Autophagy in Thymic Epithelium Shapes the T cell Repertoire and is Essential for Tolerance

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Table of contents

Summary…… 3

Zusammenfassung…… 4

1. Introduction

1.1 T cell development …….... 6
1.1.1 Overview……. 6
1.1.2 Early events in T cell development and Positive selection……. 7
1.1.3 Central tolerance…… 9

1.2 Cell Biology of Antigen Processing…… 14
1.2.1 Classical MHC class I and class II antigen loading pathways…….. 14
1.2.2 Alternative MHC class II antigen loading pathways……... 23
1.2.3 Autophagy as an endogenous antigen loading pathway…… 25

1.3 Macroautophagy-process,function and role in endogenous antigen loading……... 27
1.3.1 Molecular mechanism of autophagy…… 28
1.3.2 Role of autophagy in mammals…… 31
1.3.3 Macroautophagy and antigen presentation…… 32

1.4 Aim of the thesis……. 34

2. Results (Nedjic *et al.* Autophagy in thymic epithelium shapes the T cell repertoire and is Essential for Tolerance Nature. 2008 Sep 18; 455 (7221): 396-400)

3. Discussion…… 35

3.1 High constitutive level of autophagy in thymic epithelium…… 35

3.2 Autophagy deficient thymic epithelium - basic characterization……. 38

3.3 Autophagy and Positive selection…… 40
3.3.1 Diversity of MHCp complexes and its role in positive selection of T cells – an overview…… 40
3.3.2 Diversity of MHCII/p complexes and its role in positive selection of CD4 T cells- role of autophagy…… 43

3.4 Autophagy and MHC class II ligandome in cTEC……. 48

3.5 Medullary thymic epithelial cells and endogenous antigen loading pathways……. 49

3.6 Autophagy and tolerance induction……. 51

3.7 Proposed model and concluding remarks……... 58

4. Materials and Methods…….. 60

5. References…… 70

6. Curriculum Vitae…… 92

7. Acknowledgments…… 94

Summary

The immune system has to fulfill the remarkable task of defending the organism against a plethora of pathogens, while at the same time remaining tolerant to self. T cells are the main mediators of adaptive immune responses, as they are essential for both cellular and humoral immunity. During their development in the thymus T cells pass through tightly controlled check points, namely positive and negative selection, that ensure that only T cells expressing antigen receptors that are self Major Histocompatibility Complex (MHC) restricted and self tolerant are generated. Recognition of MHC/peptide ligands (MHCp) on cortical thymic epithelial cells (cTEC) and medullary thymic epithelial cells (mTEC) serves non-redundant functions in positive and negative selection of T cells, respectively. However, the antigen sampling and processing pathways that shuttle peptides to the MHC class II loading compartment (MIIC) in thymic epithelial cells (TEC) remain elusive. Although it is generally accepted that MHCII/peptide epitopes (MHCII/p) are generated through lysosomal processing of endocytosed polypeptides about 20% of peptides bound by MHC class II molecules of hematopoietic antigen presenting cells (APC) originate from intracellular proteins. TEC, unlike hematopoietic APC, are inefficient in capturing and/or presenting extracellular antigens, implying that TEC might primarily concentrate on their intracellular milieu for the generation of MHCII/p repertoire. Several pathways have been implicated in non-classical, endogenous MHCII loading: chaperone mediated autophagy, macroautophagy and the transporter associated with antigen processing (TAP)-dependent pathway, but respective *in vivo* significance of these processes remained unknown.

Here we addressed the role of macroautophagy (referred to as autophagy hereafter) in thymic epithelium in T cell development. Autophagy is an evolutionary conserved process responsible for the turnover of long lived cytoplasmic proteins and organelles. Autophagosomes fuse with late endosomes and lysosomes and thereby might deliver intracellular antigens to the MIIC compartment.

We were able to show that TEC, unlike most other cells in the body, display a high constitutive autophagic activity. Genetic interference with autophagy specifically in thymic epithelium led to altered positive selection of certain MHC II- but not MHC I- restricted T cell specificities. Athymic nude mice grafted with an autophagy deficient thymus displayed severe colitis and immune mediated multi-organ tissue inflammation. On the basis of these findings we propose that autophagy shuttles intracellular antigens to MIIC in TEC and in that way contributes to the efficient positive selection and tolerance induction in developing CD4 T cells.

Zusammenfassung

Aufgabe des Immunsystem ist es, den Organismus gegen eine Vielzahl von Krankheitserregern zu verteidigen und dabei die Toleranz gegenüber körpereigenen Strukturen aufrechtzuerhalten. T-Zellen sind eine der bedeutensten Komponenten der adaptiven Immunität, da sie sowohl für die humorale, als auch für die zelluläre Immunantwort essentiell sind. Während Ihrer Reifung im Thymus durchlaufen T-Zellen eine positive Selektion zur der Erkennung eigener MHC-Moleküle und eine negative Selektion, die sicherstellt, dass sich nur selbst-tolerante T-Zellen weiter entwickeln. Die Erkennung und Interaktion mit kortikalen und medullären Thymusepithelzellen (cTEC; mTEC) ermöglicht diese wichtigen Selektionsprozesse.

Auswahl und Art der Prozessierung der für die Präsentation auf Thymusepitelzellen vorgesehenen Peptide sind noch weitgehend unbekannt. Obwohl MHC II – Moleküle vorwiegend in Lysosomen mit Peptiden endozytotisch aufgenommener Polypeptide beladen werden, sind etwa 20% aller MHC II gebundenen Peptide auf hämatopoetischen APCs intrazellulären Ursprungs. Im Gegensatz dazu endozytieren und präsentieren TEC extrazelluläre Antigene äußerst ineffizient. Dies deutet auf eine Spezialisierung von TEC auf die Präsentation intrazellulärer Antigene auf MHC II Molekülen hin. Es wurden bereits mehrer Möglichkeiten für die nicht-klassische, endogene Beladung von MHC II Molekülen beschrieben, unter anderem die Chaperon-vermittelte Autophagie, Macroautophagie und der TAP-abhängige (Transporter assoziiert mit Antigen Prozessierung) Weg. Die Signifikanz dieser Prozesse in vivo ist jedoch noch unklar.

In dieser Arbeit untersuchen wir Macroautophagie (im folgenden als Autophagie bezeichnet) im Thymusepithel und ihre Bedeutung für die Entwicklung von T-Zellen näher. Autophagie ist ein evolutionär konservierter Prozess, der das Recycling langlebiger, zytoplasmatischer Proteine und Organellen ermöglicht. Autophagosomen schließen Zellbestandteile ein, verschmelzen mit späten Endosomen oder Lysosomen und liefern so intrazelluläre Antigene in das MHC II Kompartment.

Wir konnten zeigen, dass TEC, im Vergleich zu anderen Körperzellen, eine hohe, konstitutive Autophagierate aufweisen. Genetische Manipulation der Autophagie in TEC führte zu einer veränderten Positivselektion bestimmter MHC II restringierter T-Zell Spezifitäten, ohne die Selektion von MHC I restringierten T-Zellen zu beeinflussen. Athymische nude/nude Mäuse, denen ein Autophagie-defizienter Thymus transplantiert wurde, entwickelten schwere Colitis und wiesen Infiltrationen verschiedener Organe mit Lymphozyten auf. Aufgrund dieser Erkenntnisse glauben wir, dass Autophagie intrazelluläre Antigene für die Beladung von MHC II Molekülen in TEC bereit stellt und dadurch

maßgeblich zur Effizienz der Positivselektion beziehungsweise Toleranzinduktion reifender CD4 T-Zellen beiträgt.

1. Introduction

1.1 T cell Development

1.1.1 Overview

T cells are the main mediators of adaptive immune responses, thus the development of a functional and self tolerant repertoire of T cells is imperative for every healthy individual. Within the pool of mature T cells one can distinguish a number of different T cell subsets, each committed to a specialized function, that all together work in a synchronized way to efficiently eradicate different types of infections. The common characteristic of all T cells, regardless of their functional specialization, is the expression of a T cell receptor (TCR) on the cell surface. Virtually every single T cell expresses a unique TCR, hence in the mature T cell pool the repertoire of TCRs is highly diverse. This feature has evolved to enable the organism to fight the plethora of pathogens that one is likely to encounter during the life span. TCRs are not germ-line encoded, as it would take a whole genome just to transmit the genetic information for all the different TCRs found on the surface of T cells. Instead, T cell receptors are formed through a process of random somatic rearrangements of a diverse repertoire of gene segments, a process which is ultimately linked to T cell development, maturation and survival. The TCR receptor recognizes a complex consisting of a self MHC molecule (for Major Histocompatibility Complex) and a bound peptide (MHCp).

Unlike most of the hematopoietic cell types whose development takes place in the bone marrow, the vast majority of circulating T cells differentiates and maturates in the thymus, hence the abbreviation “T”. The thymus is compartmentalized into the cortical and medullary region and different cell types, i.e., cortical thymic epithelial cells (cTEC), medullary thymic epithelial cells (mTEC), dendritic cells (DC), macrophages (MΦ), B cells, fibroblasts and endothelial cells, form an unique three dimensional environment which supports distinct steps in T cell development[1,2]. T cell precursors, commonly known as common lymphoid progenitors (CLPs) enter the thymus via the blood stream at the cortico-medullary junction[3]. In the thymus, a highly complex course of proliferation, maturation and selection events takes place which results in the generation of a functional and self tolerant T cell repertoire.

However, T cell maturation is an extremely wasteful process, as more than 95% of thymocytes die during selection events on their journey through the thymus[4]. Thus, proliferation of immature thymocytes has an essential role in providing a large number of cells on which the rigorous selection processes function[5]. Such a process, demanding an enormous amount of energy and resources, is the evolutionary price that vertebrates have paid for having an immune system that is, at the population level, able to combat billions of different pathogens. Due to the random nature of TCR gene rearrangements a high number of dysfunctional or useless TCRs is formed. The process of positive selection ensures that only those T cells that express a TCR that is able to recognize self MHCp complexes with sufficient affinity, are allowed to survive. Furthermore, mechanisms of central tolerance are ensuring that positively selected thymocytes, which harbor potentially self-reactive TCRs, are either deleted or deviated into the regulatory T cell (Treg) lineage. However, central tolerance induction is not fail-safe, and indeed self-reactive T cells can be found in the periphery of any individual[6]. Importantly, Treg cells are keeping self-reactive T cells in the periphery in check. Furthermore, mechanisms of peripheral tolerance synergize with Treg mediated control of self-reactive T cells to prevent the development of autoimmunity. Thus, one can think of positive selection and central tolerance mechanisms as an evolved quality control system that ensures that only those T cells that express functional and self-tolerant TCRs are allowed to leave the thymus and seed the periphery.

1.1.2 Early events in T cell development and Positive selection

From the very moment when CLPs enter the thymus at the cortico-medullary junction the fate of the developing thymocytes is determined by soluble factors and cell-cell interactions. When T cell precursors seed the thymus their TCR gene segments are in a germ line configuration. The most immature population of thymocytes is generally defined as double negative (DN) or pro-T cells, which reflects the fact that they still do not express the co-receptor genes CD4 and CD8 which cooperatively with TCR bind to conserved regions of MHC class II and MHC class I molecules, respectively[7,8]. The DN thymocytes migrate from the cortico-medullary junction

towards the outer cortex[9] and during migration through the thymic tri-dimensional meshwork, DN cells are induced to start the process of somatic gene rearrangements[10]. Concomitantly with migration and maturation of DN population the changes in expression pattern of cell surface molecules (CD44 and CD25) take place on the basis of which the DN maturation process can be monitored. Accordingly, the DN cohort of thymocytes can be subdivided into four stages of differentiation: DN1 ($CD44^+CD25^-$), DN2 ($CD44^+CD25^+$), DN3 ($CD44^-CD25^+$) and DN4 ($CD44^-CD25^-$)[11]. It is predominantly at the DN3 stage when pro-T cells start rearranging the TCRβ locus and pass through the first developmental check point called "β selection". The TCRβ locus consists of a variable (V_β), diversity (D_β), joining (J_β) and constant (C) gene segments. It is the VDJ region of the TCRβ locus that encodes for the variable region of the TCRβ chain that forms the ligand binding domain, while the constant gene segments encode for the transmembrane regions and short cytoplasmic tails of TCR. The V, J and D segments are encoded by numerous genes that are randomly rearranged and form the variable VDJ part of TCRβ chain. The high number of gene segments encoding for the ligand binding domain of TCR is the major source of TCR receptor diversity that is found in the T cell pool. The somatic rearrangement of TCR gene segments is facilitated by the action of recombination activating gene (Rag) proteins[12-14]. Once the TCRβ chain is assembled, it pairs with the pre-TCRα chain and CD3 to form a pre-TCRα complex at the cell surface[15]. Here, the TCRβ chain is checked for its usefulness, i.e., productive rearrangement of VDJ gene segments. In the absence of signaling though pre-TCRα complex pro-T cells die by apoptosis. However, if developing thymocytes receive a signal during the process of β selection a number of changes will occur. The expression of Rag genes becomes down-regulated and subsequently chromatin remodeling at the second TCRβ locus is taking place, which results in allelic exclusion[16]. Allelic exclusion ensures that every given T cell expresses only one TCRβ chain. Furthermore, cells undergo extensive proliferation and at the late DN3 and DN4 stage in development start to rearrange their TCRα loci. However, it is in the DP stage that full-scale rearrangements on the TCRα locus take place[17]. Unlike the TCRβ locus, TCRα consists of numerous V_α and J_α gene segments, but lacks the diversity genes. The rearranged TCRα chain pairs with TCRβ to form a full TCR that is expressed on the cell surface. At this point or slightly earlier, developing T cells also start expressing their co-receptor genes, CD4 and CD8, to become double positive cells (DP) that comprise ~90% of all

thymocytes[18]. The DP cells migrate from the outer regions towards the deep cortex and interact with MHCp complexes that are displayed by thymic stromal cells. Numerous *in vitro* and *in vivo* studies have come to the conclusion that cTEC play an indispensable role in positive selection of developing T cells[19-23]. The newly assembled TCR is checked for its functionality and self-MHC restriction during the process of positive selection. In its essence, positive selection is the process whereby T cells that harbor TCR that react with adequate, low affinity with self MHCp complexes displayed by cTEC receive a survival signals and are allowed to proceed with development. In the absence of survival signals from the TCR complex immature T cells die via apoptosis, which is generally called the death by neglect pathway to highlight the absence of TCR signaling due to an inability of the receptor to recognize the ligand, i.e., a self MHCp complex[23,24]. It is estimated that around 90% of developing thymocytes undergo the death by neglect pathway[25]. However, allelic exclusion on TCRα locus is not as stringent as in the case of TCRβ locus[26]. Namely, DP T cells that express “useless” TCRs do not down-regulate RAG protein expression. Consequently, immature thymocytes can undergo several rounds of TCR gene rearrangements at the TCRα locus which increases the probability of generating a functional TCR during the life span of DP thymocytes.

Concurrent with positive selection, DP cells differentiate to become CD4 or CD8 single positive thymocytes, depending on the ability of their TCR to bind to MHCII/p or MHC class I/peptide (MHCI/p) complexes, respectively. Several theories have been put forward explaining how the “CD4 versus CD8” decision is made, but the current set of experimental data strongly supports the “signal strength” model of CD4/CD8 lineage commitment. The “signal strength” model states that the duration and/or intensity of signaling through the TCR complex dictate the CD4 versus CD8 lineage choice, i.e., has an instructive role. Thus, stronger signaling results in CD4 lineage commitment, while weaker interactions lead to differentiation into the CD8 lineage[9].

1.1.3 Central tolerance

A question that has captured immunologists for decades was how the immune system is able to combat a plethora of pathogens while at the same time it remains non-reactive to self. It was in 1959, when Burnet put forward a visionary concept

addressing this issue, the so called “clonal selection hypothesis”. The hypothesis states that lymphocytes specific for self antigens are eliminated from the repertoire to prevent autoimmune hazard[27]. A long history of research has proven Burnet´s hypothesis to be correct. The “elimination” of autoreactive lymphocytes, as proposed by Burnet, is implicated in the process of clonal deletion or negative selection which is a recessive mechanism of tolerance induction whereby developing T cells expressing a TCR that reacts with high affinity with self-MHC complexes will die by induced apoptosis[28-30].

However, in the late eighties seminal work from the Le Dourain lab has convincingly implicated the existence of dominant tolerance mechanisms operating in the thymus. An elegant set of experiments has shown that simultaneous transplantation of embryonic thymi and limb buds from quail into chicken embryos induced the acceptance of the transplanted limb, while the transplantation of a limb bud alone led to rejection of the graft. In this set up, embryonic thymi were grafted before colonization by hematopoietic precursors which highlighted the importance of the thymic epithelium in central tolerance induction. In such chimeras, T cells can be educated on either the endogenous or the transplanted quail thymus, but a recessive mechanism of tolerance operating in the quail thymus could not explain the observed phenomenon of limb acceptance. The findings argued that a transplanted quail thymus has the capacity to induce development of a special type of T cells with the ability to keep “xeno-reactive” T cells of recipient origin in a quite state[31,32]. The authors went on to show that such cohort of T cells which is able to act “*in trans*” and suppress the activity of graft-specific cells indeed exists in the periphery of chimeric animals by performing T cell transfer experiments[33,34]. In the years that followed the initial discovery of T cells with a regulatory function (hereafter referred to as Treg), Sakaguchi and colleagues have been able to show that cells mediating dominant tolerance are essential for prevention of autoimmunity, that the thymus is indispensable for the development of Treg and finally identified CD4SP cells which constitutively express the IL-2 receptor α chain (CD25) as a cell type capable of mediating suppression[35-37]. More recently, the transcription factor FoxP3 has been proposed to be the master regulator of Treg development[38-40]. Indeed, mice and humans carrying a mutation in the gene encoding FoxP3 suffer from severe autoimmunity due to a lack of Treg[41,42]. Taken together, the described studies on

Treg have convincingly shown that dominant tolerance induced in the thymus is an indispensable component of a healthy immune system.

Until a few years ago, it was generally accepted that recessive and dominant mechanisms co-operatively work in the thymus to induce the state of tolerance to ubiquitous and thymus-specific antigens, while different mechanisms of peripheral tolerance (i.e., clonal deletion and induction of anergy) keep the mature T cells reactive to tissue-specific antigens (TSA) in a silent state. Such distribution of labor between central and peripheral mechanisms of tolerance has proven not to be correct. The thymus has the capacity to express a wide range of TSA and in that way it synergizes with the peripheral tolerance machinery to ensure that the mature T cell pool of every individual is tolerant to self. This extraordinary feature of the thymus has initially been recognized as an artifact of transgenics, as it has been documented that tissue specific transgenic constructs are ectopically expressed in the thymus[43-45]. Later, Hanahan and coworkers reinterpreted these findings as an inherent characteristic of the thymus[46]. Finally, in a landmark study mTEC have been identified as the unique cell type capable of expressing a wide range of TSA in a promiscuous fashion, a feature that was not evident in cTEC or thymic DC[47,48]. Additionally, Kyewski lab went on to show that the expression of a particular TSA is not found in every single mTEC, but it is rather restricted to 1-3% of total mTEC population, suggesting that it is all mTEC together that collectively build up a mosaic of the peripheral self in the thymus ("immunological homunculus")[47,49]. MTEC appear to be a highly heterogenous group of cells, not only with the respect to the TSA profile that a particular mTEC expresses, but also based on surface expression levels of molecules involved in antigen presenting, i.e., MHC class II and CD80[50,51]. Thus, one can distinguish two subpopulations of mTEC: $mTEC^{lo}$ and $mTEC^{hi}$ expressing low to intermediate and high levels of MHC class II and co-stimulatory molecules, respectively. Curiously, the widest range of TSA expression is found within the subpopulation of cells that has antigen presentation characteristics of professional APC, i.e., $mTEC^{hi}$ [4,47,48]. It has been proposed, and recent evidence points to that direction, that $mTEC^{hi}$ are the most mature, terminally differentiated subset of mTEC with a high turn-over rate[52,53]. Although the phenomenon of promiscuous gene expression has fascinated many scientists, the regulation of this process still remains a puzzle. So far only one regulator that is essential for the

expression of some, but not all TSA has been identified, the Autoimmune regulator (Aire)[54]. The significance of Aire-driven promiscuous gene expression in mTEC^{hi} in central tolerance is highlighted by the fact that both mice and humans deficient for AIRE suffer from organ-specific autoimmunity[54, 55,56].

While it is perfectly clear that both negative selection and induction of Treg are vital for the establishment of tolerance to self, the respective contribution of different cell types to tolerance induction is still a matter of intensive research. Studies on mice with impaired development and/or organization of the medulla revealed its crucial role in tolerance induction. Thus, in Rel-B, lymphotoxin-β receptor and TRAF6 deficient mice the medullary compartment is altered, which results in the development of autoimmunity[57-58,59]. Furthermore, inhibition of thymocyte homing to the medulla in CCR7 and CCR7L deficient mice results in the break-down of central tolerance[60]. Taken together all these studies argue that the medulla plays a vital role in tolerizing the developing T cell repertoire; however the respective roles and overall input of mTEC and DC in clonal deletion and Treg induction is still unclear. Initial experiments have documented that 50% of positively selected thymocytes are eliminated from the developing repertoire by hematopoietic cells (most likely DC)[61]. However, mTEC have also tolerogenic potential, as T cells educated in the thymus where MHC class II expression is abrogated in hematopoietic system do not cause graft-versus-host disease when transferred into the lymphopenic host, while targeted deletion of MHC class II locus in both mTEC and DC leads to the development of a self-reactive T cell repertoire[62,63]. Furthermore, TSA antigen expression in mTEC is absolutely necessary for the establishment of tolerance towards peripheral antigens[54]. There are two mutually non-exclusive schools of thoughts explaining how TSA are actually presented to developing thymocytes. There is evidence arguing for cross-presentation of mTEC expressed antigen by DC, a scenario that implies that mTEC basically function as a reservoir of antigens that DC pick up for presentation to developing T cells. Indeed, a study with a TCR transgenic system revealed that DC mediated cross-presentation of mTEC derived antigen is necessary for deletion of auto-reactive CD4SP T cells. On the other hand, in the very same model deletion of CD8SP cells has been shown to be mediated by mTEC and DC[64]. The second theory states that mTEC express and autonomously present the antigens to developing T cells, and in that way directly contribute to the induction of tolerance. The deletion

potential of mTEC has been described in a system where the expression of transgenic human C-reactive protein (hCRP) was limited to 1-3% of mTEC. Using TCR transgenic animals specific for hCRP (Dep TCR transgenic) the authors documented that mTEC are able to autonomously delete hCRP specific thymocytes when the antigen is endogenously expressed[49]. Moreover, our lab has recently revealed that Aire-driven expression of cognate antigen in mTEC results in deviation of antigen specific T cells into Treg lineage and importantly mTEC mediated presentation of the antigen alone is sufficient for the induction of Treg phenotype[65]. Hence, mTEC have the capacity to process and present antigens to CD4 T cells which results in tolerance induction. It is still unclear if mTEC are dedicated “Treg inducers” or DC can also induce the deviation of self-reactive T cells into the regulatory lineage. There are studies stating that in humans DC can induce a Treg phenotype upon exposure to thymic stromal lymphopoietin (TSLP)[66]. It seems highly plausible that mTEC and DC can mediate negative selection and induce the deviation into the Treg lineage of self-reactive thymocytes.

Studies discussed above imply that the medulla plays an indispensable role in central tolerance, but the exact contribution of the cortex in tolerance induction is still a matter of debate. First reports on negative selection in TCR transgenic systems, where the fate of T cells specific for either endogenous antigen or an antigen expressed as a transgene has been followed, revealed that the self-reactive T cells are eliminated from the repertoire at the early stages of development, i.e., at the DN or DP stages[29,67-69]. Apart from deletion, other mechanisms of tolerance induction have been ascribed to cTEC, such as receptor editing. Namely, one study has suggested that upon the encounter with cognate antigen which is expressed in cTEC, self-reactive T cells down-regulate the expression of the TCRα chain which is followed by increased frequency of recombination on endogenous TCRα loci. Pairing of the newly rearranged TCRα chain with the transgenic TCRβ chain results in the generation of a novel TCR specificity[70]. However, as it will be discussed later on, T cells that express two TCRs on the cell surface may increase the chance of development of autoimmune diseases, thus the significance of receptor editing in establishing tolerance to self is questionable. Furthermore, two studies have implicated cTEC as mediators of dominant tolerance induction[71,72]. However, in studies of Bensinger et al. and Liston et al. cTEC specific MHC class II expression on

MHC class II null background was driven by K14 promoter. In this set up, the expression of MHC class II on mTEC has not be formally excluded, which is an important issue considering the fact that K14 is a marker keratin for mTEC on one hand, and inherent capacity of mTEC to promiscuously express TSA on the other. Furthermore, in our hands K14 driven expression of a transgene led to a detectable expression of protein in mTEC (unpublished observation).

Taken together, numerous reports indicated that cortex contributes to the establishment of central tolerance. However, the cortex does not have a capacity to induce complete non-reactivity to self, as T cells educated in the thymus where mTEC and DC cannot present antigens are self-reactive[63,73]. At this point, it is reasonable to assume that tolerance to ubiquitous antigens that are expressed at high levels in the cortex can be mediated by cTEC, hence a minor population of self-reactive T cells could get eliminated from the repertoire at the early stages of development.

Every single step in the development and life of T cells critically depends on the interaction of the TCR with MHCp complexes displayed by APC. Thus cell biology of antigen processing and respective MHC class I and MHC class II loading pathways have occupied attention of immunologists for decades. At present, the cellular machinery involved in antigen processing is fairly well understood, however new concepts of antigen processing are emerging which are bringing the field of antigen presentation back to the focus of immunological research. In the following paragraphs the old and new concepts of antigen processing and presentation with a particular accent on evolutionary specializations of TEC will be discussed.

1.2 Cell Biology of Antigen Processing

1.2.1 Classical MHC class I and class II antigen loading pathways

One of the landmark discoveries in immunology the significance of which cannot be highlighted enough was the elucidation of the crystal structure of the MHCI/p complex[74]. Subsequently, the crystal structure of MHCII/p complex has been resolved as well[75]. Thus, the work from the Wiley lab have paved the way to better

understanding of molecular mechanisms involved in antigen presentation and the basis of TCR/MHCp complex interactions. These studies revealed that MHC class I and MHC class II molecules share several structural features, i.e., both molecules are transmembrane proteins, consisting of an extracellular peptide binding cleft followed by a pair of immunoglobulin-like domains and a transmembrane region. MHC molecules are highly polymorphic, and the polymorphism is found in and around the region that forms the peptide binding groove. It is the polymorphism in the peptide binding cleft that allows one MHC molecule to bind to the diverse set of peptides, but on the other hand only the peptides that have features allowing complementary interactions with MHC peptide binding pockets can be found on a particular allelic version of a given MHC molecule.

