutation in Foxp3 gene. This pathological skewing towards Th1 and Th2 but not Th17 needs further investigation.
Deletion of Stk3 and Stk4 in Tregs leads to delayed Autoimmunity.

The Hippo Kinases Stk3 and Stk4 are key regulators of autophagy machinery as they inhibit the formation of new phagosomes and increase the clearance of formed ones. We crossed Foxp3^{YFPcre} mice with Stk3^{fl/fl} and Stk4^{fl/fl}. We were expecting a phenotype that resembles Vps34^{Δ/Δ}. However, the mice didn’t show any phenotypic pathology at 4 weeks (data not shown). In searching for a covert one we euthanized and analyzed these mice (Fig.4) and (Supplementary Fig.3). In a 4 weeker Stk4^{Δ/Δ}, there was no changes in Treg compartment in term of percentages as compared to wt letter mates (Fig.4a). But looking into the effector and naïve compartment we observed increased effector cells and decreased naive ones (Fig.4b).

Also there was no significant changes in cytokines expression in CD4^{+} effector Cells, CD8^{+} Cells and Treg (Supplementary Fig.3a and data not shown).
We looked into marker of proliferation Ki67 in the CD4+ cells and we did not observe any difference between Stk4Δ/Δ and (wt) letter mates (Supplementary Fig3.c). Also, in the Treg compartment no changes was observed in Helios, Nrp1, CD25, LAP, CTLA4 and ICOS (Supplementary Fig3.b,d,e). The Stk3Δ/Δ is almost identical to the wt letter mates at 4 weeks in terms of Treg percentage and in markers of activation in the T effector compartment (Fig.4c,d). However, flowing the weights of Stk4 deficient mice showed that the covert pathology (Fig.4b) start to show around day 50 of age as the Stk4Δ/Δ failed to gain weight but not the wt nor Stk3Δ/Δ (Fig.4e).

![Figure 4 Delayed Autoimmunity in Stk3Δ/Δ and Stk4Δ/Δ.](image)

**a**, a comparison of Foxp3+ cells in Foxp3YFPCre and Foxp3YFPCre Stk4Δ/Δ at 4 weeks of age. **b**, gated on CD4+ cells and plotted as naive (CD44-, CD62L+), and memory (CD44+, CD62L-). **c**, a comparison of Foxp3+ cells in Foxp3YFPCre and Foxp3YFPCre Stk3Δ/Δ at 4 weeks of age. **d**, gated on CD4+ cells and plotted as naive (CD44-, CD62L+), central memory (CD44+, CD62L+) and effector memory (CD44+, CD62L-). **e**, weights of Foxp3YFPCre and Foxp3YFPCre Stk4Δ/Δ over time n=5 per group. Plots are expressed as mean ± s.e.m and representative of two independent experiments.
12 weeks old Stk4Δ/Δ but not Stk3Δ/Δ develop severe Autoimmunity.

After observing Stk4Δ/Δ mice for 10 weeks they started to have severe dermatitis. We euthanized and analyzed these mice (Fig.5a). These mice had normal percentages in the Treg compartment (Fig.5b) but we observed generalized activation of immune response. There was an increase in the CD4+, top panel, and CD8+, lower panel, effectors and decrease in the naïve population (Fig.5c). Opposed to Vps34 deficient mice we didn’t observe any Th1 (Infγ) or Th2 (IL4) cytokines but rather we found an increase in Th17 (Fig.5d). This find also extend to the Treg compartment (data not shown). 12 weeker Stk3Δ/Δ didn’t any observable pathology. On top of that we analyzed these mice but with no significant finding (Supplementary Fig.4).

Figure 5: 12 weeks old Stk4Δ/Δ mice have Th17 Disease. a, Hematoxylin and Eosin staining for skin(X20), liver (X10) lung (X10). b, comparison of Foxp3+ cells in Foxp3YFPCre and Foxp3YFPCre Stk4Δ/Δ. c, gated on CD4+ top panel and CD8+ lower panel and plotted as naive (CD44-, CD62L+), and memory (CD44+, CD62L-). d, CD25 expression in (wt) Foxp3YFPCre and Foxp3YFPCre Stk4Δ/Δ. e, solenocytes were stimulated for 4h with 50 ng/ml PMA, 500 ng/ml ionomycin and 10 μg/ml brefeldin, gated on CD4+ Foxp3.
Discussion:

From the result above we concluded that Autophagy is crucial process for Treg survival and function. However, the role of Autophagy in LRBA deficiency requires more investigation. Since Autophagy is well known apoptotic process our observed increase in autophagy could be in result of high apoptotic rate in CD4+ cells. But that is not the case when the cells are not challenged with starvation or rapamycin since both cells i.e LRBA deficient and sufficient cell, have an equal amount of autophagic flux in steady state. On top of that, we did not observe a statistical significant change in autophagy when mTOR was inhibited by by Rapamycin. One major point is that we didn’t measure autophagy in the Foxp3+ cell but rather we used CD25+ CD127- as a Treg marker since intra-cellular staining can disrupt the autophagic dye. In any case it is logical to think that LRBA deficient cells have an increase in their autophagy because it was reported that LRBA deficient cell have decreased mTORc1 activity. It is still unknown weather autophagy in LRBA can affect Treg suppressive capacity by itself or as a secondary effect.

It’s very imperative to establish the role of Autophagy in Regulatory T Cell in light of physiological conditions and pathological ones. Since this process is very conserved and crucial for all mammalian cell. On top of that we stablished, that Tregs has a higher rate of autophagy compared to T Effector Cells hinting toward its importance. While CTLA4-Ig has improved the outcome of LRBA deficiency patient still suffer from Autoimmunity and complication of the therapy itself. Adding an adjuvant therapy targeting the defective Autophagic process in Tregs might ameliorate the disease severity and improve the outcomes.
We know from the data above and data from other groups that autophagy is crucial for Treg survival and suppressive capacity. After Knocking out \textit{Vps34} in Treg we observed a significant loss in the Treg compartment. This lose resulted in a severe autoimmune disease manifested by mice dying at very young age (less than 8 weeks). The inflammation was of multi-organ caliber and further analysis to other site of inflammation is required such as neuritis, pancreatitis, enteritis and nephropathies. Also we we have established that major compartment of lost Tregs falls under thymic Treg (Nrp1+, Helios+). It is good to establish wither the induced Tregs requires Autophagy process in a scope of a chronic infection model.

We know that T effector cells in \textit{Vps34} deficient mouse have Th1/Th2 type of inflammation. However, the exact mechanisms of skewing are not known yet especially with sparing of Th17 type inflammation. It will be of very high value to reexamine the cytokines production in vivo using different method of measurements from deferent sites such serum and broncho- alveolar fluid. And to evaluate Tregs isolated from different sites other than the spleen and lymph nodes and examine the tissue resident Tregs as the nutritional availably and oxidative stress might vary from tissue to tissue. This can clarify, in parts, the mechanisms of skewing toward Th1 or Th2 as it might be site dependent. Also, it possible to find Th17 skewing in a different organ which make the \textit{Vps34} difecincy more in line with \textit{Foxp3} deficiency. Another more assertive way, is to screen the transcriptome of \textit{Vps34} deficient cells and examine whether defective autophagy has more impact than changing the metabolic landscape of the Regulatory T cells. The increased in T effector memory phenotype among the CD4+ Cell and the loss of Treg compartment requires more investigative effort to understands Autophagy role in Treg physiology. Another intriguing finding is the loss of tTreg (Nrp1+ Helios+). A question might be raised is whether it is due to complete loss of Foxp3+ Nrp1+ Helios+ cell to become Foxp3- or
there is shift toward won regulating Helios and Nrp-1. The former might be more rational as there was a decrease in the total Treg population. Lineage tracing with Foxp3^{YFPcre} Rosa26^{eGFP} can be utilized to address this issue. The eGFP+ RFP- Cell could be the result of this phenotypic skewing.

Other than providing an internal source of nutrition, autophagy is important in clearing up reactive oxygen species (ROS). It is very imperative to determine whether the loss of Treg is due to accumulation of ROS or just metabolic defect. And it can be addressed by inhibiting the inducible nitric oxide synthase (iNOS) in nutrient full medium in-vitro. There are many ways to measure ROS in a cell and reducing the oxidative stress might rescue the phenotype. On top of that, a general measurement of apoptotic activity in Vps34 deficient regulatory T cell. We have established a high rate mitotic activity in regulatory T cells by measuring Ki67 but we still didn’t address whether the cells are completing their mitosis or just have a higher degree of apoptosis.