The MHC class I molecule is expressed on all nucleated cells in the body. A highly polymorphic MHC class I α chain (heavy chain) non-covalently interacts with the soluble protein β2-microglobulin (light chain, β2m) to form an “empty” MHC class I molecule. Unlike the α-chain, β2m is non-polymorphic and does not contribute to the formation of the peptide binding groove. The peptide binding cleft of the MHC class I molecule is in a “closed” conformation and allows for the binding of peptides that are 8-11 amino acids long[74,76]. Furthermore, peptides with C-terminal hydrophobic residues are preferentially found in a complex with MHC class I due to the specificity of the anchor pockets in the peptide binding groove. Both chains are synthesized in the endoplasmic reticulum (ER) where they are folded into a proper conformation with the help of ER-resident chaperones, such as calreticulin and calnexin[77,78,79]. It is also in the ER where peptide binding takes place, and only MHC class I molecules with bound peptide are structurally stable. Thus, one should envision MHC class I molecule as a hetero-trimeric complex, consisting of an α-chain, β2m and the peptide.

The peptides bound to MHC class I molecules are generated by the proteolytical activity of intracellular proteases, with the proteasome playing the essential role in epitope generation. MHC class I molecules are loaded with epitopes that originate from cytosolic and nuclear proteins. The proteasome is a house-keeping protease expressed in all somatic cells responsible for the degradation of ubiquitinylated proteins, accumulation of which results in cytotoxicity. It is a multimeric complex composed of a 20S barrel-like structure which is the catalytically active subunit. It

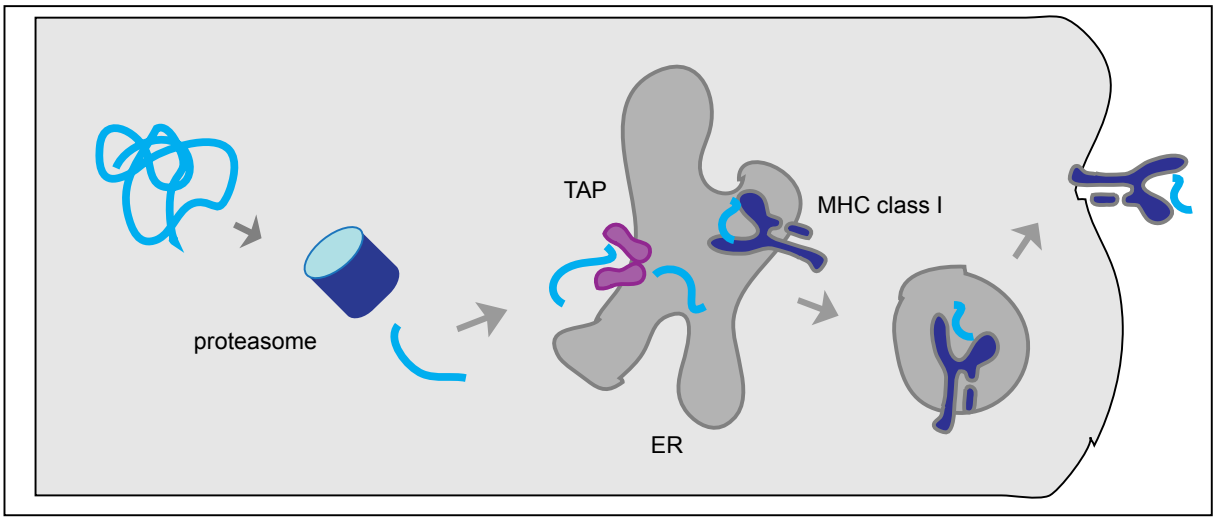
associates with the “cap”-like regulatory subunit 19S, which is responsible for the recognition and unfolding of ubiquitinylated complexes. The catalytic subunit consists of four heptameric rings, $\alpha_{(1-7)}\beta_{(1-7)}\beta_{(1-7)}\alpha_{(1-7)}$ and the hydrolytic activity is conferred to the β1, β2 and β5 subunit. The respective hydrolytic activities of the β subunits can be described as a caspase-like (cleaving after acidic residues), trypsin-like (cleaving after basic residues) and chymotrypsin-like (cleaving after hydrophobic residues). Quite elegantly, the immune system has adapted the basic cellular catabolic machinery for the generation of MHC class I peptide epitopes. Furthermore, in APC a specific isoform of the proteasome is expressed, the so called “immunoproteasome” to distinguish it from a house-keeping form, where the constitutive β subunits are replaced with “immunosubunits” LMP2 (β1i), LMP7 (β5i) and MECL1 (β2i). The hydrolytic subunits of the “immunoproteasome” have a specialized feature that allows for the generation of peptides with characteristics that are best suited for the binding to the MHC class I peptide cleft. The proteolytic activity of LMP2, LMP7 and MEL1 is chymotrypsin- and trypsin-like which leads to the generation of peptides with basic and hydrophobic amino acids at the C-terminus[80]. In the primary and secondary lymphoid organs, i.e., thymus and spleen, “immunosubunits” are constitutively expressed[81]. The analyses of LMP2 and LMP7 knock out mice have revealed that CD8 T cell repertoire is impaired, confirming the indispensable role of the “immunosubunit” mediated epitope generation for the development of a fully functional immune system[82]. Additionally, IFNγ can induce the expression of “immunosubunits” at the site of inflammation[83].

It has recently been shown that cTEC express a specific proteasomal catalytic subunit: thymus specific β5 family member (β5t)[84]. The so called “thymoproteasome” has a reduced chymotrypsin-like activity, which raises the possibility that MHC class I peptide epitopes generated in cTEC are different from those generated by constitutive proteasomes and “immunoproteasomes”. On the other hand it has been shown that the thymic stromal cells implicated in negative selection, mTEC and DC, rely on “immunoproteasomal” activity for the generation of MHC class I epitopes[81]. The expression profile of catalytic proteasomal subunits implies that the repertoire of MHCI/p complexes displayed to developing CD8 T cells in the cortex and medulla might be qualitatively and/or quantitatively different. Importantly, β5t deficient mice display significantly reduced numbers of CD8SP, while CD4SP T cell development

appears not to be impaired. The authors suggested that the block in CD8SP T cell development results from impaired positive selection due to an alteration in the MHCI/p repertoire in β5t$^{-/-}$ cTEC. However, the hypothesis awaits its formal verification.

MHC class I molecules are loaded with peptides originating from intracellular and nuclear proteins. Proteins are marked for proteasomal degradation by ubiquitination, a signal that is specifically recognized by the regulatory subunit of the proteasome. Moreover, accumulating evidence in the literature suggests that MHC class I peptide epitopes actually originate from defective ribosomal products (DRiPs). DRiPs are ubiquitinylated polypeptides that are generated as a result of an inaccurate and/or premature termination of mRNA translation[85-88]. The recent decoding of the human and mouse genome will allow for the precise assessment of the origin of MHC class I bound peptides, i.e., if they originate from “out of frame” translational products or fully assembled functional proteins in the cell.

Whatever the source of the MHC class I epitopes is, proteins and/or DRiPs, after proteasomal degradation the peptides are shuttled to the ER via the transporter associated with antigen processing (TAP). TAP is a transmembrane heterodimer consisting of TAP.1 and TAP.2 subunits and it is located in the ER membrane. The capacity of TAP to translocate peptides across the ER membrane has initially been indicated in mice with a targeted deletion of the TAP locus[89] and subsequently the mechanism of translocation has been elucidated in *in vitro* experimental set ups[90,91,92]. Apart from its role in peptide translocation, TAP is associated with the ER resident chaperone calreticulin which in turn binds to and stabilizes the “empty” MHC class I molecule. The association of TAP and calreticulin is mediated by the chaperone tapasin[79]. The topology of this supra-molecular complex allows for the efficient binding of imported peptides to the groove of MHC class I molecules. Further trimming of epitopes takes place in the ER via the action of ER-resident aminopeptidases[93]. This process ensures the generation of peptides that are 8-11 amino acids in length and thus display the structural features favorable for binding to the MHC class I cleft. Upon binding of peptides, stable MHCI/p complexes are transported from the ER via the Golgi apparatus to the cell surface for display to CD8 T cell (Graphic 1).



Graphic 1. MHC class I loading pathway. Intracellular proteins are targeted for proteasomal degradation by ubiquitination. Subsequently, peptides are transported from the cytosol to the endoplasmic reticulum (ER) via transporter associated with antigen processing (TAP). The stable peptide bound MHC class I complexes are transported to the cell surface by exocytic vesicles.

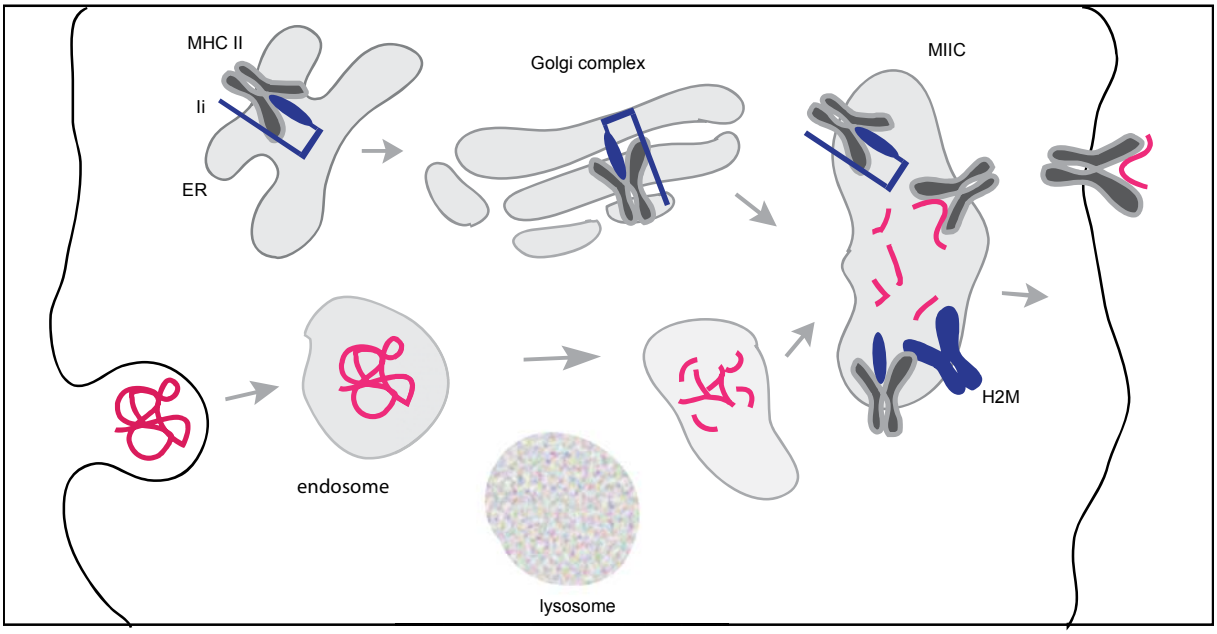
The above described model of MHC class I antigen loading was called into question by the finding that DC are able to present extracellular antigens derived from surrounding cells on MHC class I molecules and prime naive CD8 T cells during adaptive immune responses[94,95,96]. Thus, it became clear that the topological rules for MHC class I antigen loading are not as strict as it has previously been appreciated. The underlying mechanism explaining a phenomenon of so called "cross-presentation" is still a matter of intensive debate in the field. Several groups have described a model whereby internalized extracellular proteins can actively be transported from the endosomal compartment to the cytosol via (i) the action of a specific transporter protein or (ii) a poorly defined "leakage" from endosomes[97]. Once in the cytosol, the polypeptides can gain access to the MHC class I loading route after proteasomal processing and TAP-mediated translocation to the ER. However, the studies relying on the specific inhibition of proteasomal activity and/or TAP function in the phenomenon of "cross-presentation" should be carefully interpreted, as MHC class I expression levels in such experimental set-ups are reduced due to inefficient peptide loading. Recently, Neefjes and colleagues have put forward the "antigen spreading model hypothesis" that recognizes the role of gap junctions in intercellular transfer of peptides[98]. They presented conclusive evidence that antigen

synthesized in one cell can be transferred through gap junctions to the neighboring cell and presented on MHC class I via the classical MHC class I loading pathway. This model postulates that virus-infected or tumor cells may form intercellular connection channels with DC and in that way pass the information of “what is going on” in the cell. Such a sequence of events would ultimately allow for “cross-priming” of naive CD8 T cells. Nevertheless, the *in vivo* significance of gap junctions remains to be elucidated. It is, however, worth mentioning here that some groups of viruses have evolved specialized strategies that allow them to block the formation of gap junctions in infected cells[99,100]. It seems plausible that by doing so viruses can block “cross presentation” as yet another strategy of immune evasion.

Unlike MHC class I, MHC class II molecules are constitutively expressed only on professional APC, i.e., DC, B cells, macrophages and importantly on cTEC and mTEC. Thus, TEC are the only non-hematopoietic cell types that constitutively express MHC class II. Furthermore, MHC class II expression can be induced on fibroblasts, endothelial and epithelial cells at the site of inflammation in response to inflammatory cytokines, such as IFNγ.

The MHC class II molecule is a heterodimer consisting of two polymorphic polypeptide chains, MHCII-α and MHCII-β. These molecules are synthesized in the ER and assembled by the help of ER-resident chaperone proteins, such as calnexin[101]. The assembly of MHC II-α with the β chain results in the formation of the peptide binding groove which unlike in the case of MHC class I, is in an “open-end” conformation and can accommodate peptides that are 10-30 amino acids long[75, 102-104]. The premature binding of peptides to the MHC class II molecules in the ER is prevented by co-synthesis of the third component of an “immature MHC class II complex”, the invariant chain (Ii). It is the CLIP region (for class II associated invariant chain peptide) of Ii that firmly associates with the MHC class II binding groove[105,106]. Moreover, Ii contains an endosomal localization sequence which enables targeting of newly synthesized MHCII/Ii heterotrimers to the endosomal/lysosomal compartment[107-110]. In the late endosomal compartment newly synthesized MHC class II molecules meet with the machinery involved in antigen processing and MHC class II loading, more commonly known as MIIC (for MHC class II containing compartment)[111]. During trafficking to the late endosomal/lysosomal compartment, degradation of Ii chain takes place in a stepwise

fashion, and the MHC class II groove remains occupied by CLIP. Subsequently, the molecular chaperone H-2M catalyzes the dissociation of CLIP, stabilization of an "empty" MHC class II molecule and the loading of peptides to the peptide-binding groove[112-114] (Graphic 2). The H-2M molecule shows a notable structural homology to the MHC molecule itself, but unlike MHC II, H-2M is non-polymorphic. It is difficult to give an exact definition of the MIIC, however electron-microscopy studies have identified MIIC as a MHC-rich compartment with multilamellar appearance[115] and a consensus has emerged in the field that MIIC is the late endosomal compartment supplied with the machinery for efficient antigen loading[116,117].



Graphic 2. Classical MHC class II loading pathway. Extracellular antigens are captured and internalized into endosomes by APC; subsequently endosomes fuse with lysosomes which deliver proteases involved in antigen processing. On the other hand, MHC class II molecules are synthesized in the ER where they associate with invariant chain (Ii). Upon synthesis, MHC class II/Ii complexes are transported to the MHC class II loading compartment (MIIC) where they fuse with late endocytic vesicles containing processed antigens. Ii gets degraded and the remaining CLIP fragment occupies the peptide binding cleft of MHC II. The molecular chaperone H-2M catalyzes the exchange of CLIP with peptides derived from the late endosomal/lysosomal compartment.

Cathepsins, lysosomal proteases, have been shown to play a non-redundant role in Ii processing. Remarkably, cTEC and bone marrow derived APC (DC, B cells and macrophages) show different enzymatic signatures with respect to cathepsin expression. Degradation of the Ii chain in cTEC is critically dependent on Cathepsin L (CatL) activity. Thus, CatL deficient mice show accumulation of Ii chain degradation

intermediates, decreased repertoire of MHCII/p complexes as judged by an increase in MHCII/Ii derived peptide complexes on the cell surface and reduced numbers of CD4 cells in the thymus and in the periphery[118]. These findings implied that CatL plays an indispensable role in the MHC class II maturation in cTEC and in positive selection of CD4 T cells. On the other hand, Ii degradation in DC is mediated by Cathepsin S[119-121]. The involvement of different proteases responsible for MHC class II maturation in the cell types mediating positive and negative selection may imply that the peptide repertoires displayed to developing CD4 T cells in the cortex and medulla differ at least at the quantitative level, which stands in line with the discussed findings about the CD8 repertoire selection. This hypothesis is further supported by findings that a specific I-A^b/I-E$\alpha_{(52-68)}$ epitope is expressed at a lower level in cTEC then in DC[122-124]. The observations that indicate involvement of a similar set of proteases involved in antigen processing in the cell types mediating tolerance induction, i.e., mTEC and DC, and peripheral APC are intuitively understandable. Namely, in that way T cells can get checked for self-reactivity during their journey in the medulla to the very same peptide epitopes that they are exposed to in the periphery, and thus the autoimmune hazardous potential of mature T cell pool is minimized. On the other hand, the biological significance of a potentially different ligandome on cells mediating positive selection remains to be established, though it may add to the hypothesis that signaling through the TCR differs at both the quantitative and the qualitative level during the process of positive and negative selection. However, sequence analyses of peptides eluted from cTEC and peripheral APC did not reveal any major differences in the composition of respective MHCII/p repertoires[125]. Still, one has to keep in mind that the sensitivity of the techniques used for MHC class II ligandome analyses might not allow for detection of low abundance peptides that add to the diversity of a MHCII/p repertoire.

The trafficking pattern of MHC class II molecules determines the source of peptides that is presented to CD4 T cells. As outlined above, “mature” MHC class II molecules meet up on their route to the cell surface with late endosomes, and it is the late endosomal compartment of APC where MHC class II peptide loading occurs (Graphic 2). Professional APC, such as DC, are equipped with machinery that allows for efficient sampling of exogenous and cell membrane associated antigens by the process of pinocytosis, receptor mediated endocytosis and/or phagocytosis[126, 127].

The early endosomes subsequently fuse with the lysosomal compartment which is rich in hydrolytic enzymes and these fusion events ultimately result in the degradation of the ingested extracellular cargo. Thus, MHC class II molecules are primarily loaded with peptides originating from the extracellular and cell-membrane associated proteins[127].

At this point, much remains to be learned about the specific proteases involved in protein processing and peptide generation in the MHC class II loading pathway. The lysosomal proteases are the main candidates, as pharmacological inhibition of lysosomal activity results in diminished antigen presentation[126]. Indeed, in bone marrow derived APC asparagine endopeptidase has been shown to play a crucial role in degradation of microbial antigens[128]. However, the identity of enzymes involved in generation of MHC class II epitopes in thymic stromal cells is still awaiting its discovery. Follow up studies with CatL deficient mice have implicated CatL in degradation of proteins in the MHCII pathway, as CatL, Ii double knock out animals have a more severe block in positive selection of CD4 T cells then either of the single knock out animals[129,130]. The authors speculated that the underlying reason for the observed phenotype is a reduced diversity of MHCII/p complexes displayed by $CatL^{-/-}$, $Ii^{-/-}$ cTEC, though the direct proof for this hypothesis is still missing.

After binding of peptides to MHC class II molecules, the MHCII/p complexes are transported to the cell membrane. However, the precise mechanism of the MHCII/p complex delivery from MIIC to the cell surface is still enigmatic. Recent evidence suggests that the late endosomal/lysosomal compartment forms tubule-like structures and subsequently vesicles enriched with MHCII/p complexes detach from the tubular protrusions and fuse with the cell membrane[131-132] .

Intriguingly, while DC are highly proficient in sampling and presenting the extracellular milieu to developing T cells, the capacity of cTEC and mTEC to capture and/or process extracellular antigens has proven to be very low[49, 133, 134]. Thus, *ex vivo* isolated DC from mice intravenously injected with myoglobin efficiently presented the antigen to a specific T cell hybridoma, but only weak stimulation has been observed when cTEC and mTEC were used as APC. Studies in *in vitro* systems using cTEC lines have also come to the conclusion that cTEC have a rather poor ability to present exogenous antigens[135-137]. These set of data prompted the

authors to speculate that cTEC and mTEC focus their attention on the intracellular compartment for the generation of ligands for MHC class II molecules. The so called "division of labor" hypothesis has been put forward which postulates that mTEC and DC present different set of peptides to developing T cells during their educational journey through the thymus. Being highly efficient in capturing extracellular antigens, DC would preferentially use the classical, endocytic route of MHC class II antigen loading and consequently the MHCII/p repertoire of DC would mirror blood-borne proteins in the thymus. On the other hand, mTEC would present the universe of intracellular self, which importantly comprises a wide array of tissue specific antigens[138]. The nature of endogenous loading pathway/s operating in cTEC and mTEC remained unknown at that point. Studies in *in vitro* systems have implicated several possible mechanisms for the shuttling of intracellular antigens to MIIC in different APC. The nature of these endogenous MHC class II loading pathways will be subject of the following section.

1.2.2 Alternative MHC class II antigen loading pathways

Seminal work by Charles Janeway and his colleagues[103, 139] provided for the first time precise characterization of physiologically produced peptides associated with MHC class II molecules in B-cell lymphoma lines. These so called ligandome analyses relied on immunoaffinity purification of MHC class II molecules followed by acid elution and sequencing of bound peptides. Subsequent refinement of technology allowed for a high through-put MHCII/p ligandome analyses which provided first experimental evidence that challenged the classical immunological dogma of MHC class II antigen loading[102,140-142]. Specifically, these studies estimated that around 85% of peptides analyzed were derived from endogenous cellular proteins synthesized by APC itself. The vast majority of these endogenous peptides were derived from transmembrane or secreted proteins as well as proteins involved in MHC class II antigen processing. This group of proteins traffics through the endocytic compartment and display of their respective epitopes on MHC class II molecules can be easily explained by classical antigen loading pathway. However, the source of around 20% of the identified epitopes are cytosolic proteins that

normally do not intersect the endocytic machinery, i.e., mitochondrial proteins (e.g. cytochrome b5 reductase), cytoskeletal proteins (γ- Actin, F-actin capping protein), metabolical enzymes (glyceraldehyde-3-phosphate dehydrogenase, GAPDH), chaperones (Hsc70), nuclear proteins (histones) to name a few[102, 140]. The presentation of epitopes derived from intracellular and organellar proteins by MHC class II is more difficult to explain by classical, endocytic antigen loading route.

In parallel with the biochemical ligandome analyses several *in vitro* antigen presentation studies using human and murine APC in the early nineties have shown that the MHC classes II loading of ectopically expressed intracellular proteins does not require release and re-internalization of a model antigen, and consequently does not follow the endocytic route of antigen entry to the MIIC. These surprising findings revealed that MHC class II molecules sample both extracellular and intracellular pools of proteins for display to CD4 T cells. MHC class II– restricted endogenous antigen presentation has been so far documented for a wide array of different self antigens, model antigens, tumor and viral antigens, indicating that intracellular antigen loading is a phenomenon of a broad immunological significance[143-144]. Accordingly, the cell biology of intracellular antigen processing and endogenous MHC class II loading has become a field of intensive research during the last two decades. The experimental evidence coming from different laboratories revealed high complexity of cellular mechanisms involved in sampling of intracellular self for the presentation on MHC class II molecules. On the basis of these findings endogenous loading pathways can be grouped into two main categories. The first category can be defined as a TAP-dependent pathway which requires intracellular antigen processing by cytoplasmic proteases and subsequent import of protein degradation products to either ER or recycling MHC class II rich endosomes via the TAP molecule[145,146] . The second category involves a rather heterogeneous group of intracellular processes collectively known as autophagy.

1.2.3 Autophagy as an endogenous antigen loading pathway

The word autophagy originates from the ancient Greek and literally translates as self eating (*auto*=self, *phage*= to eat). There are three main autophagic pathways in higher eukaryotes that can deliver cytoplasmic constituents for lysosomal degradation: microautophagy, chaperone mediated autophagy and macroautophagy (Graphic 3).

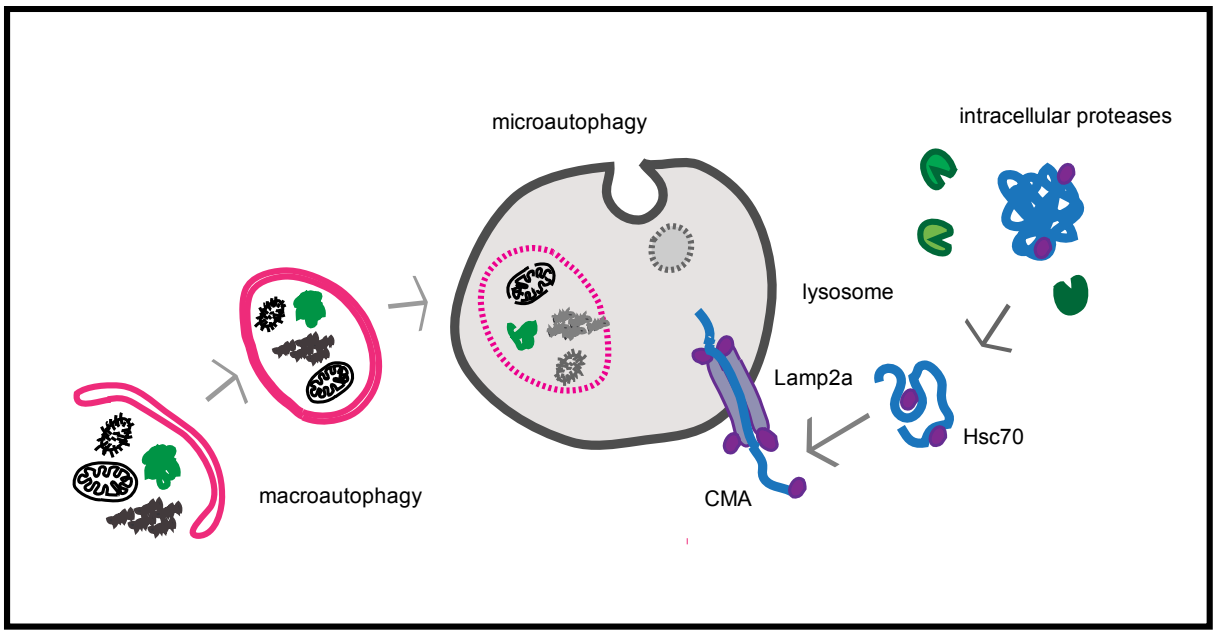
Microautophagy is a constitutively active process during which small portions of the cytosol are directly imported to the lysosomal lumen via membrane invaginations. The process has been studied mainly in yeast and cell free systems, and little is known about the molecular mechanism of microautophagy in mammals[147,148]. It is generally accepted that microautophagy is a constitutively active, nonselective catabolic pathway in the cell. To date, there is no experimental evidence implicating microautophagy in endogenous antigen loading, as the tools for targeted genetic disruption or pharmacological inhibition of the process are not available.

Chaperone mediated autophagy (CMA) is a highly selective autophagic pathway that mediates the transport of proteins and peptides to the endosomal/lysosomal compartment[149]. A chaperone, heat shock cognate protein of 70 kDa (Hsc70), is one of the essential players in CMA that specifically recognizes proteins and peptides with a particular KFERQ-like pentapetide motif. Generally, exposure of this particular motif in proteins requires either proteolytical processing by cytoplasmatic proteases and/or conformational changes of the substrate. Once Hsc70 tags the target molecule, it facilitates its delivery to the late endosome/lysosome where a specific isoform of lysosome associated membrane protein 2 (LAMP2a) translocates the substrate[150,151]. The LAMP2a protein has been proposed as a rate limiting factor in CMA[149], accordingly modulation of its expression level allows for experimental manipulation of CMA activity in the cell.

The lab of Janice Bloom has recently elegantly shown an involvement of CMA in endogenous MHC class II loading. Over expression of Hsc70 or Lamp2a in B cell lines led to superior presentation of several epitopes derived from intracellular proteins to CD4 T cells, while down-regulation of CMA resulted in diminished antigen presentation[152]. An indirect evidence for participation of CMA in endogenous antigen loading comes from the analyses of MHC class II ligandome. GAPDH is a proposed

substrate for CMA[153] and its specific epitopes have been identified as naturally occurring MHC class II ligands[141]. In this scenario, efficient presentation of intracellular antigens on MHC class II requires proteolytical processing of intracellular antigens by cytosolic proteases such as proteasome and/or calpain. The proteolysis of target proteins results in the exposure of a CMA specific pentapeptide targeting motifs that marks the peptide for delivery to late endosomes/lysosomes where the loading to MHC class II molecules takes place[152,154].

Even though the CMA pathway is a highly selective process, bioinformatical and biochemical analyses have shown that around 30% of intracellular proteins contain KFERQ-like sequence[155]. Thus the number of proteins that can be targeted for degradation via CMA is not negligible and consequently it can significantly contribute to the diversity of MHCII/p complexes in APC. Importantly, constitutive Lamp2a expression has been confirmed in a large panel of APC[152]. However, the respective *in vivo* significance of the CMA pathway for endogenous MHC class II loading in CD4 T cell development and adaptive immunity remains to be discovered.



Graphic 3. Autophagic pathways: microautophagy, chaperone mediated autophagy (CMA) and macroautophagy. Microautophagy is characterized by direct lysosomal uptake of soluble cytoplasmic proteins. In chaperone mediated autophagy (CMA), LAMP2a and Hsc70 deliver cytosolic proteins to the lysosomal lumen. Macroautophagy sequestrates cytosolic components into autophagosomes and delivers the ingested cargo for lysosomal degradation.

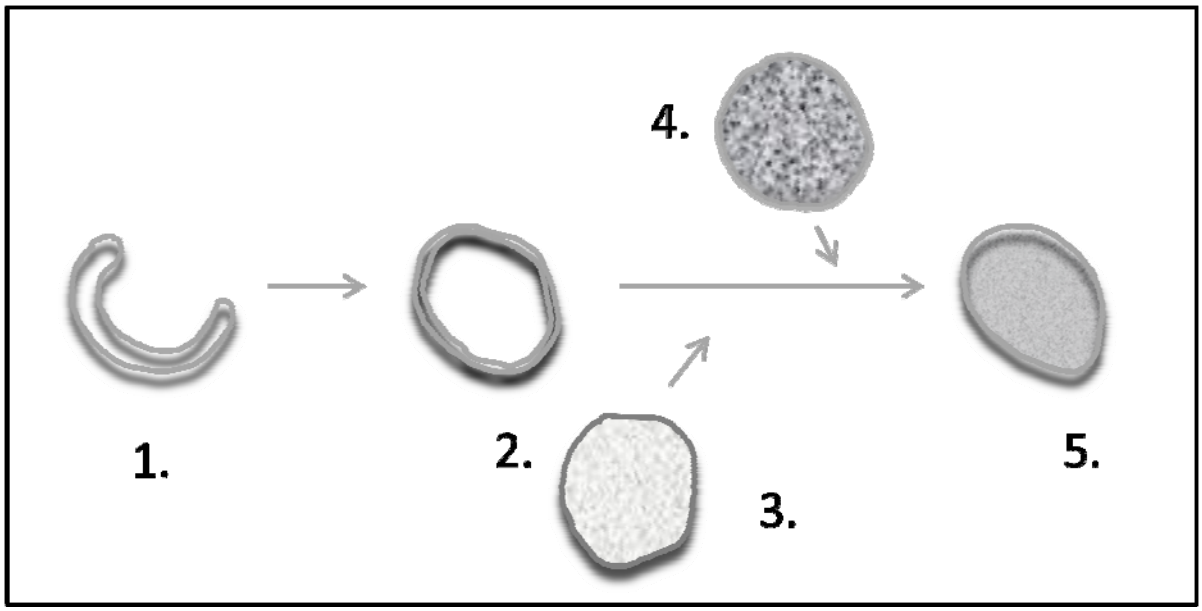
Macroautophagy is genetically best characterized autophagic pathway in eukaryotes. The implication of macroautophagy in endogenous antigen loading has been intensively studied in different *in vitro* systems and our study focuses on the role of macroautophagy in thymic epithelium. Thus, the general physiological significance, molecular mechanism and its role in antigen presentation will be discussed in the following paragraph.

1.3 Macroautophagy – process, function and role in endogenous antigen loading

Macroautophagy (hereafter referred to as **autophagy**) is an evolutionary highly conserved catabolic process within the eukaryotic phylum, which is responsible for turnover of long lived cytoplasmic proteins and aged or damaged organelles. The process has originally been identified in rat hepatocytes in the early sixties as a cellular response to glucagon treatment and starvation[156,157].

During the process of autophagy *de novo* formation of vesicles is taking place, but the origin of the membrane still remains a mystery[158, 159]. In the initiation step of autophagy a small cup shaped structure, called isolation membrane or phagophore is formed, that engulfs cytoplasmic components which are to be degraded. The isolation membrane elongates to fuse its ends and forms an immature autophagosome. The autophagosome, which is the main organelle of autophagy, is characterized by the presence of a double membrane. It is a large organelle between 0.5-1.5µm in diameter, depending on the organism and cell type[160,161]. Autophagosomes further fuse with endosomes to form mature autophagosomes or amphisomes[162-164]. It is proposed that endosomes provide the required machinery for fusion of amphisomes with lysosomes. Lysosomes deliver hydrolases that degrade ingested cytoplasmic cargo together with the inner autophagic membrane. The autophagolysosome is the only catabolic vesicle in the process of autophagy[165,166] (Graphic 4). The process is generally thought to be non selective, i.e., the isolation membrane randomly engulfs parts of the cytoplasm. However, recent studies have identified molecular players, such as p62/SQSTM1 (sequestrosome) and Alfy (autophagy-linked FYVE protein) which are involved in the

selective degradation of ubiquitinylated proteins and inclusion bodies via autophagy[167-168]. Furthermore, a selective degradation of mitochondria ("mitophagy") and invading bacteria ("xenophagy") have been described in mammals[169-171]. The respective mechanism behind selective targeting of organelles for autophagosomal degradation is still ill defined.



Graphic 4. **The process of macroautophagy**: an isolation membrane (1) enwraps a portion of cytoplasm and fuses its ends to form an autophagosome (2). Subsequently autophagosomes fuse with endosomes (3) and lysosomes (4) which results in the formation of autophagolysosome (5) where digestion of autophagic cargo is taking place.

1.3.1 Molecular mechanism of autophagy

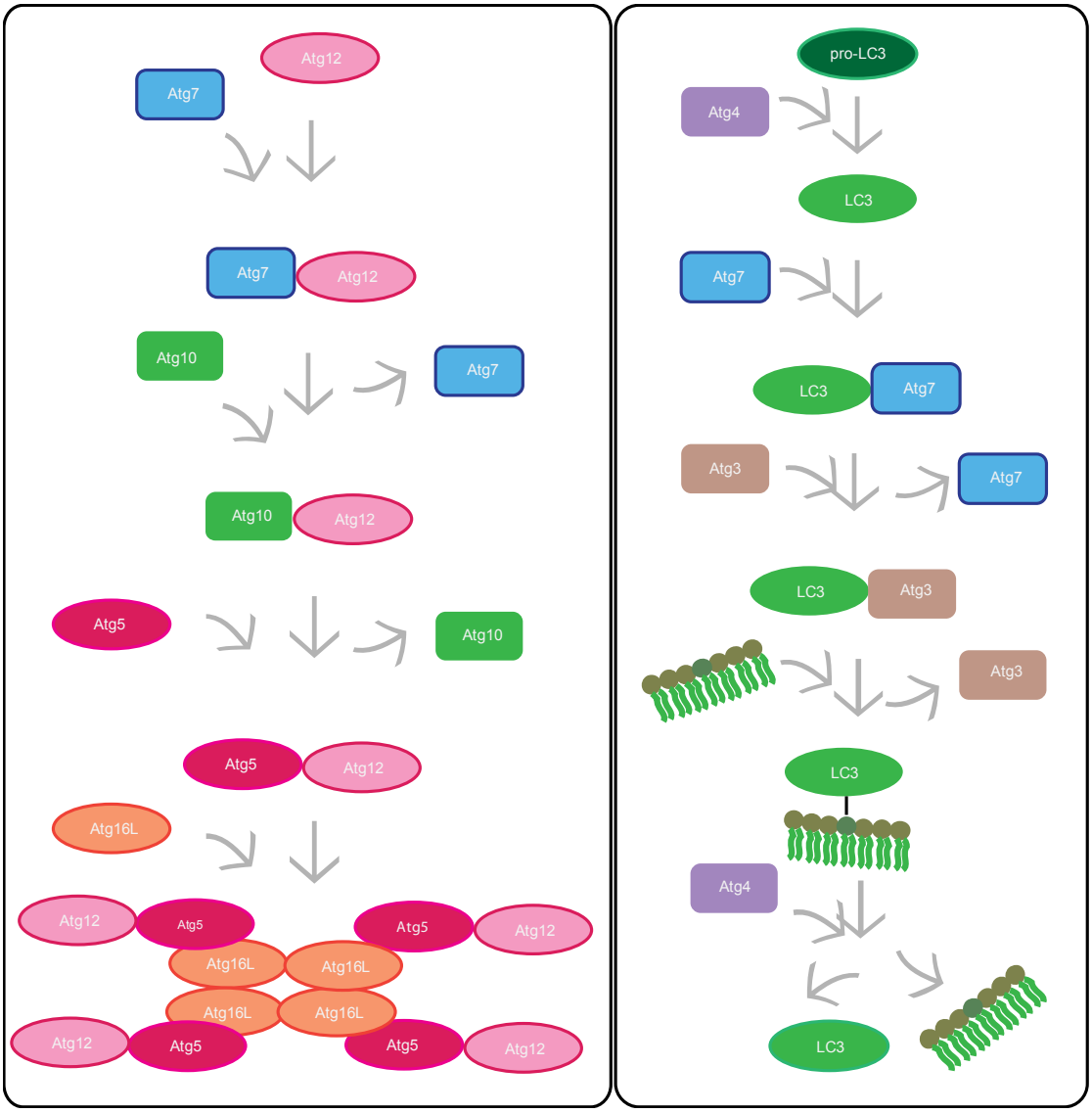
Even though autophagy has been originally described in mammals more than five decades ago, the elucidation of the underlying molecular mechanism came from genetic screens in yeast during last two decades[172, 173]. Since then, we witnessed a real explosion of knowledge regarding the molecular mechanism, regulation and physiological significance of autophagy. To date there are around 30 genes identified in yeast that play an essential role in autophagy, collectively known as Autophagy related genes (Atg)[174]. Most of the identified genes in yeast are conserved in mammals. The vast majority of Atg genes are essential players in the initiation and elongation steps of autophagosome formation. They encode for proteins involved in

conjugation reactions, which resemble enzymatic reactions described for the ubiqutin-conjugation mechanism, hence the name ubiqutin-like (Ubl) conjugation systems. There are two Ubl conjugation systems involved in autophagosome formation: Atg5-Atg12 and Atg8-PE (phosphatidylethanolamine). Mammalian homologue of yeast Atg8 ubiquitin-like modifier is microtubule associated protein 1 light chain 3 (MAP1-LC3, which will be referred to in the following as LC3).

During the initiation phase of autophagosome formation an Ubl modifier Atg12 gets covalently coupled to the Atg5 protein. This conjugation reaction requires the activity of two enzymes Atg7 (E1-like activating enzyme) and Atg10 (E2-like conjugating enzyme) (Graphic 5). The resulting Atg5-Atg12 conjugate further non-covalently interacts with Atg16L1, and ultimately forms a multimeric complex, Atg5-Atg12-Atg16L1, which is indispensable for the initiation and elongation phase of autophagosome formation[175-176]. The Atg5-Atg12-Atg16L complex is nonsimetricaly distributed on the phagophore and it primarily localizes to the outer leaflet of the membrane. Shortly before completion of autophagosome formation, the Atg5-Atg12-Atg16L complex dissociates from the membrane and recycles to the cytoplasm. Importantly, targeting of LC3 to autophagosomal membranes is absolutely dependent on Atg5-Atg12 conjugation system and accumulating evidence suggests that Atg5-Atg12-Atg16L1 complex acts as a docking site for the LC3 lipidation machinery[160, 177-178, 179]. LC3 is synthesized as an inactive precursor form: Pro-LC3. The specific cysteine protease Atg4 cleaves pro-LC3 which leads to the generation of the cytoplasmic form, LC3-I. Upon induction of autophagy, Atg7 and Atg3 enzymes mediate LC3-I protein conjugation to PE in the autophagosomal membrane which finally results in the formation of an autophagy active form of the protein: LC3-II (Graphic 5). This is so far the only known Ubl conjugation system that mediates protein lipidation[180,181]. Unlike Atg5-Atg12-Atg16L1 complex, the LC3-II protein is uniformly distributed on both inner and outer membrane and it does not dissociate from the autophagosomal vesicles until fusion with lysosomes takes place. Therefore, LC3 protein is considered to be a good autophagosomal marker. Upon formation of the autophagolysosome, the inner membrane associated LC3 portion gets degraded, while the cytosolically oriented LC3 is delipidated by Atg4 and released to the cytosol[180, 182, 183] (Graphic 5). The LC3-PE conjugation system is essential for elongation and closure of the phagophore[184]. Notably, there are at least

two more LC3 homologues in mammals, GABARAP and GATE-16[189,185-181, 186, 187]. However, the respective role of these proteins in autophagy and possible redundancy in function of mammalian Atg8 homologues in autophagosome formation has still to be elucidated.

a. b.



Graphic 5. Ubiquitin like conjugation systems involved in autophagosome formation in mammals a. Atg5-Atg12 ubiquitin like system b. LC3-PE ubiqutin like system

1.3.2 Role of autophagy in mammals

The most acknowledged role of autophagy is the supply of amino acids under nutrient-limiting conditions as exemplified by induction of autophagy under experimentally created starvation conditions[156,161] and by neonatal lethality of autophagy deficient mice[184,188,189,190]. The neonatal lethality of autophagy deficient pups is caused at least in part by an inability to sustain energy homeostasis upon a sudden termination of nutrient supplies via the placenta, the unique problem that mammals face during their development.

Apart from this evolutionary conserved role of “induced autophagy” as an adaptive response to starvation, the physiological significance of “constitutive” or basal autophagy which occurs even under nutrient rich conditions in the maintenance of cellular homeostasis has begun to be elucidated. Autophagy has been implicated as an important protein quality control mechanism in the brain and liver, as targeted ablation of autophagy in these organs resulted in the accumulation of toxic protein aggregates that ultimately led to the development of neurodegenerative disease and liver dysfunction, respectively[190-191]. Furthermore, autophagy plays a vital role during the early stages of mammalian development[192,193] and has been suggested to act as a tumor suppressive mechanism[194].

The involvement of autophagy in combating infections during innate immune responses has been repeatedly reported. Bacteria and viruses that reside in the cytosol or inside the phagosomes can be targeted for autophagosomal sequestration and subsequent degradation by lysosomal hydrolyses. In that way autophagy can contribute to the successful eradication of intracellular pathogens[170, 195-196]. Interestingly, some pathogens have evolutionary specializations which enable them to either escape from autophagic degradation or to use autophagosomal “niche” for replication in the host cells[197,198]. Thus, subversion of autophagic machinery may be another immune evasion mechanism of pathogens. Additionally, autophagy has an indispensable role in the survival and proliferation of peripheral T cells[199] and development of B cells[200], though the underlying function of autophagy in these processes remains to be resolved.

1.3.3 Macroautophagy and antigen presentation

The first experimental evidence that intuitively implicated autophagy in endogenous antigen loading came from already mentioned MHC class II ligandome analyses of B cell lines[141]. The identified MHC class II epitopes derived from intracellular self were peptides drawn from the nuclear and intracellular proteins with a long half-life, namely the known autophagy substrates. In the same line, Stevanovic and colleagues identified two epitopes derived from autophagy related protein LC3 among naturally occurring MHC class II bound peptides of Epstein Barr virus (EBV) transformed human B cell lines, which implied that the autophagic machinery can gain access to MIIC of APC. Furthermore, authors monitored changes in the displayed MHCII/p repertoire upon induction of autophagy and were able to show that the presentation of intracellular and organellar proteins is selectively enhanced when autophagy is up-regulated, while the presentation of membrane bound or extracellular proteins does not show any dependence on autophagic activity of the cell[142]. Moreover, a consensus has emerged in the field of antigen processing that the MIIC represents late endosomes/lysosomes equipped with the molecular machinery for antigen loading. As already described, during the maturation of autophagosomes, fusion with late endosomes is taking place which results in the formation of amphisomes. Electron microscopy studies revealed that the morphological appearance of amphisomes is multilamelar and multivesicular, the characteirsitcs that perfectly match ultrastructural appearance of MIIC in the cell[117, 163, 164]. Thus, one can hypothesize that in APC these two vesicular compartments intersect which results in the delivery of autophagic cargo to the MHC class II loading machinery. Recently, the laboratory of Chirstian Münz documented that the postulated cross talk between autophagy and MIIC indeed takes place in both professional and non-professional APC, such as monocyte- derived immature and mature DC, B cells and interferon γ- treated human epithelial cell lines. By taking advantage of fluorescently labeled LC3 protein, the authors were able to visualize and quantify the fusion events between autophagosomes and HLA-DM rich vesicles of APC, and they recognized that a substantial cross talk between the two pathways constitutively takes place. The immunofluorescence analyses were further supported by ultrastructural electron microscopy studies which have convincingly shown the intersection of autophagosomes and MHC class II loading machinery[201].

The first evidence that directly implicated autophagy as a functionally significant pathway for MHC class II antigen loading came from studies that relied on pharmacological inhibition of the process. Brazil *et al*. reported that inhibition of autophagy in macrophages and B cells diminishes the presentation of endogenously expressed complement C5 protein to CD4 T cells[202]. The list of endogenous antigens whose MHC class II presentation is dependent on autophagy grew in the years that followed, and now includes tumor antigens, model antigen and viral antigens[203-206]. These early studies relied on the use of chemical inhibitors of autophagy, most commonly an inhibitor of the class III PI3K complex which is essential in initiation phases of autophagosome formation, 3- methyladenine (3-MA). However, the effect of the drug is not exclusively autophagy specific, as it has some effects on endocytosis and lysosomal acidification as well, hence it may affect other catabolic pathways beside autophagy[207].

By taking advantage of small interfering RNA technology (siRNA) , Paludan *et al*. has been able to show that genetic interference with autophagy results in diminished presentation of nuclear antigen1 of EBV (EBNA1) to CD4 T cell clones in EBV transformed B cell lines[206]. This seminal work for the first time recognized autophagy dependent MHC class II presentation at the physiological expression level of antigens. Furthermore, the same group went on to show that autophagy can specifically enhance the CD4 T cell responses, as targeting of influenza matrix protein 1 (MP1) to autophagosomes enhanced stimulation of CD4, but not CD8 MP1-specific T cell clones [201].

Overall, in above discussed studies the convincing evidence that autophagy can intersect with MIIC in APC and feed the enwrapped cytosolic cargo to MHC class II loading system has been presented. However, the *in vivo* evidence for the involvement of autophagy in antigen presentation during T cell development and adaptive immune responses is still missing.

1.4 Aim of the thesis

MHC class II antigen loading pathways in thymic epithelium remain poorly defined[49]. Strikingly, analyses of GFP-LC3 autophagy reporter mice have identified the thymus as a rather unique site of starvation independent autophagy[161]. The emerging evidence for the existence of endogenous MHC class II antigen loading pathways in different subsets of human and murine APC[152,201,208] and the outstanding observation that autophagy is constitutively active in the thymus irrespective of nutrient conditions encouraged us to hypothesize that TEC have subverted this ancient catabolic process for the generation of MHC class II ligands from intracellular proteins.

At the moment when this study has been initiated a good TEC-specific Cre transgenic mouse line has not been available, therefore we could not take advantage of Cre/LoxP technology and conditional knock out studies to specifically evaluate the role of autophagy in thymic epithelium. To circumvent the problem of neonatal lethality of autophagy deficient animals[189], we took advantage of the embryonic thymus transplantations. This approach allowed us to create a “full conditional Atg5 knock out” in thymic epithelium and to check for the role of macroautophagy in the T cell repertoire selection and tolerance induction. We planned to assess the efficacy of positive selection by transplanting Atg5$^{-/-}$ E15 thymi under the kidney capsule of a large panel of TCR transgenic mouse strains and to check for the tolerogenic potential of Atg5$^{-/-}$ thymic epithelium by generating chimeric nu/nu $^{\text{thymus Atg5-/-}}$ animals.

LETTERS

Autophagy in thymic epithelium shapes the T-cell repertoire and is essential for tolerance

Jelena Nedjic[1]†, Martin Aichinger[1]†, Jan Emmerich[1]†, Noboru Mizushima[2] & Ludger Klein[1,3]

Recognition of self-antigen-derived epitopes presented by major histocompatibility complex class II (MHC II) molecules on thymic epithelial cells (TECs) is critical for the generation of a functional and self-tolerant CD4 T-cell repertoire. Whereas haematopoietic antigen-presenting cells generate MHC-II–peptide complexes predominantly through the processing of endocytosed polypeptides[1], it remains unknown if and how TECs use unconventional pathways of antigen presentation. Here we address the role of macroautophagy, a process that has recently been shown to allow for endogenous MHC II loading[2–6], in T-cell repertoire selection in the mouse thymus. In contrast to most other tissues, TECs had a high constitutive level of autophagy. Genetic interference with autophagy specifically in TECs led to altered selection of certain MHC-II-restricted T-cell specificities and resulted in severe colitis and multi-organ inflammation. Our findings indicate that autophagy focuses the MHC-II–peptide repertoire of TECs on their intracellular milieu, which notably comprises a wide array of otherwise strictly 'tissue-specific' self antigens[7,8]. In doing so, it contributes to T-cell selection and is essential for the generation of a self-tolerant T-cell repertoire.

Thymic epithelial cells (TECs) are the only non-haematopoietic cell types that constitutively express MHC II. Cortical TECs (cTECs) have predominantly been implicated in positive selection of thymocytes carrying self-MHC-restricted T-cell receptors (TCRs)[9], whereas medullary TECs (mTECs) fulfil a non-redundant role in tolerance induction by 'promiscuously' expressing otherwise tissue-restricted antigens (TRAs)[7,8]. Notably, tolerance induction towards mTEC-derived antigens can operate through direct antigen recognition on mTECs[10] or by cross-presentation by thymic dendritic cells[11].

MHC-II–peptide ligands are primarily generated through processing of exogenous proteins[1]. TECs, however, show a remarkably poor efficacy in capturing and processing extracellular antigens[12], suggesting alternative pathways of MHC II loading. Candidate routes through which endogenous loading of MHC II molecules may be achieved are a TAP-dependent pathway[13], chaperone-mediated autophagy[14] and macroautophagy[2–6].

Macroautophagy is a bulk protein degradation process that is upregulated to sustain metabolic fitness during food deprivation[15]. Autophagosomes fuse with lysosomes for degradation of their cargo, and it is conceivable that at this point an intersection with the MHC II pathway occurs[2]. Because the thymus has been identified as a site of unusual starvation-independent autophagic activity[16], we hypothesized that this phenomenon may facilitate endogenous MHC II loading in TECs and thus contribute to positive selection and/or tolerance induction.

Using green fluorescent protein (GFP)–LC3 autophagy reporter mice[16] (Supplementary Fig. 1a), we confirmed that the thymus had robust, starvation-independent autophagic activity (Fig. 1a). In contrast, in many other tissues—for example, muscle—detectable autophagy is induced only upon starvation (Fig. 1a and Supplementary Fig. 1b). Absence of punctae in thymi of reporter mice with a targeted disruption of *Atg5* (autophagy-related gene 5)[17,18], an essential component of autophagosome formation, indicated that these structures were not unspecific aggregates (Supplementary Fig. 1c).

When purified thymic stromal cells were analysed (Fig. 1b), the highest frequency (68.5 ± 2.9%) of autophagy-positive cells (≥5

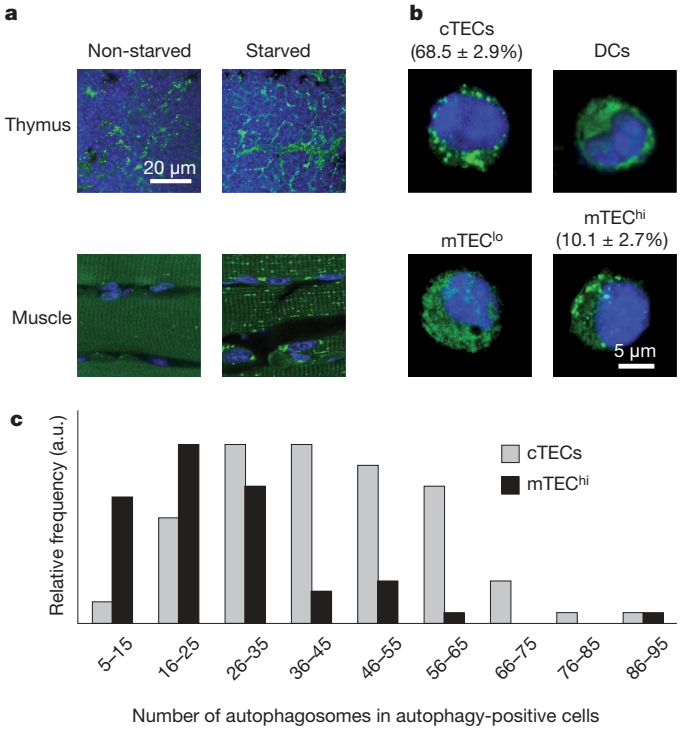


Figure 1 | Constitutive autophagy in TECs. a, Sections of the thymus and muscle of fed and starved GFP–LC3 mice were stained with anti-GFP Alexa 488 antibody (green) and 4,6-diamidino-2-phenylindole (DAPI; blue). Images are representative of $n = 6$. **b**, Thymic stromal cells from GFP–LC3 mice were fixed onto glass slides and stained with anti-GFP Alexa 488 and DAPI. Typical autophagy-positive cTECs and mTEC^{hi} are shown. The average frequencies ± s.d. of cTECs and mTEC^{hi} scoring autophagy positive (that is, ≥5 autophagosomes) in two independent preparations are indicated. Less than 1% of dendritic cells (DCs) and mTEC^{lo} were positive. **c**, Relative distribution of the number of autophagosomes in autophagy-positive cTECs and mTEC^{hi}; a.u., arbitrary units.