The Stk3 and Stk4 are the initiation enzymes in the Hippo pathway. Up to three month of age, mice with Stk3 deficient Treg didn’t express any phenotypic or major molecular abnormality. We hypothesized that Stk4 is the major paralogue in regulatory T cells. Sorting a wt regulatory T cell and measuring the expression of Stk3 using a quantitative PCR or plotting the protein itself, are different ways to test the hypothesis. On the other hand, Stk3 phenotype could appear later in life i.e more than 3 months of age.

Mice with Stk4 deficient Tregs developed autoimmunity after 10 weeks indicating that the Hippo pathway is indispensable for Treg function. We observed no changes in Treg percentage wise but we observed an activation of CD4^{+} effector and CD8^{+} cells. On top of that
we observed a decrease in the naive cells population compared to the wt. For that we hypothesized that Stk4 deficient Treg are defective in their suppressive activity. To test this hypothesis in-vivo adoptive transfer of Stk4 deficient Treg along with wt T effector into a Rag deficient mice and observe for inflammation or in-vitro suppression assay.

The Stk4 enzyme lies on top of deferent pathway one of which is Autophagy. We don’t know whether the observed Autoimmunity is due to defective autophagy or defective Foxo3 and Yap-taz phosphorylation. To answer this conundrum crossing mice with Stk4 deficient regulatory T cells with floxed constitutively active Foxo3 and Yap-taz alleles. This might postpone the phenotype but with superimposed non-physiological metabolic defect. Measurement of autophagic flux (Appendix B) in Stk4 deficient Tregs is necessary to determine the mechanistic etiology behind the observable autoimmunity.

In conclusion, Autophagy is critical for Treg Cells survival and homoeostasis. Where defective Autophagy can impair Treg function, many useful therapeutic strategies can be implemented. Targeting Autophagy with small molecules can be useful adjuvants in cancer therapy. Not only affecting Treg cells but also the growing tumor cells. On the other hand, activating mTORc1 and inhibiting mTORc2 in Treg cells can be useful in treating autoimmune disorders. However, a lot is unknown about the effects of Autophagy on Treg metabolism, functionality and transcriptional profiling. Further characterization and elucidation for the mouse models used is needed to understand the mechanism in which autophagy affect Treg cells.
References:


Appendix A: Supplementary Figures

Supplementary Figure 1 Method Validation for Autophagy measurement. **a**, measurement of Autophagic flux in from Foxp3RPF mice gated on CD4+ cell. **b**, Same as **a** gated on RFP+ cell. **c**, method validation for human cell gated on CD4+ Cell. **d**, schematic representation of the mouse model use.

Supplementary Figure 2 Conanical markers of Regulatory T cells in Vps34Δ/Δ mice. **a**, CTLA4. **b**, LAP. **c**, ICOS. **d**,CD25. Plots are expressed as mean ± s.e.m and representative of two independent experiments.
**Supplementary Figure 3** Cellular characterization of 4 weeks old Stk4Δ/Δ mice. 

**a**, solenocytes were stimulated for 4h with 50 ng/ml PMA, 500 ng/ml ionomycin and 10 μg/ml brefeldin, gated on CD4⁺ Foxp3⁺. 

**b**, analysis of Foxp3⁺ cells for expression of Nrp1 and Helios. 

**c**, Solenocyte with intra cellular staining for Ki67 and Foxp3, plots are percentages of total among Foxp3⁺ cells.

**d**, and **e**, Conanical markers of Regulatory T cells in Stk4Δ/Δ. Plots are expressed as mean ± s.e.m

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**Supplementary Figure 4** Cellular characterization of 12 weeks old Stk3Δ/Δ mice. 

**a**, Percentages of T effector and Treg among CD4⁺ splenocyte. 

**b**, analysis of Foxp3⁺ cells for expression of Nrp1 and Helios. 

**c**, solenocytes were stimulated for 4h with 50 ng/ml PMA, 500 ng/ml ionomycin and 10 μg/ml brefeldin, gated on CD4⁺ Foxp3⁺. Plot are expressed as mean ± s.e.m
Appendix B: Autophagy Dynamic.

Using the LC3\textsuperscript{RFP, GFP} to assess the dynamic of Autophagy. After forming the labeled Autophagosome GFP\textsuperscript{+}RFP\textsuperscript{+}, GFP is sensitive to the Lysosomal proteases while RFP is resistant. Utilizing this model, we can assess the rate of formation and the rate of degradation.