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autophagosomes) was found among cTECs. In mTEC subsets[19,20], 10.1 ± 2.7% of mature MHC II^{hi} CD80^{hi} mTECs (mTEC^{hi}) scored positive, whereas autophagy was negligible among immature MHC II^{lo} CD80^{lo} mTECs (mTEC^{lo}), demonstrating a co-segregation of autophagy with 'promiscuous' antigen expression[21]. The number of autophagosomes in positive cells was scattered around maxima of about 35 or 20 in cTECs or the mTEC^{hi} subset, respectively (Fig. 1c).

Although autophagy has been implicated in developmental processes[15,22], *Atg5*$^{-/-}$ mice are born at Mendelian ratios without evident developmental abnormalities[17]. However, neonatal lethality of *Atg5*$^{-/-}$ mice, at least in part caused by a perinatal metabolic crisis, precluded a direct assessment of T-cell selection and tolerance in this system. To assess the capacity of *Atg5*$^{-/-}$ thymi to support T-cell development beyond the perinatal phase, we transplanted embryonic *Atg5*$^{-/-}$ thymi under the renal capsule of normal adult recipients. When analysed after 6 to 8 weeks, *Atg5*$^{-/-}$ thymi were significantly smaller than wild-type controls (Fig. 2a).

Several observations suggested that the reduced cellularity of *Atg5*$^{-/-}$ grafts was not caused by aberrant differentiation of the epithelial compartment. Thus, *Atg5*$^{-/-}$ grafts were properly compartmentalized into cortical and medullary areas (Fig. 2b) and contained cTECs and mTECs at normal frequencies (Supplementary Fig. 2). Expression of MHC I and II on *Atg5*$^{-/-}$ epithelial cells was essentially normal; however, a slight decrease in MHC II density was observed on cTECs (Fig. 2c). *Atg5*$^{-/-}$ mTECs showed the typical biphasic MHC II expression indicative of normal segregation into mTEC^{lo} and mTEC^{hi} subsets, and promiscuous antigen expression was intact (Fig. 2d).

Next we considered whether the reduced cellularity of *Atg5*$^{-/-}$ grafts may have resulted from perturbed T-cell development. Ratios of thymocyte subsets including CD25^{+} Foxp3^{+} regulatory T cells were normal in H-2^{d} *Atg5*$^{-/-}$ (Fig. 3a) or H-2^{b} *Atg5*$^{-/-}$ thymi (Supplementary Fig. 3a). TCR-α or TCR-β V-region use by CD4 single-positive (SP) thymocytes was also normal (Supplementary Fig. 3b, c), indicating that *Atg5*$^{-/-}$ deficiency in TECs was compatible with development of a polyclonal T-cell repertoire containing the main T-cell lineages.

Positive selection is remarkably flexible regarding the requirements for the composition of MHC–peptide (MHCp) ligands on cTECs, as exemplified by the efficient selection of polyclonal CD4 T cells in mice expressing a markedly reduced spectrum of MHCp complexes[23,24]. If autophagy were to contribute to the generation of MHC II ligands on TECs, its absence would be expected to have rather subtle consequences. Two predictions followed from these considerations. First, selection of particular monoclonal MHC-II-restricted TCR specificities should be affected in *Atg5*$^{-/-}$ thymi. Second, if the reduced cellularity of *Atg5*$^{-/-}$ thymi was caused by altered T-cell selection, a 'permissive' monoclonal TCR specificity should rescue the organ size. We tested these predictions by analysing the selection of several MHC-II-restricted transgenic TCRs (that is, TCR-HA restricted to I-E^{d}, AND restricted to I-E^{k}, DO11.10 restricted to I-A^{d}, and DEP and SEP restricted to I-A^{b}). In *Atg5*$^{-/-}$ H-2^{d} lobes grafted into TCR-HA transgenic mice, both the frequency of CD4 SP cells expressing the TCR-HA receptor and the cellularity of lobes were significantly reduced as compared to controls (Fig. 3b). Similarly, *Atg5*$^{-/-}$ H-2^{b} lobes grafted into SEP TCR transgenic hosts had reduced cellularity, and the decreased frequency of CD4 SP cells together with an increased expression of endogenous TCR-α chains indicated that there was selective pressure against this TCR (Supplementary Fig. 4). In contrast, the AND TCR seemed to be selected as efficiently in the *Atg5*$^{-/-}$ to AND grafts as in the wild-type to AND controls; this finding coincided with equal cellularities (Fig. 3c). For the *Atg5*$^{-/-}$ to DEP (Supplementary Fig. 5) and the *Atg5*$^{-/-}$ to DO11.10 (Supplementary Fig. 6) transplants, we likewise observed efficient positive selection of the respective TCR and rescue of thymic cellularities. Of note, the frequency of clonotype-positive CD4 SP cells was significantly increased in the *Atg5*$^{-/-}$ to DO11.10 lobes, indicating that *Atg5* deficiency could also be beneficial for the selection of particular specificities. Transplantation of *Atg5*$^{-/-}$ lobes into mixed (DO11.10 *Rag2*$^{-/-}$/TCR-HA *Rag2*$^{-/-}$) bone marrow chimaeras accentuated the opposing consequences of epithelial *Atg5* deficiency for these TCRs (Supplementary Fig. 6d). In contrast to these observations with MHC-II-restricted TCRs, selection of three MHC-I-restricted transgenic TCRs (P14 restricted to H-2D^{b}, OT-I restricted to H-2K^{b} and HY restricted to H-2D^{b}) in *Atg5*$^{-/-}$ or wild-type lobes was indistinguishable, both with respect to expression of the transgenic TCR chains and thymic cellularity (Fig. 3d and Supplementary Fig. 7; see also Supplementary Table 1 for a summary). Taken together, these findings were consistent with autophagy in TECs specifically shaping the MHC-II-restricted T-cell repertoire, probably through the generation of particular MHC-II–peptide ligands. This underscores the notion that distinct pathways of antigen presentation in TECs may be essential for the generation of the 'normal' T-cell repertoire[25,26].

Evidence that autophagy can indeed mould the composition of MHC II ligands was recently obtained by mass spectrometric analysis of MHC-II-bound peptides of B-lymphoblastoid cell lines in the presence or absence of autophagy[6]. Quantitative considerations rendered such a global assessment of the 'MHC ligandome' impossible for limited numbers of *ex vivo* isolated TECs. To visualize whether a particular MHC-class-II-bound peptide on cTECs was affected by genetic interference with autophagy, we used a monoclonal antibody (Y-Ae) that recognizes I-A^{b} when occupied by a I-Eα-derived peptide[27]. The I-E$\alpha_{52\text{-}68}$–I-A^{b} complex is abundant on haematopoietic antigen-presenting cells (~10% of all I-A^{b}–peptide complexes)[27], probably as a result of co-sorting of I-E and I-A molecules. Notably, however, this complex is comparatively underrepresented on cTECs, and it was hypothesized that this was due to competition

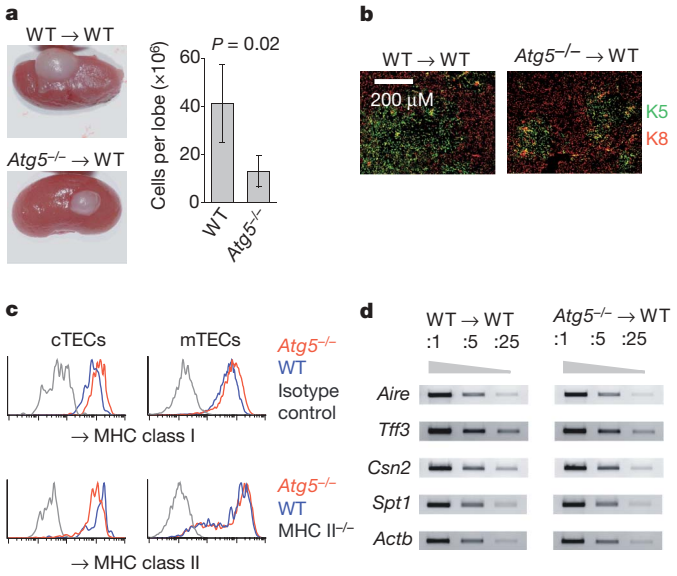

Figure 2 | Epithelial differentiation in the absence of *Atg5*. **a**, Thymi from wild-type (WT) or *Atg5*$^{-/-}$ embryos were transplanted under the kidney capsule of wild-type recipients and analysed 6 weeks later. The average cellularities ± s.d. are shown; $n = 5$. **b**, Normal compartmentalization of *Atg5*$^{-/-}$ grafts in cytokeratin-8-positive (red; K8) cortical and cytokeratin-5-positive (green; K5) medullary regions; $n = 8$. **c**, Expression of MHC class I and II on cTECs or mTECs from *Atg5*$^{-/-}$ to wild-type (WT) grafts (red), or wild-type to wild-type grafts (blue). Staining controls are depicted in grey; data are representative of three experiments. **d**, RT–PCR analysis of autoimmune regulator (*Aire*) and TRA expression in mTECs isolated from *Atg5*$^{-/-}$ to wild-type, or wild-type to wild-type grafts. Expression of β-actin (*Actb*), intestinal trefoil factor (*Tff3*), casein 2 (*Csn2*) and salivary protein 1 (*Spt1*) are also shown (5-fold serial dilutions). Data are representative of two experiments.

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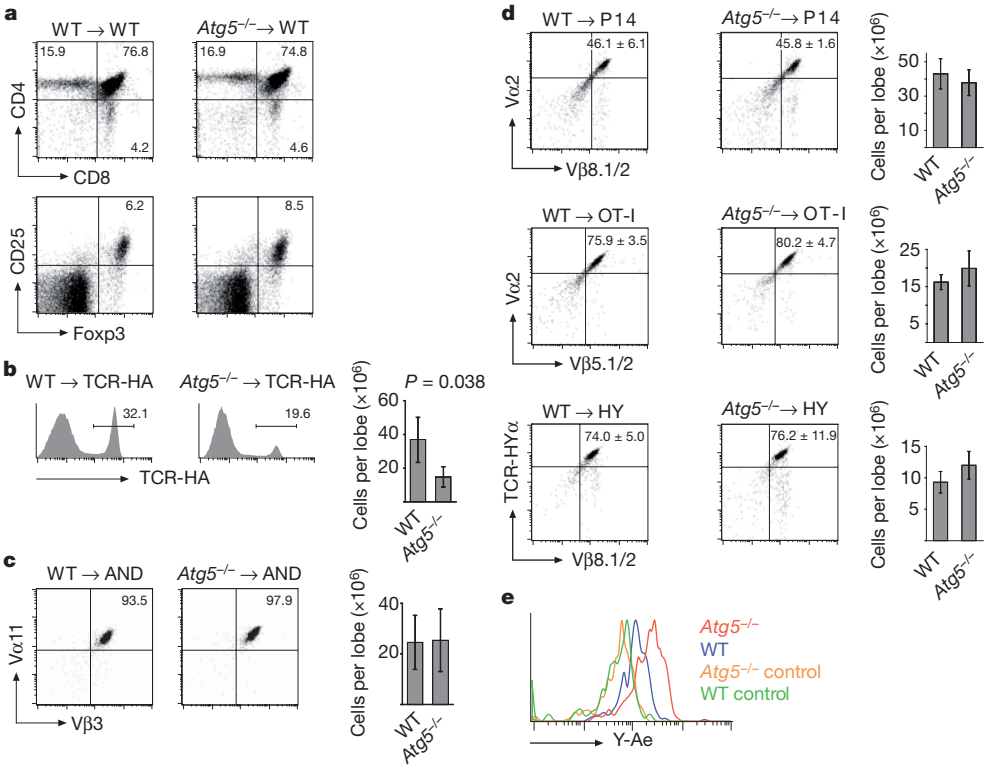

Figure 3 | *Atg5* deficiency modulates selection of MHC-II-restricted TCR specificities and the abundance of a specific MHCp complex. **a**, Polyclonal T-cell development in *Atg5*$^{-/-}$ to wild-type (WT), or wild-type to wild-type grafts. The top dot plots show CD4 and CD8 staining; the bottom dot plots show expression of CD25 and Foxp3 by CD4 SP thymocytes; $n \geq 5$. **b**, *Atg5*$^{-/-}$ or wild-type lobes were grafted into TCR-HA transgenic recipients. Graft cellularity ± s.d. and the expression of the transgenic TCR on CD4 SP cells were analysed after 6 weeks. The frequency ± s.d. of TCR-HA^{+} cells among CD4 SP cells was 32.1% ± 3.2% in wild-type to TCR-HA, and 19.6% ± 4.3% in *Atg5*$^{-/-}$ to TCR-HA lobes; $P = 1.8 \times 10^{-8}$ ($n \geq 14$). **c**, *Atg5*$^{-/-}$ or wild-type lobes were grafted into AND transgenic mice. Graft cellularity ± s.d. and the expression of the transgenic TCR (Vβ3–Vα11) on CD4 SP cells were analysed after 6 weeks. The frequency ± s.d. of Vβ3^{+} Vα11^{+} CD4 SP cells was 93.5% ± 0.2% in wild-type to AND lobes ($n = 3$) and 97.9% ± 1.1% ($n = 6$) in *Atg5*$^{-/-}$ to AND lobes ($P = 9 \times 10^{-4}$). **d**, Thymi from wild-type or *Atg5*$^{-/-}$ embryos were grafted into P14, OT-I or HY TCR-transgenic recipients. The dot plots show the expression of the transgenic TCR V-segments on CD8 SP cells. Thymus cellularities ± s.d. are shown in the respective bar diagrams. Data are representative of $n = 4$ (P14), $n \geq 4$ (OT-I) and $n \geq 2$ (HY). **e**, Thymi from wild-type or (BALB/c × C57BL/6)F_1 *Atg5*$^{-/-}$ embryos were transplanted into (BALB/c × C57BL/6)F_1 recipients. Six weeks after grafting, expression of the I-Eα_{52-68}–I-A^{b} complex on cTECs was analysed using the monoclonal antibody Y-Ae. *Atg5*$^{-/-}$ cTECs are shown in red; wild-type cTECs are shown in blue. Control stainings are shown in orange (*Atg5*$^{-/-}$) and green (wild type). Data are representative of two independent experiments.

for I-A^{b} binding by peptides that may be expressed and/or processed in a cTEC-specific manner. When we analysed cTECs from F_1 (BALB/c × C57BL/6) *Atg5*$^{-/-}$ lobes, we found an increased abundance of the I-Eα_{52-68} –I-A^{b} complex as compared to wild-type controls, whereas total MHC II levels showed an inverse behaviour (Fig. 3e, compare with Fig. 2c). These findings provided direct evidence that *Atg5* deficiency causes a quantitative shift in the MHC II ligandome of cTECs. We propose that in *Atg5*$^{-/-}$ cTECs, a set of epitopes that rely on autophagy for endogenous MHC II loading may not compete with the Eα peptide, which itself may easily gain access to MHC II by other pathways.

To address whether the T-cell repertoire selected by *Atg5*$^{-/-}$ TECs was self-tolerant, *Atg5*$^{-/-}$ or *Atg5*$^{+/+}$ thymi were grafted under the kidney capsule of athymic mice (designated *nu/nu*$^{Atg5-/-}$ or *nu/nu*^{WT}, respectively). The peripheral repertoire of CD4 T cells was similar with respect to Vα and Vβ use in both sets of chimaeras (Supplementary Fig. 8). However, the frequency of CD4 T cells with an activated phenotype (CD62L^{lo} and CD69^{+}) was significantly increased in the *nu/nu*$^{Atg5-/-}$ chimaeras (Fig. 4a). Between 4 and 6 weeks after grafting, wasting began in *nu/nu*$^{Atg5-/-}$ but not *nu/nu*^{WT} chimaeras, and most of the *nu/nu*$^{Atg5-/-}$ chimaeras had to be killed between 8 to 16 weeks after grafting (Fig. 4b). Recipients of *Atg5*$^{-/-}$ thymi had patches of flaky skin, and inspection of internal organs showed a massively enlarged colon, atrophy of the uterus, complete absence of fat pads and in many cases enlarged lymph nodes (Fig. 4c). Histological examination demonstrated inflammatory infiltrates in the colon, liver, lung, uterus and Harderian gland (Fig. 4d and Supplementary Table 2). Chimaeras generated with 5 days deoxyguanosine treatment of embryonic lobes or by transplantation immediately after preparation yielded essentially identical outcomes, excluding organ-culture-related epithelial tissue damage or carry-over of *Atg5*$^{-/-}$ haematopoietic cells as critical factors for disease development. The latter point was bolstered by the fact that *Atg5*$^{-/-}$ to wild-type fetal liver chimaeras did not show any signs of autoimmunity (Supplementary Fig. 9; ref. 28).

Adoptive transfer of 1×10^{7} purified CD90^{+} T cells from *nu/nu*$^{Atg5-/-}$ chimaeras into *nu/nu* recipients recapitulated essentially all aspects of autoimmunity as observed in donor chimaeras (Supplementary Fig. 10). When transferred separately, CD4^{+} T cells from *nu/nu*$^{Atg5-/-}$ chimaeras elicited autoimmune symptoms more efficiently than CD8^{+} T cells, in line with *Atg5*$^{-/-}$ deficiency in thymic epithelium primarily perturbing the selection of the CD4 T-cell repertoire (data not shown).

Taken together, we propose that perturbations in both positive and negative selection caused by *Atg5* deficiency in TECs may synergize to fuel autoimmunity. First, alterations in the composition of MHCp ligands on cTECs may affect positive selection, potentially creating a mildly lymphopenic environment that might foster autoimmunity. Second, impaired presentation of TRAs by mTECs may allow for exit into the periphery of autoreactive thymocytes that would normally be deleted or deviated into the regulatory T cell lineage.

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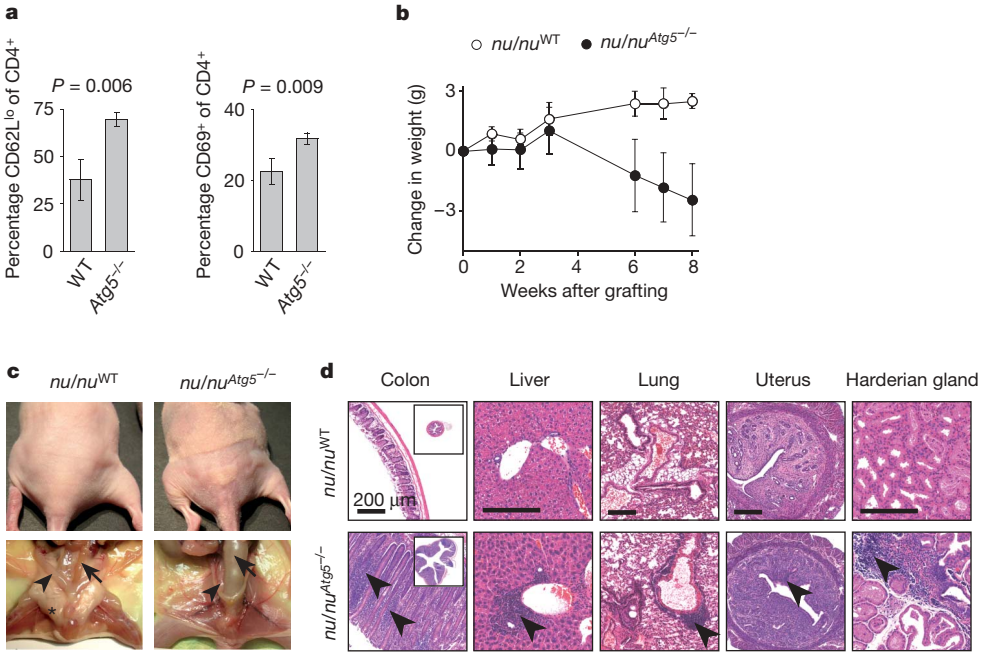

Figure 4 | *Atg5* deficiency in thymic epithelium causes colitis and multiorgan lymphoid infiltration. *Atg5*$^{-/-}$ or wild-type (WT) lobes were grafted into athymic (*nu/nu*) mice (*nu/nu*$^{Atg5-/-}$ or *nu/nu*^{WT}, respectively). Data are representative of three experiments with $n \geq 4$. **a**, Expression of activation markers on lymph node CD4 T cells in *nu/nu*$^{Atg5-/-}$ or *nu/nu*^{WT} chimaeras. **b**, The changes in body weight over time are shown. **c**, The appearance of *nu/nu*$^{Atg5-/-}$ or *nu/nu*^{WT} chimaeras. The colon (arrow) and uterus (arrowhead) are highlighted; note the absence of fat pads (asterisk) in *nu/nu*$^{Atg5-/-}$ chimaeras. **d**, Haematoxylin and eosin staining of organs from *nu/nu*$^{Atg5-/-}$ or *nu/nu*^{WT} chimaeras. Arrows indicate infiltrates.

The organ selectivity of the autoimmune manifestations observed here remains to be explained. Of note, the Harderian gland itself has a substantial level of constitutive autophagy (Supplementary Fig. 11). Thus, autophagy-dependent epitope display in the periphery, if not counterbalanced by an analogous tolerogenic mechanism in the thymus, may be critical for immune-mediated tissue destruction. Along these lines, the human *ATG16L1* gene was recently identified as a susceptibility locus for Crohn's disease[29,30]. Our data offer an explanation for the association of an autophagy-related gene with breakdown of intestinal immune homeostasis.

METHODS SUMMARY

Animals. All transgenic mouse strains have been described elsewhere. Animal protocols were approved by local authorities.

Antibodies and flow cytometry. Flow cytometry was performed according to standard procedures. See full Methods for antibodies.

GFP–LC3 analyses. Before analyses, mice were anaesthetized and perfused through the left ventricle with 4% paraformaldehyde. Organs were collected and fixed with sucrose. Tissue samples were embedded in optimal cutting temperature (OCT) medium and sectioned at 5 µm before staining with an anti-GFP antibody. For quantification, purified thymic stromal cells were obtained from enzymatically digested thymi by a combination of density fractionation and FACS sorting. Stromal cells were fixed onto slides and processed for staining with an anti-GFP antibody.

Thymus transplantation. Embryonic thymi were transplanted under the kidney capsule of female animals. *Atg5*$^{-/-}$ embryos and *Atg5*$^{+/+}$ (wild-type) controls within individual experiments were obtained from the same *Atg5*$^{+/-}$ pregnant female.

Histopathology. Organs were immersion-fixed and embedded in paraffin blocks. Sections were stained with haematoxylin and eosin and automatically scanned using the Zeiss MIRAX SCAN system.

Statistical analyses. All statistical analyses were performed using the two-tailed Student's *t*-test with unequal variance.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Contributions J.N. was involved in all experiments (assisted by J.E., M.A. and L.K.). M.A. carried out the analysis of TRA expression. J.N., N.M. and L.K. designed experimental strategies. J.N. and L.K. wrote the manuscript. All authors discussed and commented on the contents of this manuscript.

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METHODS

Animals. *Atg5*$^{-/-}$ and GFP–LC3 mice have been described previously[17,16]. BALB/c$^{nu/nu}$ animals were purchased from Taconic Farms. The AND TCR transgenic and B10.A animals were obtained from Jackson Laboratories. The TCR-HA, DO11.10, SEP and DEP TCR transgenic animals were described elsewhere[31,32]. All mice were bred and maintained in individually ventilated cages in the animal facility of the Research Institute of Molecular Pathology under specific pathogen-free conditions. All animal studies were approved by local authorities (MA58) and were performed according to Austrian regulations.

Antibodies and flow cytometry. Biotin-conjugated monoclonal antibody Y-Ae was a gift from B. Kyewski. The following monoclonal antibodies were used: biotin-conjugated antibody to CD11c (HL3), CD62L (MEL14), CD80 (16-10A1), Vβ3 (KJ25) and Vβ14 (14-2); fluorescein isothiocyanate (FITC)-conjugated antibody to CD62L (MEL14), CD90.1 (HIS51), H-2K^b (AF6-88.5), Vα8.3 (KT50), Vβ3 (KJ25), Vβ4 (KT4), Vβ5.1,5.2 (MR9-4), Vβ6 (RR4-7), Vβ8.1/2 (MR5-2), Vβ8.3 (1B3.3) and Vβ13 (MR12-3); phycoerythrin-conjugated antibody to CD69 (H1.2F3), Ly51 (BP-1), Vα2 (B20.1), Vα11.1,11.2 (RR8-1), Vβ2 (B20.6), Vβ7 (TR310), Vβ8.3 (1B3.3) and Vβ10 (B21.5); CyChrome-conjugated antibody to CD8 (53-6.7) and CD45 (30-F11); phycoerythrin-Cy7 conjugated antibody to CD25 (PC61); allophycocyanin-conjugated antibody to CD4 (RM4-5) and CD8 (52-6.7); allophycocyanin-indotricarbocyanin-conjugated antibody to CD4 (GK1.5); and phycoerythin-Cy7-conjugated streptavidin and FITC-conjugated streptavidin (Becton Dickinson). Phycoerythrin-conjugated monoclonal antibody to TCR-HYα (T3.70) was purchased from eBiosciences. *Ulex europaeus* agglutinin (UEA-1) was purchased from Sigma Aldrich and conjugated to Alexa 647 in our laboratory. Monoclonal antibodies specific for TCR-HA (6.5), DO11.10 (KJ1-26), EpCAM (G8.8), Vβ8 (F23.1), pan-MHC class II (P7.7) and CD4 (GK1.5) were purified from hybridoma supernatants and conjugated to biotin, Alexa Fluor 488 or Alexa Fluor 647 in our laboratory. Surface staining was performed according to standard procedures at a density of 1×10^6 to 3×10^6 cells per 50 μl. FoxP3 intracellular staining was carried out according to the manufacturer's recommendations with phycoerythrin- or allophycocyanin-conjugated monoclonal antibodies to mouse FoxP3 (FJK-16s, eBiosciences). A FACSCanto (Becton Dickinson) with FACSDiva software (Becton Dickinson) was used for data acquisition and the Flow Jo software was used for data analysis.

Immunofluorescence. Frozen sections (5 μm) were fixed in cold acetone, and then washed and blocked for 30 min with 10% (v/v) FCS in PBS. Sections were permeabilized in 0.1% (v/v) Tween in PBS for 10 min, and stained overnight at 4 °C with biotinylated anti-keratin 8 antibody (TROMA-1, Developmental Studies Hybridoma Bank). Sections were incubated with secondary antibody for 90 min at room temperature and, after washing three times for 15 min each in 0.1% (v/v) Tween in PBS, were blocked with anti-rat serum (Jackson Immuno Research Laboratories) for 30 min at room temperature. After washing, sections were incubated with anti-keratin 5 antibody (Covance) for 2 h at room temperature. Secondary reagents were streptavidin-Cy3 (Jackson Immuno Research Laboratories) and Alexa-Fluor-488-conjugated anti-rabbit antibody (Molecular Probes). Nuclei were counterstained with ProLong Gold antifade reagent with DAPI (Molecular Probes). Samples were analysed with an inverted confocal LSM 510 laser scanning confocal microscope (Zeiss).

GFP–LC3 analyses. GFP–LC3 mice and wild-type controls were maintained on a normal diet or kept without food for 48 h before analyses, with free access to drinking water. Before analyses, mice were anaesthetized and perfused through the left ventricle with 4% paraformaldehyde in PBS. Organs were collected and further fixed with the same fixative for 4 h, followed by treatment with 15% sucrose in PBS for 4 h at room temperature and then with 30% sucrose solution overnight at 4 °C. Tissue samples were embedded in OCT (Sakura Finetek Europe B.V.) and stored at −70 °C. The samples were sectioned (5 μm), and stained with Alexa-Fluor-488-conjugated anti-GFP antibody (Molecular Probes) overnight at 4 °C. Samples were mounted using ProLong Gold antifade reagent with DAPI.

Quantification of autophagic activity in thymic stromal cells. Thymi from two-week-old GFP–LC3 mice were cut into small pieces and digested at 37 °C in IMDM containing 0.2 mg ml^{-1} collagenase (Roche), 0.2 mg ml^{-1} dispase I (Roche), 2% FCS, 25 mM HEPES (pH 7.2) and 25 μg ml^{-1} DNase I, followed by incubation in 5 mM EDTA for 5 min. Cells were washed and resuspended in Percoll (GE Healthcare), followed by a layer of Percoll and PBS as the upper phase. Gradients were spun for 30 min at 1,350*g* in the cold, and low density cells were collected from the upper interface, washed and then stained for FACS sorting. Stromal cells were sorted according to CD45, Ly51, EpCAM, CD80 and CD11c expression (cTECs: CD45$^-$Ly51$^+$EpCAM$^+$; mTEC^{lo}: CD45$^-$Ly51$^-$EpCAM$^+$CD80^{lo}; mTEC^{hi}: CD45$^-$Ly51$^-$EpCAM$^+$CD80^{hi}; dendritic cells: CD45$^+$CD11c$^+$) and fixed on to poly-L-lysine-coated slides (Sigma Aldrich) with ice cold acetone. Cells were permeabilized in 0.1% (v/v) Tween in PBS for 10 min, and blocked with 10% (v/v) FCS in PBS for 30 min at room temperature. Cells were incubated overnight at 4 °C with Alexa-Fluor-488-conjugated anti-GFP antibody and mounted with ProLong Gold antifade reagent with DAPI. A total of 1,000 dendritic cells, 1,845 cTECs, 1,232 mTEC^{hi} and 245 mTEC^{lo} have been analysed for determination of the percentage of autophagy-positive cells. Cells with more than five autophagosomes (that is, GFP-positive punctae between 0.5 and 1.5 μm in diameter) were scored as positive. For quantification of the number of autophagosomes per cell, a total of 81 autophagy-positive cTECs and 51 autophagy positive mTEC^{hi} have been recorded and analysed. Cells were analysed with an inverted confocal LSM510 Zeiss microscope by taking *Z* stacks of 0.5 μm thickness and subsequently the number of autophagosomes in the whole cell was determined.

Purification and adoptive transfer of T cells. Pooled cell suspensions of spleen and lymph nodes (mesenteric, inguinal, axillary, brachial, superficial cervical, deep cervical and lumbar) from nu/nu$^{Atg5-/-}$ and nu/nu^{WT} chimaeras were subjected to erythrocyte lysis. Cells were then incubated with Fc-receptor-blocking antibody (2.4G2) and were stained with biotin-anti-CD90.2 (30-H-12). After incubation with streptavidin microbeads (Miltenyi Biotec), CD90$^+$ cells were positively selected on midi-MACS columns. 1×10^7 CD90$^+$ cells were injected in a volume of 200 μl in PBS into the lateral tail veins of BALB/c$^{nu/nu}$ animals. Animals were analysed 6–12 weeks after transfer.

Thymus transplantation. Embryonic thymi (E15; with or without deoxyguanosine treatment) were transplanted under the kidney capsule of female animals. *Atg5*$^{-/-}$ embryos and *Atg5*$^{+/+}$ (wild type) controls in individual experiments were obtained from the same *Atg5*$^{+/-}$ pregnant female after mating to a *Atg5*$^{+/-}$ male.

Deoxyguanosine treatment. E15 thymic lobes were placed on 0.45 mm membrane filters (Milipore) supported by Gelfoam (Pharmacia&Upjohn) and were incubated for 5 days with 10% (v/v) IMDM supplemented with 1.35 mM 2-deoxyguanosine before transplantation.

Bone marrow chimaeras. For mixed bone marrow chimaeras, T-cell-depleted TCR-HA *Rag2*$^{-/-}$ CD45.1$^{+/+}$ and DO11.10 *Rag2*$^{-/-}$ CD45.1$^{+/-}$ bone marrow were mixed at a ratio of 1:1 and a total of 1×10^7 cells were injected into the lateral tail vein of irradiated BALB/c recipients (2×450 rad). Animals were transplanted with *Atg5*$^{-/-}$ or *Atg5*$^{+/+}$ (wild-type) lobes 4 weeks after bone marrow reconstitution. Grafts were analysed 6 weeks after transplantation.

Fetal liver chimaeras. Fetal livers were harvested from E15 embryos. A total of 5.5×10^6 fetal liver cells were injected into the lateral tail veins of irradiated (2×550 rad) CD45.1 C57BL/6 recipients. Chimaeras were analysed 12 weeks after reconstitution.

Semiquantitative RT–PCR. mTECs were isolated from 4–5 transplanted thymi as described above and total RNA was isolated using the high pure RNA isolation kit (Roche). RNA was reverse transcribed using the iScript RT kit (Biorad). PCR reactions were carried out in a final volume of 25 μl using the Advantage 2 polymerase mix (Clontech) and Advantage 2 PCR buffer SA (Clontech) according to the manufacturers' recommendations. dNTPs (Fermentas) were used at a final concentration of 200 μM and primers were used each at 400 nM final concentration.

Primers were *Aire* forward: 5′-GAGTCACAGCACCTTCCTCTT-3′ (438 base pairs (bp)); *Aire* reverse: 5′-GGGACAGCCGTCACAACA-3′ (438 bp); *Spt1* forward: 5′-TGAAACTCAGGCAGATAG-3′ (383 bp); *Spt1* reverse: 5′-GAGGGAG AATAGTCAGGAT-3′ (383 bp); *Tff3* forward: 5′-CTGGCTAATGCTGTTGG TG-3′ (377 bp); *Tff3* reverse: 5′-TGTTGGCTGTGAGGTCTTT-3′ (377 bp); *Csn2* forward: 5′-TCATCCTCGCCTGCCTTGT-3′ (401 bp); *Csn2* reverse: 5′-GCGGAGCACAGTTTCAGAGTT-3′ (401 bp); *Actb* forward: 5′-GGTGGG AATGGGTCAGA-3′ (380 bp); *Actb* reverse: 5′-GAGCATAGCCCTCGTAG AT-3′ (380 bp).

All amplicons span at least one intron. Cycling conditions were as follows: 94 °C for 3 min; ×31 (*Actb*), ×34 (*Aire*), ×36 (*Csn2*/*Tff3*), ×38 (*Spt1*) 94 °C for 20 s, 56 °C for 30 s, 72 °C for 30 s; 72 °C for 5 min. Samples were run on a 1.5% (w/v) agarose and ethidium bromide gel in TAE buffer.

Histopathology. Organs were collected from donor animals and immersion-fixed with either 4% paraformaldehyde overnight at 4 °C or in an IHC zinc fixative (Becton Dickinson) for 48 h at room temperature. Samples were embedded in paraffin blocks. Sections (3–5 μm thick) were stained with haematoxylin and eosin and automatically scanned using the Zeiss MIRAX SCAN system. Subsequently, sections were analysed 'blind' using the Zeiss MIRAX VIEWER software.

Statistical analyses. All statistical analyses were performed using the two tailed Student's *t*-test with unequal variance.

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Supplementary Figure 1

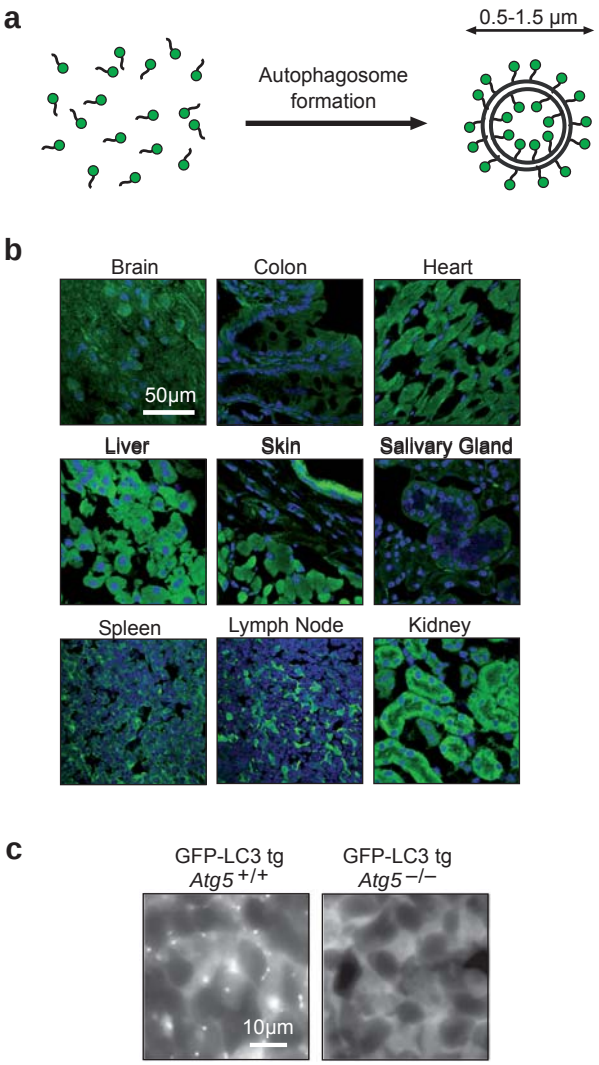


Figure S1. Constitutive autophagy is low to undetectable in a large panel of organs. a, Schematic of how GFP-LC3 fusion proteins are incorporated into autophagosomes and can be used to monitor autophagic activity. *b*, Tissue-sections of organs from normally fed GFP-LC3 mice were assessed for autophagic activity by staining with anti-GFP Alexa 488 antibody (green) and DAPI (blue). Note that the diffuse cytoplasmic green fluorescence in all organs is caused by ubiquitous expression of GFP-LC3, whereas GFP+ autophagosomes, i.e. punctate structures of 0.5-1.5 µm in diameter, are largely absent. Data are representative of n ≥ 3. **c**, Thymus sections (E15.5) of GFP-LC3 mice on Atg5+/+ (WT) or Atg5–/– background were assessed for the presence of GFP+ puncta as described above. Genetic interference with the enzymatic cascade leading to autophagosome formation abrogates formation of green puncta, indicating that these structures are true autophagosomes and not unspecific aggregates.

Supplementary Figure 2

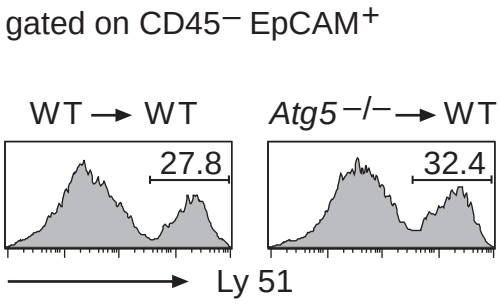


Figure S2. Normal ratio of mTEC and cTEC in Atg5 deficient thymic grafts. H-2k Atg5–/– or Atg5+/+ (WT) E15 lobes were grafted into mice expressing the I-Ak restricted AND T cell receptor. Note that in these experiments, we used AND TCR-transgenic hosts rather than WT hosts in order to exclude distortions in the cTEC / mTEC ratio eventually caused as a secondary effect of the reduced organ size in WT hosts. Eight weeks after grafting, lobes were harvested and TEC-enriched cell suspensions were prepared by digestion with collagenase / dispase follwed by density fractionation. Cells were stained with antibodies to CD45 (pan-hematopoietic), EpCAM (G8.8; pan-epithelial) and Ly51 (cTEC). Histograms show expression of Ly51 on gated CD45–EpCAM+ cells (total TEC). Numbers in plots indicate the frequency of cTEC among total TEC.

Supplementary Figure 3

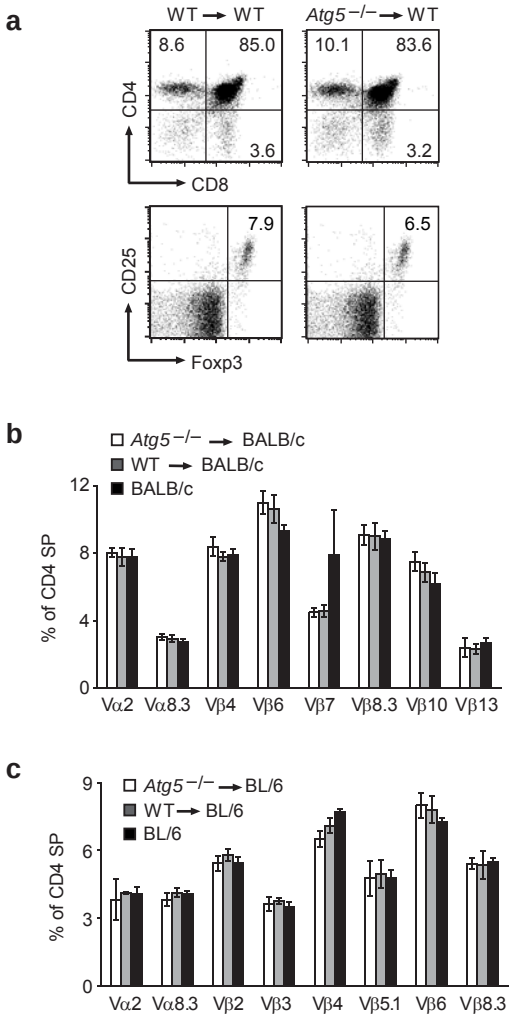


Figure S3. Polyclonal T cell selection by H-2b and H-2d Atg5 deficient thymic epithelium. Thymus lobes from Atg5+/+ (WT) or Atg5–/– E15 embryos on H-2b background were transplanted under the kidney capsule of H-2b WT recipients. **a**, FACS-analysis of T cell development in Atg5–/– → WT or Atg5+/+ (WT) → WT grafts. Upper dot plots show CD4 versus CD8 staining (representative of n = 14 and n = 8, respectively), lower dot plots show expression of the regulatory T cell markers CD25 and Foxp3 (intracellular staining) on gated CD4 single-positive thymocytes (representative of n = 5 each). **b,c** Comparison of TCR V-region usage by CD4 SP cells from Atg5–/– → WT (white bars), Atg5+/+ (WT) → WT grafts (grey bars) and normal WT thymi on H-2d (**b**) or H-2b (**c**) background. Data are representative of 3 lobes analyzed individually for each genotype.

Supplementary Figure 4

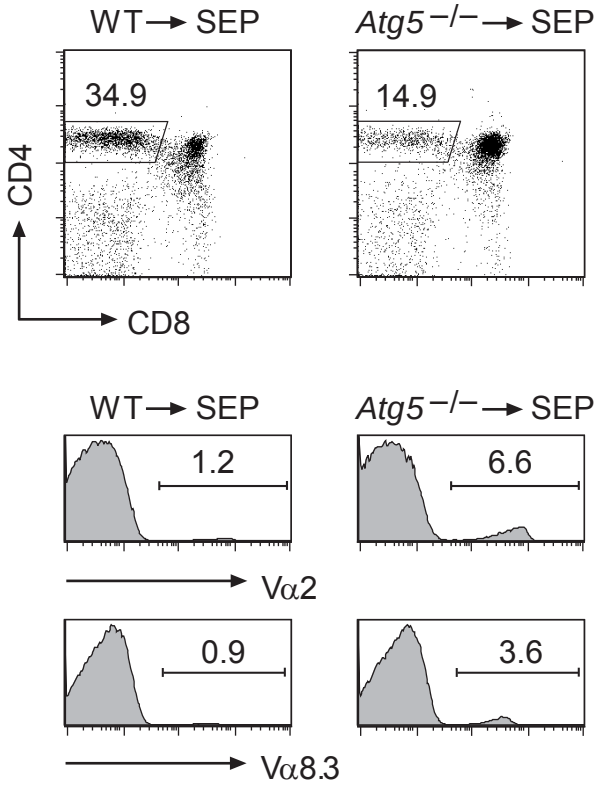


Figure S4. Impaired selection of SEP TCR transgenic T cells by Atg5 deficient thymic epithelium. Thymus lobes from Atg5+/+ (WT) or Atg5–/– E15 embryos on H-2b background were transplanted under the kidney capsule of H-2b SEP TCR-transgenic recipients (Vβ8.3 / Vα4). The dot plots show CD4 versus CD8 staining of thymocytes. Lacking a Vα4 specific staining reagent, we assessed the expression of endogenously rearranged α-chains as an indirect measure of the selection efficiency in this system. The histograms show expression of endogenously rearranged TCR α-chains (Vα2 and Vα8.3) on gated CD4 SP cells. The frequency of cells expressing individual endogenously rearranged α-chains varied widely between individual Atg5–/– grafts, but consistently was substantially higher than in WT lobes. Data are representative of n ≥ 6.

Supplementary Figure 5

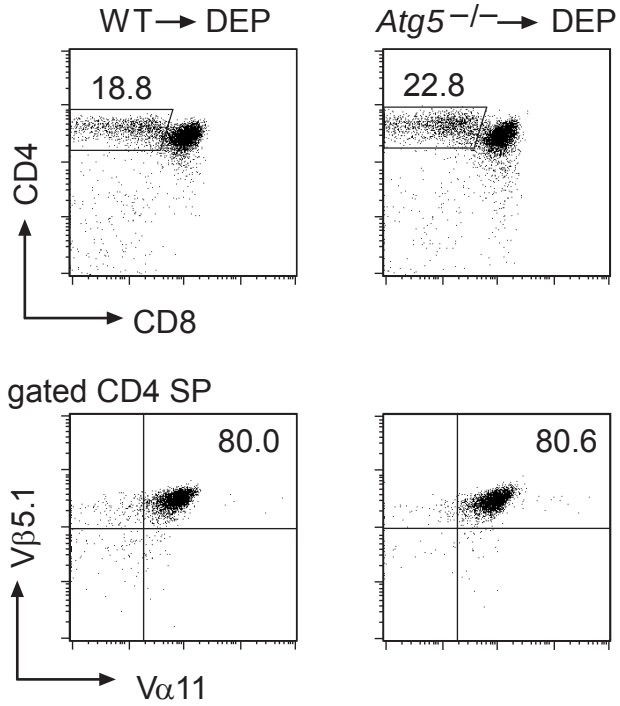


Figure S5. Normal selection of DEP TCR transgenic T cells by Atg5 deficient thymic epithelium. Thymic lobes from Atg5+/+ (WT) or Atg5–/– E15 embryos on H-2b background were transplanted under the kidney capsule of H-2b DEP TCR-transgenic recipients (Vβ5.1/ Vα11). The upper dot plots show CD4 versus CD8 staining of thymocytes. The lower dot plots show expression of the transgenic TCR V-segments on gated CD4 SP cells. Data are representative of n = 5 each.

Supplementary Figure 6

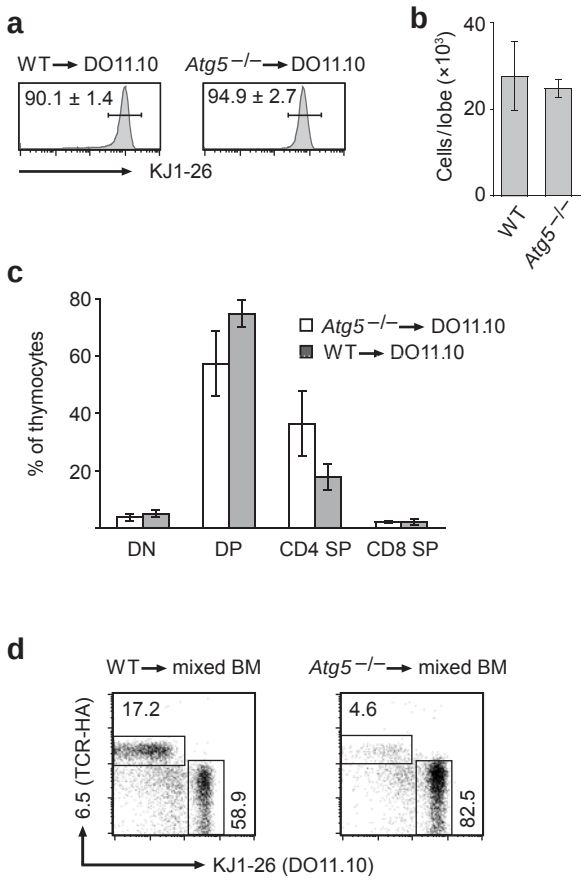


Figure S6. Enhanced selection of DO11.10 TCR transgenic T cells in the absence of Atg5 in TEC. Thymus lobes from Atg5+/+ (WT) or Atg5–/– E15 embryos on H-2d background were transplanted under the kidney capsule of DO11.10 TCR-transgenic recipients. (n = 5 for Atg5–/– → DO11.10 and n = 9 for Atg5+/+ (WT) → DO11.10 grafts). **a**, Expression of the DO11.10 TCR on gated CD4 SP thymocytes as assessed by staining with the clonotypic antibody KJ1-26. Numbers indicate the frequency ± SD of KJ1-26+ CD4 SP cells (P = 0.008). **b**, Cellularity of Atg5–/– → DO11.10 and Atg5+/+ (WT) → DO11.10 grafts. **c**, Frequency of thymocyte subsets. Note the significantly increased frequency of CD4 SP cells (P = 0.001) at the expense of the DP compartment (P = 0.002) in Atg5–/– → DO11.10 grafts. **d**, Competitive development of TCR-HA and DO11.10 thymocytes in Atg5–/– or Atg5+/+ (WT) lobes. BALB/c mice were lethally irradiated and reconstituted with a 1 : 1 mixture of bone marrow cells from TCR-HA rag2–/– and DO11.10 rag2–/– mice. These chimeras then received grafts of Atg5–/– or Atg5+/+ (WT) lobes under the left or right kidney capsule, respectively. The dot plots show expression of the two transgenic TCRs on gated CD4 SP thymocytes. Data are representative of three chimeras.

Supplementary Figure 7

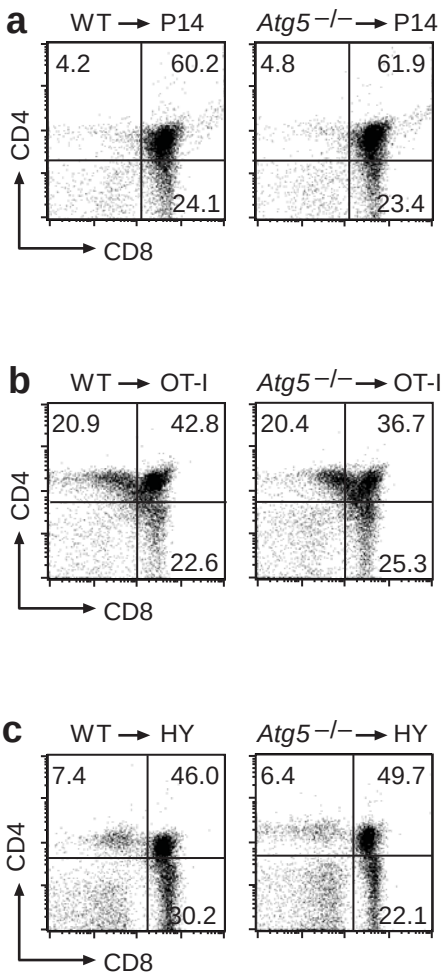


Figure S7. Unaltered selection of MHC class I restricted transgenic TCRs by Atg5–/– TEC. Thymus lobes from Atg5+/+ (WT) or Atg5–/– E15 embryos on H-2b background were transplanted under the kidney capsule of (**a**) P14, (**b**) OT-I or (**c**) HY TCR-transgenic recipients. The dot plots show CD4 versus CD8 staining of thymocytes.

Supplementary Figure 8

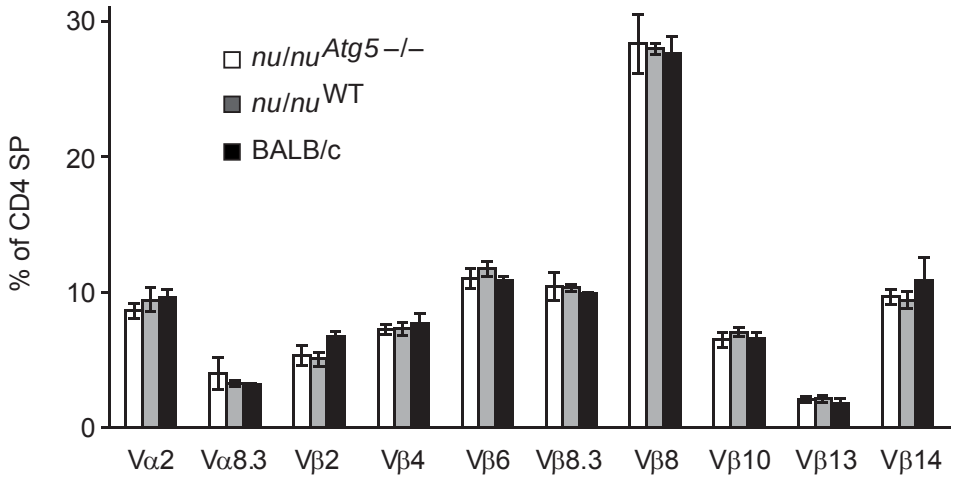


Figure S8. V-region usage by lymph node CD4+ cells from Atg5–/– → nu/nu or Atg5+/+ → nu/nu chimeras. Comparison of TCR V-region usage by lymph node CD4+ cells from Atg5–/– → nu/nu (white bars), Atg5+/+ (WT) → nu/nu grafts (grey bars) and normal WT (BALB/c) thymi on H-2d background as assessed by flow cytometric analysis eight to twelve weeks after grafting (n = 6 for each genotype).

Supplementary Figure 9

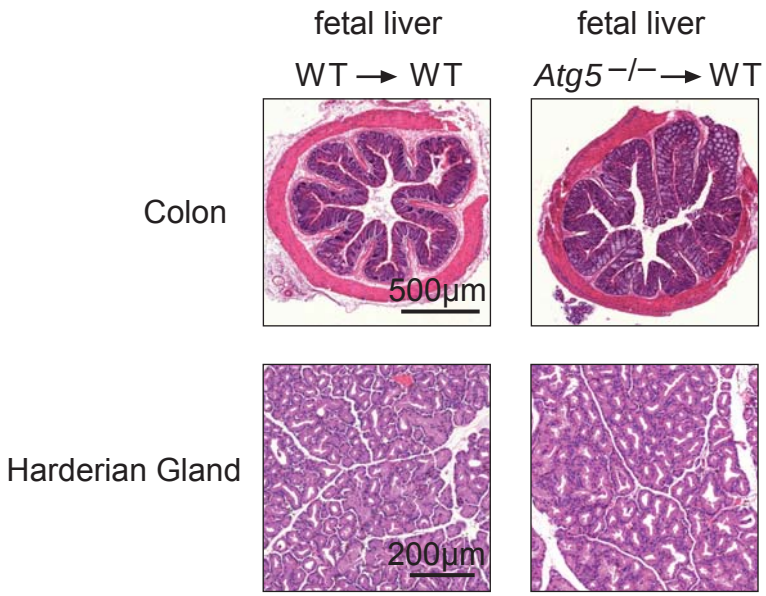


Figure S9. Atg5 deficiency in hematopoietic cells does not cause autoimmunity. Lethally irradiated (2 x 450 rad) WT animals were reconstituted with Atg5–/– or Atg5+/+ (WT) fetal liver cells. These chimeras did not show any signs of autoimmunity twelve weeks after reconstitution. Representative histological sections (H&E) of colon and harderian gland are shown (n = 3 each).

Supplementary Figure 10

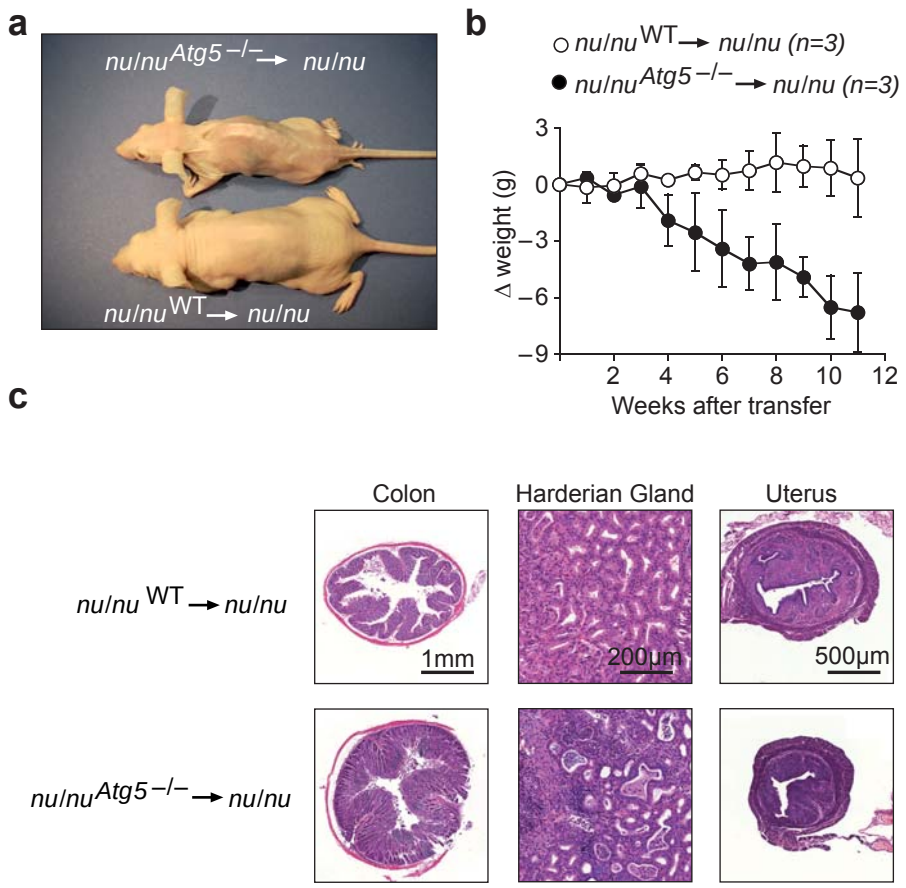


Figure S10. Adoptive transfer of purified T cells from Atg5–/– → nu/nu chimeras recapitulates autoimmunity. a, 1 × 10^7 purified CD90+ T cells from nu/nuAtg5–/– or nu/nuWT chimeras eight weeks after thymus grafting were adoptively transferred into nu/nu recipients. Depicted is the typical external appearance of recipients ten weeks after T cell transfer (representative of two independent experiments, n = 5). **b**, Changes in body weight (Δ weight) were monitored up to eleven weeks. **c**, H & E staining of sections of representative organs from recipients of nu/nu$^{Atg5–/–}$ or nu/nu^{WT} T cells.

Supplementary Figure 11

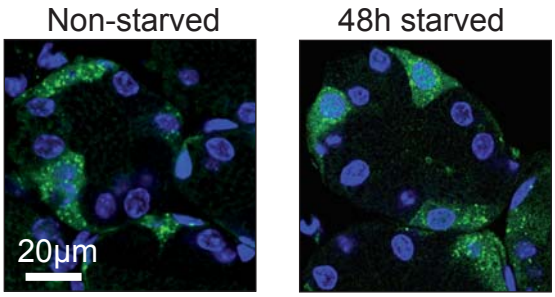


Figure S11. Constitutive autophagy in the Harderian gland. Tissue-sections of the Harderian gland of normally fed (non-starved) GFP-LC3 mice and GFP-LC3 mice that had been deprived of food for 48 hours (starved) were stained with anti-GFP Alexa 488 antibody (green) and DAPI (blue) to visualize autophagic activity.

Supplementary Table 1

MHC II restricted TCR tansgenes

	Restriction Element	Organ Size	Positive Selection
TCR-HA	I-E^d	↓	↓
DO11.10	I-A^d	=	↑
AND	I-E^k	=	↑
SEP	I-A^b	↓	↓
DEP	I-A^b	=	=

MHC I restricted TCR tansgenes

	Restriction Element	Organ Size	Positive Selection
P14	D^b	=	=
OT-I	K^b	=	=
HY	D^b	=	=

Table S1. Selection of transgenic TCRs in *Atg5*$^{-/-}$ thymi as compared to WT controls. Positive Selection: ↑ and ↓ indicate a higher or lower frequency of clonotype positive CD4 or CD8 SP cells, respectively. = indicates that the frequency of clonotype positive SP cells was similar in *Atg5*$^{-/-}$ thymi and WT controls.

Organ Size: ↓and = indicate lower or equal cellularity, respectively, of *Atg5*$^{-/-}$ thymi as compared to WT controls.

Supplementary Table 2

	nu/nu ^{WT}	*nu/nu* $^{Atg5-/-}$
Colon	0 / 16	13 / 15
Harderian Gland	5* / 16	12 / 16
Uterus	2** / 11	10 / 12
Liver	4 / 16	9 / 16
Lung	4 / 16	9 / 15

Table S2. Infiltrates in *nu/nu*^{WT} or *nu/nu*$^{Atg5-/-}$ chimeras. * indicates a mild infiltration without histopathological changes of the Harderian gland (as opposed to manifest signs of tissue destruction in *nu/nu*$^{Atg5-/-}$ chimeras). ** indicates focal accumulation of mononuclear cells around the Fallopian tube in the absence of thinning of the organ (as opposed to infiltration throughout the organ in *nu/nu*$^{Atg5-/-}$ chimeras).

3. DISCUSSION

3.1 High constitutive level of autophagy in thymic epithelium

For a long time autophagy has been recognized as a basic cellular response to starvation, with a main role in sustaining the energy status of the cell during stressful conditions[157, 209]. Typically, the basal level of autophagy is low (Supplementary Figure 1b), but the process gets up-regulated upon food withdrawal in order to sustain metabolic homeostasis ("induced" autophagy) (Figure 1a-lower panel). However, here we presented data arguing that the process of autophagy is differentially regulated among several organs and cell types in mice, as basal autophagy levels as well as cell response to starvation treatment widely differs (Figure 1, Supplementary Figure 1b). These findings are in agreement with previously published studies of Ohsumi and colleagues[161].

Extensive *in vivo* analyses of autophagy reporter mice (GFP-LC3) have documented a high constitutive level of autophagy in the thymus, even under nutrient-rich conditions, a pattern that has been observed in only two more cell types: podocytes and lens epithelial cells[161]. Here, we identified a new cell type showing the similar pattern of autophagic activity, namely the subset of cells in the Harderian gland (Supplementary Figure 11). Interestingly, the basal level of autophagy in the thymus is higher in neonates than in adult thymi[161]. However, it remains to be answered if up-regulation of autophagy during the neonatal period in the thymus is a result of a physiological starvation that happens in mammals shortly after birth when nutrient supply via placenta is suddenly terminated[189], or if it is related to the specific immunological function of the organ.

In order to precisely assign autophagic activity to a particular population of thymic stromal cells we performed a detailed quantification of autophagy on sorted cells (purity ≥98%). We documented that the highest autophagic activity is confined to the cTEC subset with ~70% of cells being autophagy positive. Autophagy positive cTEC contained an average of about 35 autophagosomes per cell (Figure 1c), which corresponds to approximately 1-2% of total cytosolic volume being engulfed in

autophagosomes in the steady state. In mTEC^{lo} autophagic activity has not been detected, while ~10% of the mTEC^{hi} population scored positive for autophagy. Interestingly, high autophagic activity co-segregated with a subpopulation of mTEC that has optimal antigen presentation capacity: high MHC class II and CD80 expression level, and at the same time expresses a wide array of TSA[48,52, 53]. The more detailed characterization of autophagy positive mTEC^{hi} subset will be the subject of our future investigation. One of the most interesting tasks would be to verify if autophagy positive cells are indeed Aire^{+} mTEC. Unlike TEC, less than 1% of thymic DC scored positive for autophagic activity by our monitoring assay (Figure 1b).

Our data are in a good agreement with immunofluorescence analyses of thymic sections from paraformaldehyde perfused autophagy reporter mice. Staining with epithelial markers revealed co-segregation of autophagosomes with cytokeratin positive cells in the cortex and medulla[161]. Importantly, perfusion with paraformaldehyde prevents the artificial induction of autophagy during the experimental procedure which excludes the possibility that observed high numbers of autophagosomes in *ex vivo* isolated TEC is a consequence of autophagy up-regulation during the isolation procedure of thymic epithelium.

Autophagy is not commonly up-regulated in different epithelial tissues, as we have not observed significant autophagic activity in epithelial cells in other organs, i.e., skin, salivary gland, colon. Furthermore, it appears not to be a general characteristic of lymphoid organs, as lymph node and spleen scored negative for autophagy (Supplementary Figure 1b).

The caveat of the autophagy monitoring system that relies on posttranslational modifications of LC3 protein is that it allows for visualization of autophagosomes and amphisomes, while the final maturation step – formation of autophagolysosomes cannot be observed due to degradation of luminal portion of LC3 and covalent decoupling of LC3 from the outer autophagolysosomal membrane[210]. Therefore, the accumulation of autophagosomes in the steady state in GFP-LC3 reporter animals can indicate (i) high numbers of newly forming vesicles implying high level of autophagy in the cell, (ii) blockage in the fusion with lysosomes which would reflect

accumulation of immature autophagosomes or (iii) the inhibition of lysosomal degradation of autophagic cargo which would lead to accumulation of late autophagic vacuoles. The most accurate method to monitor autophagic activity in the cell is immunoelectron microscopy[207]. The method allows for unambiguous delineation of autophagosomes, which are characterized by the presence of a bilayered membrane, and autophagolysosomes, which are defined as single membrane vesicles with electron dense material. Furthermore, on the basis of electron density of autophagolysosomes one can estimate the activity of lysosomal proteases. Electron microscopy of thymic epithelium has shown numerous proteolyticaly active autophagolysosomes in TEC[161], an observation that allows us to interpret the high numbers of autophagosomes in TEC from GFP-LC3 reporter mice as a sign of high constitutive autophagic activity.

There are reports stating that over-expression of LC3 protein can lead to the formation of nonspecific aggregates which can be falsely interpreted as newly forming autophagosomes[211, 212]. To check for this possibility, we bred GFP-LC3 mice on an autophagy deficient background and analyzed the formation of autophagosomes in neonates. In GFP-LC3 Atg5$^{-/-}$ mice only the diffuse cytoplasmic staining has been observed (Supplementary Figure 1c), confirming that green dots observed in thymic epithelium are indeed autophagosomes, and not unspecific protein aggregates.

As already mentioned, there are three homologues of the yeast Atg8 protein in mammals: LC3, GATA16 and GABARAP. Upon induction of autophagy all mammalian Atg8 homologues pass through the same posttranslational modification steps that ultimately lead to covalent coupling of proteins to the lipids in autophagosomal membranes[181]. Histological analyses of GFP-GATA16 and GFP-GABARAP reporter mice have also identified the thymus as a rather unique organ displaying high constitutive level of autophagy irrespective of nutrient supply (I.Tanida and E.Kominami, unpublished results). These preliminary analyses of new autophagy reporter mice further support the notion that high constitutive autophagy is a physiological characteristic of the thymic epithelium.

The observed pattern of autophagic activity begs the question what the role of autophagy in TEC is. In the following sections, I will discuss our work that strongly supports the hypothesis that autophagy in TEC contributes to the generation of MHC class II epitopes derived from intracellular proteins and in that way contributes to positive selection and tolerance induction of developing T cell repertoire.

3.2 Autophagy deficient thymic epithelium - basic characterization

As thymic epithelial cells display high constitutive autophagic activity, we reasoned that genetic interference with autophagy in TEC might cause alterations in basic cellular functions. However, a number of different parameters reflecting TEC organization and function are unaffected in Atg5$^{-/-}$ grafts. Thus, immunofluorescence analyses of thymic grafts documented a proper formation of cortical and medullary compartments (Figure 2b), and FACS analyses further confirmed that percentages of cTEC and mTEC in Atg5$^{-/-}$ transplants are similar to WT (Supplementary Figure 2). MTEC properly segregate into already described mTEC^{lo} and mTEC^{hi} subpopulations (Figure2c-lower panel). Furthermore, promiscuous expression of Aire-dependent and Aire-independent tissue specific antigens in mTEC (Figure 2d) as well as expression of MHC class I and II molecules in Atg5$^{-/-}$ cTEC and mTEC is intact (Figure 2c). It is, however, worth mentioning that we observed a slight, but reproducible, decrease in MHC class II expression and quite opposite slightly enhanced expression of MHC class I molecules on Atg5$^{-/-}$ cTEC. The effects of autophagy on MHC loading and expression will be further discussed in the following sections.

Recent studies have implicated autophagy as an important intracellular protein quality control mechanism, as autophagy deficient hepatocytes[190] and neuronal cells[213,191] accumulate ubiquitinylated protein aggregates, which ultimately leads to impaired cellular function and toxicity. The molecular mechanism of such selective targeting of proteins for autophagic degradation is currently unknown, although several proteins, such as p62/SQSTM1 and Alfy, have been shown to play important roles in targeting ubiquitinylated aggregates for autophagosomal degradation[167, 168, 214]. It has been proposed that autophagic clearance of intracellular proteins might be especially important in post-mitotic or slowly dividing cells as they do not have the luxury of diluting toxic aggregates in the course of cell growth and division.

Additionally, expression of aggregation prone proteins may be elevated in hepatocytes and neuronal cells. However, the histological inspection of thymi from Atg5$^{-/-}$ neonates revealed that TEC do not accumulate ubiquitinylated proteins and inclusion bodies (N.Mizushima, personal communications). Therefore it appears that in TEC ubiquitinylated intracellular proteins are more efficiently targeted for proteasomal degradation and/or proteolysis by other cytosolic proteases involved in processing of intracellular proteins, which in turn can compensate for a deficiency in autophagic clearance of protein aggregates. The similar observation has been made in lens epithelial cells, which like TEC, display high level of autophagy. Additionally, dramatic degradation of organelles and cellular remodeling occurs during the process of lens differentiation. However, autophagy, even though constitutively active at the high level, is not essential for the elimination of organelles or ubiquitinylated proteins during the development of lens fibers[215]. Hence, one can conclude that different cell types have adapted autophagic process to fulfill diverse physiological tasks, and that proteolytic dependence on the autophagy does not show the same pattern among tissues.

Autophagy has been shown to act as either (i) a cell survival mechanism or as (ii) a cell death pathway (type II programmed cell death) depending on cell types and/or environmental conditions[216,277,278]. To this end we were able to show that the transplanted Atg5$^{-/-}$ grafts survive as long as WT grafts when transplanted under the kidney capsule of WT animals, whereby the survival time was monitored for 5 months. A way to address the putative role of autophagy in survival of TEC would be the inspection of apoptotic markers in Atg5$^{-/-}$ thymic grafts. However, studies of apoptosis with *ex vivo* isolated TEC are very difficult because of the induction of high levels of Annexin V and caspase activation as a consequence of the complicated TEC isolation procedure. Another approach to characterize cell death rate of TEC in the absence of autophagy would be to perform *in situ* TUNNEL assay analyses and quantify the number of dying TEC using immunofluorescence methodology. This fine delineation of effects of autophagy on the physiology of TEC will be the subject of future investigations.

Taken together, our findings suggest that the viability and development of TEC is not globally affected by the absence of Atg5. Interestingly, similarly to TEC, interference

with autophagy in DC does not impair their viability or the general antigen presentation capacity, i.e., MHC class I or II expression, but it is indispensable for (i) innate recognition of viral particles and IFNα response in plasmacytoid DC (pDC) in mice and (ii) it contributes to endogenous antigen loading of intracellular pathogen-derived and model antigens in human monocyte-derived immature and mature DC[196,201, 206]. Thus, it seems that autophagy arms professional APC to better perform their immunity-related functions while at the same time house-keeping roles of autophagy in these cell types can be substituted by other catabolic pathways.

3.3 Autophagy and Positive selection

3.3.1 Diversity of MHCp complexes and its role in positive selection of T cells – an overview

Peptides are an essential part of MHC complexes and are required for stable expression of both MHC class I and class II molecules. Substantial insight regarding the contribution of a diverse repertoire of peptides on MHC molecules expressed on thymic stromal cells in selecting T cells has been gained during the early 90-ies. Three different theories have been put forward to describe the role of MHC bound peptide epitopes in TCR/MHCp interactions[23]. In the first scenario peptides play just a structural role by stabilizing MHC complexes and thereby conferring a proper conformation for interactions with TCR. The other two theories recognize the role of TCR interactions with side chains of peptides bound to the MHC, but differ in respect to the specificity of this interaction. While one of the models implies that the interaction of TCR with MHC bound peptides is degenerate, i.e., one MHCp complex would be able to select millions of different TCR specificities, the second model proposes that those interactions are highly specific and further suggest that diversity of peptide epitopes bound to MHC molecules is essential for the selection of a polyclonal T cell repertoire[23,217,218].

The first direct experimental evidence for the role of peptides in T cell selection came with a wave of studies on CD8, and subsequently CD4 T cell selection. The elucidation of mechanisms involved in antigen processing and loading to MHC class I and II molecules, made it possible to genetically modulate essential players

responsible for the generation of a diverse repertoire of MHC bound peptides. Using TAP ($TAP^{-/-}$) and β2-microglobulin ($\beta 2m^{-/-}$) deficient animals, several research laboratories set out to study the role of peptides in selection of the CD8 T cell repertoire[89,219,220]. $TAP^{-/-}$ and $\beta 2m^{-/-}$ animals have a very low surface expression of MHC class I molecules due to (i) an inefficiency in peptide shuttling to the ER where the MHC class I peptide loading takes place ($TAP^{-/-}$) or (ii) defects in MHC class I complex assembly caused by the lack of the light chain molecule ($\beta 2m^{-/-}$). The number of CD8 T cells is greatly reduced both in the thymus and periphery of those animals, but the respective influence of low MHC class I expression level versus diminished diversity of MHCI/p complexes on the selection of CD8 T cells could not be delineated in these *in vivo* settings. However, in the fetal thymic organ cultures (FTOC) MHC class I expression in $TAP^{-/-}$ or $\beta 2m^{-/-}$ system can be restored upon exogenous adding of peptides or β2-microglobulin and peptides, respectively. Furthermore, in FTOC with $TAP^{-/-}$ or $\beta 2m^{-/-}$ one can experimentally modulate the diversity of peptides added to the system and evaluate the contribution of peptide diversity to CD8 T cell selection. These experiments revealed that polyclonal CD8 T cell development is more efficient on a diverse mixture of MHCI/p complexes and implied that a plethora of MHCI/p complexes is required for development of diverse repertoire of CD8 T cells[221,222]. Furthermore, studies on the selection of particular TCR specificities revealed that only peptides related to the natural antigenic peptide of a respective TCR can induce positive selection, while unrelated peptides are not able to do so[223-224]. These findings talk in favor of a scenario where specific TCR/MHCp interactions are taking place during the process of positive selection and outline the importance of diversity of MHC bound peptides in selection of a full, polyclonal T cell repertoire.

It was also in the early 90-ies that several groups have generated invariant chain (Ii) deficient animals ($Ii^{-/-}$)[109,110,225]. $Ii^{-/-}$ animals have a 5-7 fold reduced expression of surface MHC class II and reduced numbers of CD4 T cells in the thymus and peripheral lymphoid organs. Due to the impairment in MHC II antigen loading and the structural instability of class II complex in this system, APC in $Ii^{-/-}$ mouse express either empty MHC II molecules or MHC II molecules weakly bound to the peptide. Analyses of selection of particular TCR specificities on $Ii^{-/-}$ background has shown a developmental block at the DP stage of only some TCR specificities, while other TCR

specificities did not show any problems with positive selection in the absence of Ii, implying that MHCII/p diversity is essential for the selection of at least some TCRs[226]. However, as both peptide diversity and MHC II expression level in $Ii^{-/-}$ animals is severely reduced, one cannot separate the influence of decreased MHC II expression versus severely reduced peptide diversity on the observed phenotype. To circumvent this problem, the question has been addressed by generating animals that express a very limited repertoire of MHCII/p complexes, while the surface MHC II expression remains unchanged. Two mouse models have been created that fulfill these criteria, H-$2M^{-/-}$ animals and the so called "single ligand" mouse model[227, 228]. The molecular chaperone H-2M facilitates the dissociation of the CLIP peptide from the MHC class II peptide binding groove and subsequent loading of antigenic peptides. Therefore, in H-$2M^{-/-}$ animals the vast majority of MHC class II molecules are occupied with a CLIP peptide (~97%)[229]. In a "single ligand" model mice deficient for endogenous MHC II (MHC $II^{-/-}$) and invariant chain ($Ii^{-/-}$) transgenically express a class II molecule (I-$A\beta^b$) that is covalently linked to the I-$E\alpha_{(52-68)}$ peptide- AbEp. Consequently, in the AbEp mouse, virtually all MHC class II molecules are loaded with the I-$E\alpha_{(52-68)}$ peptide. Analyses of both mouse models gave essentially the same results. Those animals are able to support bulk CD4 T cell development, though the number of CD4 T cell is reduced both in the thymus and the periphery. Subsequent analyses of the CD4 T cell receptor diversity have documented the selection of a broad, polyclonal T cell repertoire with respect to TCR Vβ segment usage and sequence analyses of the complementary determining region-3 (CDR-3). However, three different TCR specificities (SMARTA, Dep and AND TCRs) have displayed a complete developmental block at the DP stage when selected on an H-$2M^{-/-}$ background. Results discussed above would imply a rather redundant role of peptides in the selection of CD4 T cells. One single MHCII/p complex would be able to support selection of a highly diverse, though not complete CD4 repertoire[230]. These findings are quite opposite to the findings on positive selection of CD8 T cells. "Single ligand" mouse models imply that positive selection of CD4 cells is a rather promiscuous process with regard to the specificity of peptide/TCR interactions, while CD8 TCR/MHCp interactions are suggested to be highly specific [224,231].

Such a redundant role of peptides in selecting a broad CD4 T cell repertoire has been challenged by more elegant analyses of positive selection in H-$2M^{-/-}$ FTOC and

findings of Barton *et al*. in another “single ligand” mouse model, where similarly to AbEp mouse, ~95% of MHC II molecules are occupied with the I-E$\alpha_{(52-68)}$ peptide[232, 233]. By using specific monoclonal antibodies in FTOC the authors blocked the interactions of developing T cells with either MHCII/CLIP or MHCII/I-Eα complex. The only MHCII/p complexes developing T cells could react with in these FTOC are remaining low abundance MHCII/p complexes. Interestingly, positive selection worked equally efficient when the interactions with a “single ligand” have been blocked, but was significantly diminished if H-2M$^{-/-}$ FTOC were treated with a monoclonal antibody that is specific for MHC II/non-CLIP epitopes. Those data argue that the positive selection in “single ligand” mouse models actually occurs on the remaining minute amounts of MHCII/p complexes that contribute to the diversity of MHC class II ligandome and support the hypothesis that a diverse repertoire of MHC II bound peptides is absolutely necessary for the development of polyclonal CD4 T cells.

3.3.2 Diversity of MHCII/p complexes and its role in positive selection of CD4 T cells – role of autophagy

We speculated that autophagy could be one of the alternative endogenous MHC class II loading pathways in TEC that shuttles intracellular proteins to MIIC. In that way, autophagy would contribute to the diversity of the MHCII/p repertoire of cTEC that is presented to developing T cells during the process of positive selection. Nevertheless, autophagy should have rather subtle consequences on the diversity of MHCII/p repertoire in comparison to “single ligand” mouse models as other antigen loading pathways are still operative in Atg5$^{-/-}$ cTEC. Therefore, one would speculate that the changes, if evident, in the diversity of the CD4 T cell repertoire would be rather subtle in autophagy deficient animals and that it would affect only particular T cell specificities which selection is mediated by autophagy-dependent MHC class II epitopes.

Experiments designed to test the efficacy of positive selection on autophagy deficient thymic epithelium revealed that a broad repertoire of T cells is selected. Developing T cells properly proceed through DN, DP, CD4SP and CD8SP stages of development and percentages of cells falling into those categories are same as in WT (Figure 3a

and Supplementary Figure 3a). Furthermore, athymic, nude animals grafted with Atg5$^{-/-}$ embryonic thymi have a fully reconstituted T cell compartment in peripheral lymphoid organs. The selected CD4 T cell repertoire appears to be broad and polyclonal, as judged by unimpaired usage of a large panel of Vβ and Vα TCR segments both in the thymus (Supplementary Figure 3b and 3c) and periphery (Supplementary Figure 8).

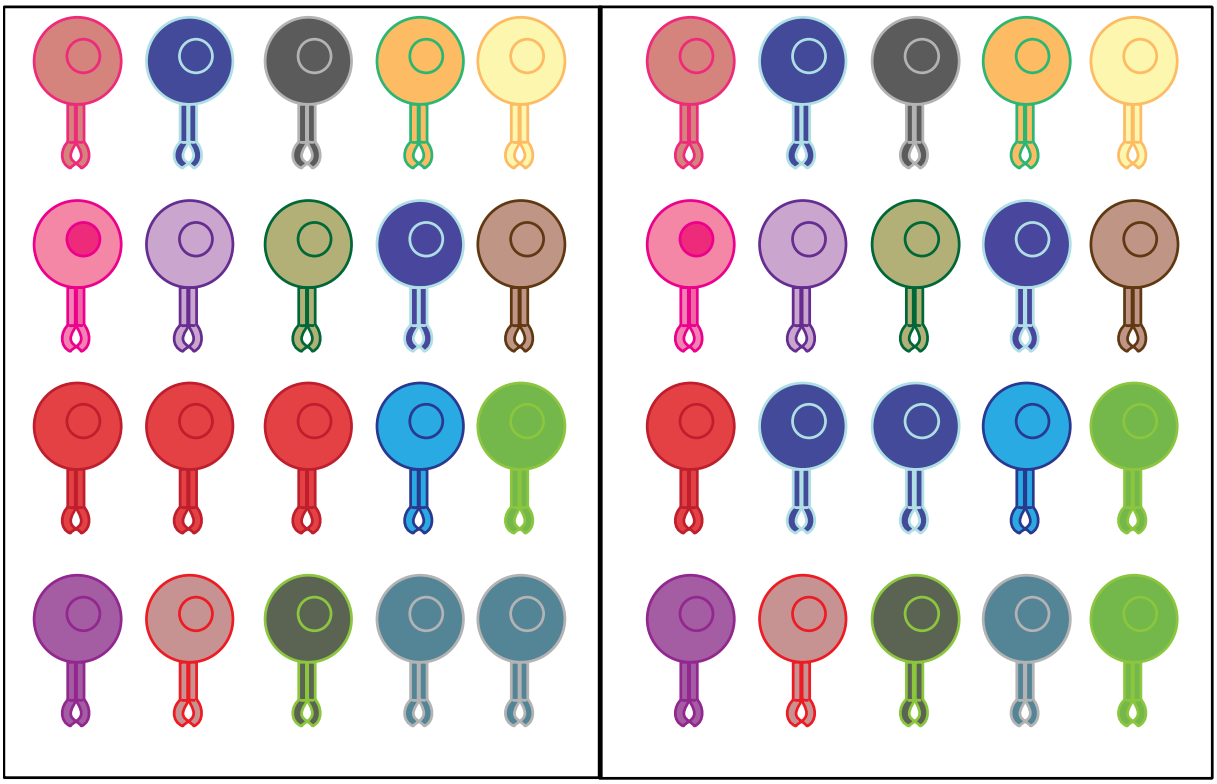
To get a closer insight into the diversity of a selected T cell repertoire we performed in depth analyses of Atg5$^{-/-}$ cTEC capacity to select different TCR specificities, by taking advantage of already established TCR transgenic mouse models. Those analyses revealed that positive selection of particular MHC class II- restricted TCRs is altered on Atg5$^{-/-}$ thymic epithelium (Supplementary Table 1). Interestingly, different T cell specificities display opposite outcomes of positive selection on Atg5$^{-/-}$ epithelium. While TCR-HA and Sep specific T cells show less efficient positive selection on Atg5$^{-/-}$ thymic epithelium, i.e., reduced numbers of CD4SP T cells expressing the transgenic TCR (TCR-HA system) and increased frequency of CD4SP with endogenously rearranged Vα TCR segments (Sep TCR system), (Figure 3b and Supplementary Figure 4), positive selection of DO11.10 TCR transgenic animals seems to be more efficient on Atg5$^{-/-}$ cTEC (Supplementary Figure 6a and 6c). The increased secondary TCRα gene rearrangements in Sep TCR transgenic animals may indicate that cells attempt to produce a receptor that can be more efficiently selected by available MHCII/p complexes and thus escape death by neglect. Efficacy of positive selection of AND and Dep TCR transgenic cells is comparable between WT and Atg5$^{-/-}$ epithelium (Figure 3c and Supplementary Figure 5). Curiously, the peptide mediating positive selection of AND TCR, gp250, has been recently identified in *in vitro* system. The gp250 peptide is derived from a sortilin family-related multifunctional endocytic receptor protein. The protein is expressed in a wide array of APC, including cTEC (Allen P., unpublished data). As an endosomal protein, gp250 can intersect with MIIC by following conventional rules of MHC class II antigen loading. Thus, loading of the gp250 peptide to the MHC class II should be independent of autophagy. Notwithstanding the fact that respective role of gp250 peptide in *in vivo* selection of AND TCR transgenic animals has not been shown, this finding captured our attention as AND TCR specificity has shown autophagy-independent positive selection outcome which stands in line with the assumption that

the ligand mediating its selection follows the classical, endocytic antigen loading route.

Experiments with mixed bone marrow chimeras where animals expressed only two TCR specificities, TCR-HA and DO11.10, recapitulated our findings from the analyses of positive selection in the single TCR transgenic system. In this "competitive" system positive selection of cells expressing DO11.10 TCR is enhanced on the account of reduced numbers of cells expressing TCR-HA in Atg5$^{-/-}$ epithelium (Supplementary Figure 6d). These findings imply competition among T cells with different specificities for selecting ligands during positive selection, which is in agreement with published data[234]. Thus, the selection of some MHC class II-restricted TCRs critically relies on autophagy-dependent epitopes (TCR-HA, Sep TCR). On the other hand, the absence of autophagy-dependent epitopes in the MHC class II ligandome may favor selection of TCRs that specifically recognize epitopes drawn from non-autophagic substrates (DO-11.10 TCR).

The finding that some MHC class II- restricted TCR transgenics can be positively selected on autophagy deficient cTEC, while the selection of TCRs with other specificities is substantially affected, shows that the general ability of Atg5$^{-/-}$ cTEC to mediate positive selection of developing T cells is not impaired. As already mentioned, we observed a slight, but reproducible, down-regulation of MHC class II expression in Atg5$^{-/-}$ cTEC. This finding may imply that the loading of peptides to the MIIC is impaired in Atg5$^{-/-}$ cTEC, as peptide binding to the MHC class II groove is essential for the stabilization and expression of MHC class II molecules on the cell surface. In contrast to described "single ligand" mouse models[233,228,229], we did not observe any differences in percentages of T cells at different developmental stages in Atg5$^{-/-}$ grafts transplanted to WT animals, and neither of the tested TCR transgenic models displayed a block in development at the DP stage. Those findings are in agreement with our proposed hypothesis that reduction of peptide diversity in autophagy deficient cTEC is not as severe as in a "single ligand" mouse models, and thus changes in efficacy of T cell positive selection are rather subtle. Nevertheless, the T cell repertoire selected on Atg5$^{-/-}$ thymic epithelium, though highly diverse, is different to the repertoire of T cells selected on WT cTEC (Graphic 6).

a. b.



Graphic 6. The role of autophagy in positive selection of CD4 T cells. In WT mice (a.) a highly diverse repertoire of MHCII/self peptide complexes enables the selection of a full, polyclonal T cell repertoire. In autophagy deficient TEC (b) the diversity of MHCII/self peptide complexes is reduced which results in the selection of an altered repertoire of CD4 T cells (i.e., reduced frequency of TCR specificities which positive selection depends on autophagy-dependent MHC class II epitopes-red cells, and increased frequency of TCR specificities that are positively selected on autophagy-independent epitopes-green and blue cells in the graphic).

The cellularity of Atg5$^{-/-}$ thymic grafts transplanted to WT mice was significantly reduced (Figure 2a). This finding was in contrast to “single ligand” mouse models, where the overall thymic cellularity did not show significant changes[227,228]. The possible explanation for the discrepancy in the results might lie in differences in the experimental set up used. We conducted our experiments using embryonic thymus transplantation, while in above listed studies straight forward analyses of animals have been done. The possible influence of experimental manipulation on the overall thymic physiology in our set up will be addressed by performing conditional knock out studies with the recently described Foxn1-Cre mouse line[235].

We reasoned that reduced cellularity of Atg5$^{-/-}$ grafts mirrors impaired positive selection of a diverse, polyclonal set of T cell specificities that critically depend on the MHC class II epitopes generated via the autophagic route. In agreement with our interpretation, efficiency of positive selection in TCR transgenic systems correlated with the size of the grafted thymi. Atg5$^{-/-}$ thymi had significantly lower cellularity in TCR transgenic mice that displayed impaired positive selection, TCR-HA and Sep TCR (Figure 3b), while there was no difference in the cellularity of Atg5$^{-/-}$ and WT lobes in the systems where positive selection worked as efficient as on WT epithelium, AND TCR, Dep TCR, DO11.10 TCR (Figure 3c, Supplementary Figure 6a). Furthermore, neither of three MHC class I- restricted TCR transgenic animals tested, P14, OT-I and H-Y TCR transgenic, showed neither a difference in the efficacy of positive selection on WT versus Atg5$^{-/-}$ epithelium nor a difference in the cellularity of transplanted lobes (Figure 3d). These data are consistent with the hypothesis that autophagy specifically molds the MHC class II, but not the MHC class I repertoire. In line with our findings, it has been convincingly shown that targeting of intracellular proteins to autophagosomes enhanced specifically CD4, but not CD8 T cell responses in *in vitro* cultures[201]. Furthermore, efficient positive selection of MHC class I- restricted TCR transgenic T cells on autophagy deficient epithelium adds another proof that there is no global effect of autophagy deficiency on the positively selecting thymic environment.

It is worth mentioning here that even though we have not observed any differences in neither bulk CD8SP T cell selection (Figure 3a) nor in the positive selection of particular MHC class I- restricted TCR specificities (Figure 3d) we deem it likely that autophagy might have an indirect role in generation of MHCI/p complexes. It is known that the availability of proteasomal substrates can critically influence whether a particular antigen is going to be presented on MHC class I[88, 236]. As already mentioned in the introduction to MHC class I antigen loading, ubiquitinylated proteins are substrates for proteasomal degradation. On the other hand, although autophagy is generally considered to be a bulk, non-selective catabolic pathway, the specific autophagic uptake of ubiquitinylated proteins and aggregates has been described[167, 168, 214]. Thus, it seems reasonable to speculate that autophagy may compete with the proteasomal degradation system for available intracellular proteins. Consequently, the shuttling of peptides into the MHC class I loading pathway might be more efficient

in autophagy deficient thymic epithelium. The slightly increased MHC class I expression level on Atg5$^{-/-}$ cTEC is in agreement with the above mentioned hypothesis (Figure 2c-upper panel). In this scenario, the influence of autophagy on the peptide repertoire presented on MHC class I molecules might be opposite to its effect on the generation of the MHCII/p repertoire. Autophagy might broaden the repertoire of epitopes presented on MHC class II and at the same time limit the diversity of the peptides presented on MHC class I molecules.

In light of experimental evidence from our and other laboratories discussed above one can conclude that a highly diverse MHCp repertoire is essential for the generation of millions of different T cell specificities[232, 233]. A T cell repertoire that is selected on cTEC displaying reduced diversity of MHCII/p complexes is altered (Graphic 6), which supports the proposed theory that during the process of positive selection highly specific interactions between TCR and MHCp complexes are taking place. However, it still remains to be resolved if a particular TCR can be selected on only one specific MHCp complex or the selection of one TCR specificity can be driven by several different ligands.

3.4 Autophagy and MHC class II ligandome in cTEC

Comparable expression levels of MHC class II between Atg5$^{-/-}$ and WT cTEC (Figure 2c) argues that the observed defect in the CD4SP T cell repertoire selection is caused by alterations in MHC class II ligandome on autophagy deficient epithelium. Unfortunately, it is not possible to conduct direct cTEC ligandome analysis due to technical limitations[141,142], hence we were unable to directly perform characterization of a peptide repertoire displayed by Atg5$^{-/-}$ and WT cTEC. Nonetheless, we observed a quantitative difference in the expression of a particular MHC II/p complex, I-Eα$_{(52-68)}$ bound to I-A^{b} molecule in Atg5$^{-/-}$ cTEC (Figure 3). To our knowledge, this is the first study showing changes in the MHCII/p repertoire in autophagy deficient APC in an *in vivo* system. The difference in the expression level of a particular MHCII/p complex on Atg5$^{-/-}$ cTEC strongly supports our hypothesis that autophagy specifically shapes repertoire of peptides presented by MHC class II molecules on thymic epithelial cells.

Our findings are in good agreement with previous *in vivo* and *in vitro* studies which together came to the conclusion that cTEC have a remarkably low capacity to present exogenous antigens to T cells, while being capable of presenting intracellular antigens[49,135,137]. Here we show data arguing for a scenario in which autophagy allows for shuttling of intracellular proteins to the MIIC compartment of cTEC.

Recent experimental evidence implies that the activation of autophagy also affects the activity of lysosomal proteases involved in MHC class II antigen processing. Up-regulation of autophagy in B cell lines led to a time-dependent down regulation of cathepsin activity resulting in the generation of longer antigenic peptides from the same source proteins[142]. Lysosomal proteolysis in professional APC is very carefully balanced, as efficient degradation would ultimately lead to the complete destruction of antigenic protein fragments. Accordingly, professional APC in peripheral lymphoid organs, such as DC and B cells, have attenuated lysosomal proteolysis in comparison to less efficient APC like macrophages. Therefore, highly active lysosomal proteases have a negative influence on the antigen presentation capacity of the cell[237-239]. The observed effect of autophagy on lysosomal proteolysis in the study by Dengjel *et al.* can contribute to a more efficient generation of antigenic peptides with the optimal properties for the biding to the MHC class II groove. It is tempting to speculate that concomitant with delivering of intracellular antigen to MIIC in TEC, autophagy also influences the other aspects of antigen processing such as lysosomal proteolysis. In that way, autophagy would on one hand contribute to the diversity of MHCII/p repertoire and on the other, it would make them more efficient in generating peptides that are suitable for MHC class II binding.

3.5 Medullary thymic epithelial cells and endogenous antigen loading pathways

The existence of endogenous MHC class II antigen loading pathways in mTEC and its importance for tolerance induction have been implicated more than a decade ago in the studies with transgenic animals expressing a model antigen in the nucleus[240, 241]. By targeting the expression of a model antigen to the nucleus of either mTEC or DC the authors have shown that tolerance to MHC class II epitopes can be achieved only if the antigen is expressed in mTEC. Furthermore, the study suggested that the

cross presentation of mTEC derived antigen by DC is not necessary for antigen presentation, at least in *ex vivo* and *in vitro* assays. Thus, endogenous loading of nuclear antigens on MHC class II has been proposed to be a peculiar feature of mTEC. Similar observations came from studies of transgenic animals expressing a model antigen in mitochondrial membrane, though in this model system the antigen was ubiquitously expressed in all thymic stromal cells[68]. Using elegant bone marrow chimeras the authors were able to document that TEC can directly process and efficiently present mitochondrial antigens to developing CD4 T cells which results in the clonal deletion of antigen specific cells. However, the nature of a respective endogenous MHC class II loading pathway remained a mystery.

The high constitutive level of autophagy in cTEC and mTEC^{hi} described in our study implies autophagy as an endogenous loading pathway that allows for presentation of nuclear and mitochondrial antigens in the above described systems. Furthermore, undetectable autophagy in DC may explain the observation of Oukka *et al.* that DC are unable to process and present nuclear antigens on MHC class II. Curiously, there is now accumulating evidence in the literature supporting suggested explanation, i.e., autophagy has been implicated as a pathway involved in endogenous loading of nuclear antigens in an *in vitro* system[204] and peptide elution analyses upon induction of autophagy revealed up-regulation of nuclear proteins derived epitopes on MHC class II[142]. It is an intriguing question how the nuclear antigens are actually targeted to autophagosomes. Non-selective degradation of nuclear proteins would be hazardous for cell survival, and so far chaperones involved in a specific targeting of nuclear proteins for autophagosomal degradation have not been identified. It may be possible that MHC class II epitopes derived from nuclear antigens actually originate from DRiPs, as it has been proposed for MHC class I bound epitopes[242, 243]. DRiPs are ubiquitinylated polypeptides[86], thus they can be specifically recognized by p62/SQSTM1 and subsequently targeted to autophagosomes via p62 interaction with LC3 protein[167, 214]. Similarly, selective autophagosomal degradation of mitochondria has been repeatedly documented in different systems[169]. Hence, the model systems described by Kosmatopoulus and Hedrick groups provide an excellent tool for testing the role of autophagy in endogenous antigen loading in mTEC and its part in tolerance induction.

Our analyses of autophagic activity using GFP-LC3 reporter animals would imply that autophagy operates as an endogenous loading pathway in a subpopulation of mTEC ($mTEC^{hi}$) that are fully equipped for efficient antigen presentation (Figure 1). These findings may shed light on the observed different antigen presentation characteristics of mTEC-like cell lines[135-137] and *in situ* antigen presentation features of mTEC[49,244]. While mTEC-like cell lines have proven to be efficient in presenting exogenous antigens, *in vivo* studies claim that mTEC are inefficient in capturing and/or processing extracellular proteins. Thus, the expression of a transgenic human C reactive protein (hCRP) by only few mTEC has been shown to be sufficient for the efficient deletion of autoreactive T cells, and furthermore the dependence on DC mediated cross presentation of mTEC derived antigens for the tolerance induction has been elegantly excluded in the study. On the other hand, if a model antigen has been introduced in the system as a serum-borne protein, tolerance induction was completely dependent on hematopoietic APC. Thus, these data argue that mTEC have a rather low capacity to sample the serum-borne antigens *in vivo*, but are efficient in presenting endogenous antigens to developing T cells[49]. According to the "terminal differentiation model" the $Aire^{+}mTEC^{hi}$ subset of mTEC is the most mature one and expresses the highest number of tissue restricted antigens[4]. However, the rapid turnover of terminally differentiated mTEC *in vivo* on one hand[52, 53] and the impaired survival of $CD80^{hi}$ mTEC in thymic re-aggregation cultures[52] led to the suggestion that promiscuous expression of a wide array of genes has an apoptotic effect which in turn precludes making stable mTEC-like cell lines representing the $mTEC^{hi}$ population. Thus, mTEC-like cell lines might rather mirror the characteristics of immature mTEC. It feels justified to conclude that the respective antigen loading characteristic among mTEC subtypes might be different. As we observed high autophagic activity in $mTEC^{hi}$ subsets, we would like to suggest here that it is this subpopulation that primarily concentrates its MHC class II ligandome on cytosolic proteins.

3.6 Autophagy and tolerance induction

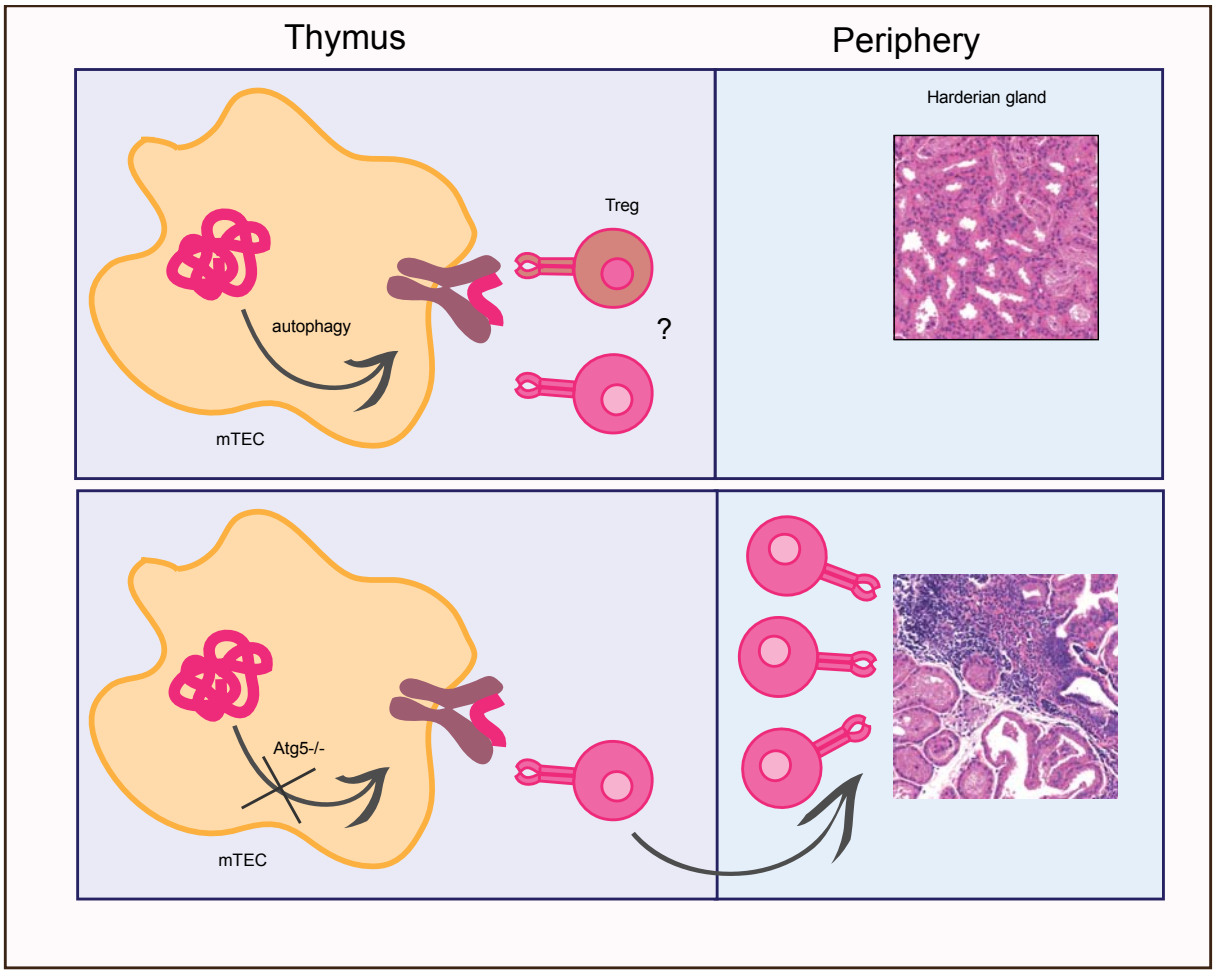
Transplantation of autophagy deficient thymi to athymic, nude animals ($nu/nu^{thymus\ Atg5^{-/-}}$ chimeras) resulted in development of CD4 T cells with an activated phenotype ($CD62L^{lo}CD69^{hi}CD44^{hi}$) and inspection of peripheral organs from these chimeras

revealed massive lymphocytic infiltration in colon, uterus, Harderian gland, liver and lung. Furthermore, animals developed wasting disease that typically set in around 4-6 weeks after grafting of Atg5$^{-/-}$ lobes. Accordingly, inspection of animals revealed total loss of fat pads in nu/nu $^{\text{thymus Atg5-/-}}$ chimeras (Figure 4). Taken together, animals displayed autoimmunity-like disease which implicated that the tolerogenic potential of autophagy deficient thymic epithelium was impaired. Importantly, adoptive transfer of CD90 MACS purified peripheral cells from nu/nu$^{\text{thymus Atg5-/-}}$ chimeras into nude recipients recapitulated essentially all aspects of autoimmunity observed in original chimeras (Supplementary Figure 10). To exclude a potential contribution of "carry-over" Atg5$^{-/-}$ hematopoietic cells to the development of the autoimmune like disease in nu/nu$^{\text{thymus Atg5-/-}}$ chimeras, we created bone marrow chimeras where WT animals have been reconstituted with Atg5$^{-/-}$ fetal liver cells. Inspection of peripheral organs revealed no signs of lymphocytic infiltration (Supplementary Figure 9). Thus, Atg5$^{-/-}$ hematopoietic cells do not contribute to the development of autoimmunity in our model.

Recent studies in humans have implicated a strong genetic linkage between the autophagy-related genes ATG16L1 and IRGM (Immunity-Related GTPase, family M) and the development of Crohn´s disease, a form of inflammatory bowel disease (IBD) with a high incidence in the European and North American population[245-247]. A study by Kuballa and colleagues suggested that impaired clearance of bacterial micro-flora in autophagy deficient intestinal epithelium might have a role in the immunopathogenesis of Crohn´s disease[248]. Concerning the well established role of autophagy in clearance of intracellular pathogens this explanation seems plausible[170,171,195,249]. Our study offers another, mutually non exclusive explanation for the role of autophagy in the development of IBD, namely the breakdown of central tolerance to intestinal antigens. However, it has recently been shown that mice hypomorphic for Atg16L1 protein expression do not develop autoimmune responses directed towards intestinal tissue, but rather display a specific impairment in the function of Paneth cells[176]. The discrepancy in observed phenotypes with different strains of autophagy null animals and experimental set ups might be explained by the fact that in Atg16L1 hypomorphic mice autophagy is still active at the basal level, as mice survive after birth and do not show a typical autophagy null phenotype[184,189,190,188]. This explanation is in agreement with previous observations which suggested

that only small amounts of Atg5 protein are required for autophagy[193,250]. Thus, the basal level of autophagy operating in mTEC of Atg16L1 hypomorphic mice might be sufficient for the induction of tolerance to tissue specific antigens. On the other hand, autophagy has been shown to be indispensable for proliferation and survival of mature T cells in the periphery. Additionally, autophagy deficient T cells display impaired TCR signaling in response to antigen stimulation[199]. Therefore, lack of intestinal inflammation in Atg16L1 hypomorphic mice might further be explained by the inability of auto-reactive T cells to respond to self antigens in the periphery. Nevertheless, as Crohn´s disease is a highly complex genetic disorder on one hand[251] and autophagy has been shown to play an important role in both innate and adaptive immune responses on the other[188,249,252], we might be just at the tip of an iceberg in elucidating its role in different aspects of IBD pathology.

We would like to suggest that the underlying reason responsible for the breakdown of immunological tolerance presented in this study is the interference with endogenous antigen loading pathways in Atg5$^{-/-}$ mTEC (Graphic 7). MTEC play an indispensable role in T cell tolerance induction by promiscuously expressing tissue specific antigens[4,54,59,253,254]. Autophagy enables mTEC to present intracellular antigens to developing T cells, hence interference with autophagy in mTEC allows self-reactive T cells to bypass the mTEC-dependent central tolerance mechanisms and escape to the periphery. At this point it is an unresolved issue whether the ability of Atg5$^{-/-}$ mTEC to delete self-reactive thymocytes (negative selection) or to induce FoxP3 Treg is affected. We observed equal percentages of CD25^{+}FoxP3^{+} positive cells in Atg5$^{-/-}$ grafts on both Bl/6 and BALB/c backgrounds (Figure3 and Supplementary Figure3). However, it remains to be determined if the repertoire of Treg in nu/nu$^{\text{thymus Atg5-/-}}$ and nu/nu$^{\text{thymus WT}}$ chimeras is the same.



Graphic 7. The role of autophagy in central tolerance induction. Autophagy shuttles intracellular tissue specific antigens expressed in mTEC to the MIIC and by doing so it contributes to their tolerogenic capacity. Interference with autophagy abolishes endogenous antigen presentation in mTEC which results in the escape of self-reactive T cells to the periphery and development of autoimmune disease.

Additional factors might contribute to the development of autoimmunity in the nu/nu$^{thymus\ Atg5\text{-}/\text{-}}$ chimeras. One of the candidates would be a homeostatic proliferation of T cells induced by lymphopenia in nude animals[255,256]. However, as our control nu/nu$^{thymus\ WT}$ chimeras did not show any signs of wasting and immune-mediated tissue destruction (Figure 4) nor were the CD90 T cells from nu/nu$^{thymus\ WT}$ chimeras able to cause autoimmunity in athymic recipients (Supplementary Figure 10), one can exclude lymphopenia as a sole reason for the autoimmunity in our experimental set up. The potentially reduced T cell output from Atg5$^{-/-}$ grafts coupled with homeostatic expansion of self-reactive T cells that escape the thymic censorship and populate the periphery in nu/nu$^{thymus\ Atg5\text{-}/\text{-}}$ chimeras has to be taken into account. However, we observed in our initial experiments with transplantation of two Atg5$^{-/-}$

grafts into nude animals (nu/nu$^{2xthymus\ Atg5-/-}$) recapitulation of the disease phenotype, which developed with faster kinetics, i.e., earlier onset of disease (data not shown). Thus, these data would argue that impaired T cell output is not the underlying reason for a breakdown of central tolerance, but lymphopenia driven homeostatic expansion of self-reactive T cells can contribute to the aggressiveness of autoimmunity observed in our study.

Reduced diversity of MHCII/p complexes on Atg5$^{-/-}$ cTEC and altered positive selection of CD4 T cell repertoire may also contribute to development of autoimmunity in nu/nu$^{thymus\ Atg5-/-}$ chimeras. Thymocytes that fail to be positively selected can undergo a second round of TCRα rearrangement, as positive selection signaling is necessary for down-regulation of Rag expression[26, 257, 258]. A successful secondary rearrangement increases the chance for a T cell to get positively selected and escape death by neglect. Importantly, if the secondary TCRα rearrangement takes place on the second Vα allele, and not on the already rearranged one, T cells will have two different antigen specificities, i.e., two TCRα chains pairing with the same TCRβ chain[234,259-262]. It has been suggested that T cells with dual specificities increase the risk of autoimmunity, as they can be selected through one TCR chain, but the second TCR might be self-reactive. Furthermore, the competitive pairing of two different TCRα chains with the same TCRβ chain results in reduced expression of TCR on the cell surface. The low expression of an self-reactive receptor may enable the T cell to bypass thymic censorship and escape to the periphery[70,263,264]. Thus, secondary rearrangements increase the success rate of positive selection, but it comes with the prize of an elevated risk for the development of autoimmune diseases. The incomplete repertoire of MHCII/p complexes on Atg5$^{-/-}$ cTEC results in limited positively selecting niches for some TCR specificities and thus may cause more frequent secondary Vα gene rearrangements then in WT animals. Indeed, we observed such a phenomenon in the selection of Sep TCR transgenic T cells. If educated on autophagy deficient epithelium, Sep TCR transgenic T cells show increased expression of endogenously rearranged TCRα chains (Vα2 and Vα8.3), implying the existence of dual-specificity T cells (Supplementary Figure 4). However, lacking anti-clonotypic or Vα4 antibody, we were not able to formally show that endogenously rearranged and transgenic Vα chains are co-expressed in the same group of T cells. Nevertheless, the observed higher frequency of secondary TCRα

rearrangements can theoretically lead to increased numbers of T cells expressing two different TCR specificities on the cell surface and in that way can synergize with a failure of mTEC-dependent tolerance induction to govern autoimmunity observed in nu/nu$^{thymus\ Atg5-/-}$ chimeras. Therefore, it will be of utmost importance to precisely assign a role of autophagy in cTEC versus mTEC for the development of a self-MHC restricted, functionally competent and self tolerant T-cell repertoire. However, at the moment we are unable to address this question due to the lack of good cTEC and mTEC specific Cre transgenic mouse lines that would allow us to conduct conditional knock out studies by taking advantage of Cre/LoxP technology.

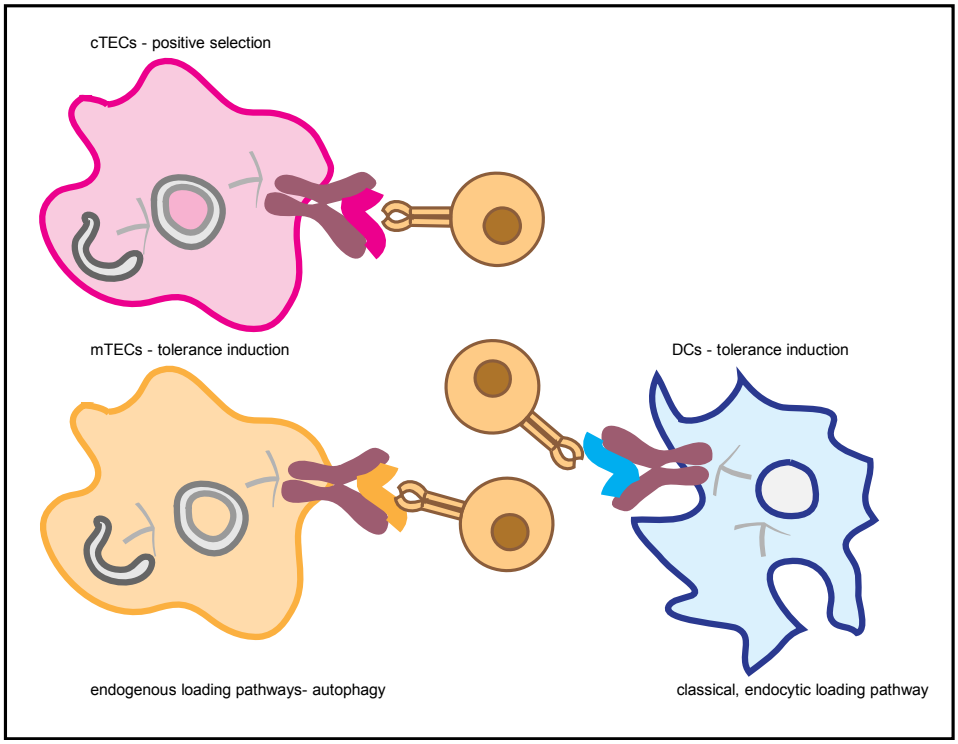
As a note of caution, however, it should be said that the autoimmune response observed in our experimental set up could be directed against Atg5 protein-derived epitopes. T cells specific for Atg5 epitopes can bypass the mechanisms of central tolerance induction as Atg5 protein is not expressed in TEC. We consider this possibility unlikely, as Atg5 protein is constitutively expressed in all peripheral organs. Hence, we would expect a larger panel of organs to be affected in nu/nu $^{thymus\ Atg5-/-}$ chimeras if the autoimmunity was caused by Atg5 specific T cells. However, to formally exclude this possibility one would have to create a transgenic animal expressing a mutated form of the Atg5 protein, whereby the full length protein would still be expressed, but its autophagy-related function would be compromised [160]. In that way, TEC would be able to present most of the Atg5 derived epitopes and escape of self-reactive, Atg5 specific T cells to the periphery would be precluded. Furthermore, one can alternatively address this question by analyzing the immunological phenotype of the other autophagy null animals, such as already described Atg7, Atg3 and Atg16L knock outs[188,184,190] .

An important issue arising from our observations concerns the potential mechanism that primes naive, self reactive T cells in the periphery of nu/nu$^{thymus\ Atg5-/-}$ animals. The main players in the initiation phases of adaptive immune responses are DC. How are then “vetoed” T cells, specific for TSA expressed and presented by mTEC that escape thymic censorship on Atg5$^{-/-}$ thymic epithelium, activated in the periphery? One can think of a scenario in which DC pick up TSA in peripheral organs from apoptotic cells and migrate to the draining lymph nodes where they can prime self-reactive T cells. The classical endocytic route of MHC class II loading, i.e., the

engulfment of apoptotic bodies by DC in the periphery, would theoretically allow for the generation of the same peptide epitopes from TSA as autophagic delivery to MIIC, as both pathways converge to the same proteolytic machinery. Once activated in the lymph nodes, self-reactive T cells will home to the peripheral organs where they can recognize cognate antigens. But here the new question arises: how are the antigens presented to self-reactive T cells at the site of inflammation? High constitutive autophagic activity in the Harderian gland under normal physiological conditions on one hand, and severe immune-mediated histo-pathological destruction of the gland in the nu/nu$^{\text{thymus Atg5-/-}}$ animals on the other is an interesting observation offering possible answer (Supplementary Figure 11). In the affected organs, inflammatory cytokines, such as IFNγ, might lead to the up-regulation of (i) MHC class II molecules and (ii) autophagy on fibroblasts, epithelial and endothelial cells[195, 265]. By up-regulating autophagy and MHC class II, stromal cells in targeted organs might allow for delivery of intracellular protein to MIIC and subsequent antigen presentation, as it has been convincingly shown for human epithelial cell lines[201] . Curiously, in studies of psoriasis in humans it has been documented that CD4 T cells with cytolytic activity frequently surround MHC class II positive epithelial cells[266]. Thus, it seems reasonable to speculate that similar modes of antigen presentation in peripheral organs occur in our experimental set up once the self-reactive T cells find themselves in the target organs.

3.7 Proposed model and concluding remarks

“The division of labor” hypothesis has been proposed nearly a decade ago, which postulates that mTEC and DC sample and present a non overlapping pool of self antigens to developing T cells. However, the nature of the pathway/s that enables TEC to concentrate their MHC class II ligandome on the intracellular milieu (Graphic 8) remained undefined at the time. Here we presented evidence that autophagy can operate as an endogenous MHC class II loading pathway in TEC. In that way it contributes to the diversity of MHCII/p complexes in cTEC that are displayed to the developing thymocytes during positive selection and at the same time broadens the repertoire of MHCII/p complexes in mTEC, which are displayed to thymocytes during the process of tolerance induction in the medulla.



Graphic 8. Proposed model of different MHC class II antigen loading routes in thymic stromal cell subtypes. Autophagy delivers intracellular proteins to MIIC of cortical and medullary thymic epithelial cells (cTEC and mTEC) and in that way broadens the repertoire of MHCII/p complexes that is presented to developing CD4 T cells during the processes of positive selection and tolerance induction. Dendritic cells (DC) efficiently sample extracellular proteins and generate MHC class II epitopes via the classical antigen loading pathway.

We focused our research on the role of macroautophagy, as this pathway has been shown to display an atypically high activity in TEC and the genetic tools for studying and manipulating macroautophagy were available. However, other alternative pathways of MHC class II antigen loading can as well operate in the thymic epithelium and/or DC and in that way contribute to T cell development. Chaperone mediated autophagy is an interesting candidate, as it was convincingly shown in *in vitro* assays that it can contribute to MHC class II loading[152]. However genetic manipulation of essential players in CMA, Hsc70 and LAMP2a, affects other basic cellular processes. LAMP2 deficient animals display a general problem in the process of endosomal/lysosomal fusion and accumulation of early autophagosomes in a large panel of tissues which makes analyses of CMA contribution to MHC class II loading very difficult to interpret[267,268]. In the same line, Hsc70 is involved in many basic cellular processes. As a molecular chaperone Hsc70 binds numerous proteins thereby mediating both folding and degradation[269]. One possible approach to study CMA in antigen loading would be the generation of miRNA transgenic animals specific for LAMP2a isoform of the LAMP2 protein, whereby the CMA knock down phenotype could be analyzed. The list of other putative endogenous loading pathways includes microautophagy, which is a genetically poorly characterized process, and TAP dependent MHC class II loading. The contribution of these endogenous antigen loading pathways for the generation of a highly diverse MHCII/p repertoire on thymic APC remains to be elucidated in the years that follow.

4. Materials and Methods

Animals. *Atg5*$^{-/-}$ and *GFP-LC3* mice have been described previously [161, 189]. BALB/c $^{nu/nu}$ and *HY* TCR transgenic animals were purchased from Taconic Farms. *AND* and *OT-I* TCR transgenic and B10.A animals were obtained from Jackson Laboratories. *AND*, *P14, OT-I, HY, TCR-HA*, *DO11.10*, *Sep* and *Dep* TCR transgenic animals were described elsewhere[223, 270, 271, 29, 244, 272, 273]. *P14 TCR* transgenic animals were a kind gift from Prof. Penninger. All mice were bred and maintained in individually ventilated cages in the animal facility of the Research Institute of Molecular Pathology (Vienna, Austria) under specific pathogen-free conditions. All animal studies were approved by local authorities (MA58) and were done according to Austrian regulations.

Genotyping

DNA samples for the genotyping were prepared by 5h digestion of tail pieces at 55°C in the tail-buffer followed by thermal inactivation of proteinase K by incubation of the reaction mixture for 5 min at 95°C. *Atg5*$^{-/-}$ animals were genotyped using primers LK39 5´-GAATATGAAGGCACACCCCTGAAATG-3´ and LK40 5´-ACAACGTCGAGCACAGCTGCGCAAGG-3´ for the wild type allele, and LK40 and LK41 5´- GTACTGCATAATGGTTTAACTCTTGC-3´ for the knock-out allele. The *Atg5*$^{+/+}$ allele gives a PCR product of 351bp, while the *Atg5*$^{-/-}$ allele gives a PCR product of 574bp. Amplification conditions were as for the Atg5 program as detailed in the supplementary methods. *GFP-LC3* transgenic animals were genotyped using primers LK35 5´-TCCTGCTGGAGTTCGTGACCG-3´ and LK36 5´-TTGCGAATTCTCAGCCGTCTTCATCTCTCTCGC-3´ for the transgenic allele, and LK37 5´-TGAGCGAGCTCATCAAGATAATCAGGT-3´ and LK38 5´-GTTAGCATTGAGCTGCAAGCGCCGTCT-3´for the wild type allele. The *GFP-LC3*$^{+/+}$ allele gives a PCR product of ~400bp; while the wild type allele gives a PCR product of ~500bp. Amplification conditions were as for the TD 54x30 program as detailed in the supplementary methods. *AND TCR* transgenic animals were genotyped using primers AND5 5´- GACTTGGAGATTGCCAACCCATATCTAAGT-3´and AND3 5´-TGAGCCGAAGGTGTAGTCGGAGTTTGCATT-3´ using amplification conditions as for the TD54x30 program. The transgenic allele gives a PCR product of ~410bp. *Dep TCR* transgenic animals were genotyped using primers LK28 5´-CGAGAGGAAGCATGTCTAAC-3´ and LK29 5´- ACCGCGGTCATCCAACACAG-3´.

Amplification conditions were as for the TD54x30 program, and the transgenic allele gives a PCR product of ~650bp. *Sep TCR* transgenic animals were genotyped using primers LK44 5´-AGTCGCGAGATGGGCTCC-3´ and LK45 5´-CGCGGTCATCCAACACAG-3´ using amplification conditions as for the TD54x30 program. The transgenic allele gives a PCR amplicon of ~550bp. *TCR-HA TCR* transgenic animals were genotyped using primers TCR-HA5 5´- ACA AGG TGG CAG TAA CAG GA-3´and TCR-HA3 5´- ACA GTC AGT CTG GTT CCT GA-3´using amplification conditions as for the TD54x30 program. The transgenic allele gives a PCR product of ~800bp. DO11.10 TCR transgenic animals were genotyped using primers DO11.10-5 5´- CAGGAGGGATCCAGTGCCAGC-3´and DO11.10-3 5´-TGGCTCTACAGTGAGTTTGGT-3´. The transgenic allele gives a PCR product of ~290bp using TD54x30 PCR program. *OT-I TCR* transgenic animals were genotype using primers JN40 5´- AAGGTGGAGAGAGACAAAGGATTC-3´and JN41 5´-TTGAGAGCTGTCTCC-3´. The amplifications conditions were as for the OT-I program as detailed in the supplementary methods. The transgenic allele gives a PCR product of ~300bp. *P14-TCR* transgenic animals were genotyped using primers JN46 5´- CGAGGATCCTTTAACTGGTACACAGCACG-3´and JN47 5´-CTGACCTGCAGTTATGAGGACAGCAC-3´. The amplification conditions were as for the P14 PCR program as detailed in the supplementary methods. The transgenic allele gives a PCR amplicon of ~200bp. *HY-TCR* transgenic animals were genotyped using primers HY-1 5´- GCTTTGAGGCGGAGTTTAGG-3´and HY-2 5´-GCTCACTGTCAGCTTTGTCC-3´. The amplification conditions were as for the HY program as detailed in the supplementary methods. The transgenic allele give a PCR product of ~300bp. *Rag2*$^{-/-}$ animals were genotyped using primers RAG2-5´ 5´-GCAACATGTTATCCAGTAGCCGGT-3´ and RAG2-3´ 5´-TTGGGAGGACACTCACTTGCCAGT- 3´ for the wild type allele, and RAG2-5 and RAG2-int 5´ - GTATGCAGCCGCCGCATTGCATCA-3´ for the knock out allele. Amplification conditions were as for the TD54x30 program. The wild type allele gives a PCR product of ~605bp, while the knock out allele gives a PCR product of ~1000bp. Male embryos were genotyped using primers MA329 5´-TTGTCTAGAGAGCATGGAGGGCCATGTCAA-3´ and MA330 5´-CCACTCCTCTGTGACACTTTAGCCCTCCGA-3´ that are specifically amplifying Y-chromosome linked SRY antigen. The amplification conditions were as for the TD54x30 and male allele gives a PCR product of ~400bp.

Antibodies and flow cytometry. Biotin-conjugated monoclonal antibody (mAb) Y-Ae was a kind gift of Prof. B. Kyewski. Biotin-conjugated mAbs to CD62L (MEL14), CD80 (16-10A1), CD11c (HL3), Vβ3 (KJ25) and Vβ14 (14-2), fluorescein isothiocyanate (FITC)–conjugated mAbs to CD62L (MEL14), CD90.1 (HIS51), H-2K^b (AF6-88.5), Vα8.3 (KT50),Vβ3(KJ25),Vβ4 (KT4), Vβ5.1,5.2 (MR9-4), Vβ6 (RR4-7), Vβ8.1/2 (MR5-2), Vβ8.3 (1B3.3), Vβ13 (MR12-3), phycoerythrin (PE)-conjugated mAbs to CD69 (H1.2F3), Ly51 (BP-1), Vα2 (B20.1),Vα11.1,11.2 (RR8-1), Vβ2 (B20.6),Vβ7 (TR310),Vβ8.3 (1B3.3),Vβ10 (B21.5), CyChrome-conjugated mAbs to CD8 (53-6.7), CD45 (30-F11), phycoerythrin-Cy7 conjugated mAb to CD25 (PC61), allophycocyanin (APC)-conjugated mAbs to CD4 (RM4-5) and CD8 (52-6.7), allophycocyanin-indotricarbocyanin-conjugated mAb to CD4 (GK1.5), PE-Cy7-conjugated streptavidin and FITC–conjugated straptavidin were purchased from Becton Dickinson. PE-conjugated mAb to TCR-HYα (T3.70) was purchased from eBiosciences. *Ulex europaeus* agglutinin (UEA-1) was purchased from Sigma Aldrich and conjugated to Alexa 647 in our lab. mAbs specific for TCR-HA (6.5), DO11.10 (KJ1-26), EpCAM (G8.8), Vβ8 (F23.1), pan-MHC class II (P7.7) and CD4 (GK1.5) were purified from hybridoma supernatants and conjugated to biotin, Alexa Fluor 488 or Alexa Fluor 647 in our lab. Surface staining was done according to standard procedures at a density of $1x10^6$ to $3x10^6$ cells per 50 µl. FoxP3 intracellular staining was done according to the manufacturer's recommendations with phycoerythrin- or allophycocyanin-conjugated mAb to mouse FoxP3 (FJK-16s, eBiosciences, San Diego, CA). A FACSCanto (Becton Dickinson) with FACSDiva software (Becton Dickinson) was used for data acquisition and the Flow Jo software was used for data analysis.

Intracellular staining. As previously mentioned, Foxp3 intracellular staining was performed according to the manufacturer's recommendations using PE- or APC-conjugated anti-mouse Foxp3 mAb (eBiosciences). In brief, 5×10^6 cells were centrifuged at 2000 r.p.m. for 5 minutes at 4 °C in FACS tubes and stained in 100 µl FACS buffer (1x PBS, 2% FCS, 1 mM EDTA) containing appropriate cell surface markers, i.e., anti-CD4,CD8 and CD25 mAbs. Cells were washed with 1 ml FACS buffer, centrifuged as before and resuspended in 1 ml 1X Fixation/Perm buffer from Ebioscience Foxp3 staining kit. Cells were incubated either overnight or for 3h at 4 ° C in the dark. Cells were subsequently washed two times with 1 ml Permeabilization

Buffer from eBioscience Foxp3 staining kit and resuspended in 50 µl 2 % rat serum diluted in Permeabilization buffer. Cells were incubated in the dark on ice for 15 minutes. Appropriate volume of PE conjugated anti-mouse Foxp3 mAb from eBiosciences was added to the cell suspension and the cells were incubated on 4°C in the dark for 45 minutes. Cells were washed in 1 ml Permeabilisation buffer and resuspended in 200 µl FACS buffer before analysis.

Immunofluorescence. Frozen sections (5µm) were fixed in cold acetone, washed and blocked for 30 min with 10% (vol/vol) FCS in PBS. Sections were permeabilized in 0.1% (vol/vol) Tween in PBS for 10 min, and stained overnight at 4°C with biotinylated anti keratin 8 antibody (TROMA-1, Developmental Studies Hybridoma Bank). Sections were incubated with secondary antibody for 90 min at room temperature, and after washing 3 times for 15 min each in 0.1% (vol/vol) Tween in PBS were blocked with anti-rat serum (Jackson Immuno Research Laboratories, Inc.) for 30 min at room temperature. After washing, sections were incubated with anti-keratin 5 antibody (Covance) for 2 h at room temperature. Secondary reagents were straptavidin-Cy3 (Jackson Immuno Research Laboratories,Inc.) and Alexa Fluor 488-conjugated anti-rabbit antibody (Molecular Probes). Nuclei were counterstained with ProLong Gold antifade reagent with 4, 6-diamidino-2-phenylindole (DAPI) (Molecular Probes). Samples were analyzed with an inverted confocal LSM 510 laser scanning microscope (Carl Zeiss AG, Germany).

GFP-LC3 analyses. GFP-LC3 mice and WT controls were maintained on normal diet or kept without food for 48 hours before analyses with free access to drinking water. Prior to analyses, mice were anaesthetized and perfused through the left heart ventricle with 4% paraformaldehyde (PFA) in PBS. Organs were harvested and further fixed with the same fixative for 4h, followed by treatment with 15% sucrose in PBS for 4h at room temperature and then with 30% sucrose solution overnight at 4°C. Tissue samples were embedded in O.C.T. (Sakura Finetek Europe B.V.) and stored at -70°C. The samples were sectioned (5µm), and stained with Alexa Fluor 488 conjugated anti-GFP antibody (Molecular Probes) overnight at 4°C. Samples were mounted using ProLong Gold antifade reagent with DAPI.

Quantification of autophagic activity in thymic stromal cells. Thymi from two week old GFP-LC3 mice were cut into small pieces and digested at 37 °C in IMDM

containing 0.2 mg/ml Collagenase (Roche), 0.2 mg/ml Dispase I (Roche), 2% FCS, 25mM Hepes (pH 7.2) and 25 µg/ml DNase I, followed by incubation in 5 mM EDTA for 5 min. Cells were washed and resuspended in Percoll™ (GE Healthcare) (ρ 1.115), followed by a layer of Percoll™ (ρ 1.050) and PBS as upper phase. Gradients were spun for 30 min at 1350g in the cold and low density cells were harvested from the upper interface, washed and stained for FACS sorting. Stromal cells were sorted according to CD45, Ly51, EpCAM, CD80 and CD11c expression (cTEC = $CD45^-Ly51^+EpCAM^+$; $mTEC^{lo}$ $CD45^-Ly51^-EpCAM^+CD80^{lo}$, $mTEC^{hi}$ $CD45^-Ly51^-EpCAM^+CD80^{hi}$; DC = $CD45^+CD11c^+$) and fixed onto poly-L-lysine–coated slides (Sigma Aldrich) with ice cold acetone. Cells were permeabilized in 0.1% (vol/vol) Tween in PBS for 10 min, and blocked with 10% (vol/vol) FCS in PBS for 30 min at room temperature. Cells were incubated overnight at 4°C with Alexa Fluor 488 conjugated anti-GFP antibody and mounted with ProLong Gold antifade reagent with DAPI. A total of 1000 dendritic cells, 1845 cTEC, 1232 $mTEC^{hi}$ and 245 $mTEC^{lo}$ have been analyzed for determination of the percentage of autophagy positive cells. Cells with more than 5 autophagosomes (= GFP positive punctae between 0.5 and 1.5 µm in diameter) were scored positive. For quantification of the number of autophagosomes per cell, a total of 81 autophagy positive cTEC and 51 autophagy positive $mTEC^{hi}$ have been recorded and analyzed. Cells were analyzed with an inverted confocal LSM510 Zeiss microscope by taking Z stacks of 0.5 µm thickness and subsequently the number of autophagosomes in the whole cell volume was determined.

Purification and adoptive transfer of T cells. Pooled cell suspensions of spleen and lymph nodes (mesenteric, inguinal, axillary, brachial, superficial cervical, deep cervical and lumbar) from $nu/nu^{Atg5-/-}$ and nu/nu^{wt} chimeras were subjected to erythrocyte lysis. Cells were then incubated with Fc-receptor-blocking antibody (2.4G2) and were stained with biotin-anti-CD90. After incubation with streptavidin microbeads (Miltenyi Biotec), $CD90^+$ cells were positively selected on midi-MACS columns. $1x10^7$ $CD90^+$ cells were injected in a volume of 200µl in PBS into the lateral tail veins of $BALB/c^{nu/nu}$ animals. Animals were analyzed 6-12 weeks after transfer.

Thymus transplantation. Embryonic thymi (E16.5-E15.5) (with or without dGuo treatment) were transplanted under the kidney capsule of female animals. Recipient animals were anesthetized with ketamine/xylazine prior to transplantation. *Atg5*$^{-/-}$

embryos and *Atg5*$^{+/+}$ (WT) controls within individual experiments were obtained from the same *Atg5*$^{+/-}$ pregnant female after mating to an *Atg5*$^{+/-}$ male. For the analyses of efficacy of positive selection in *HY-TCR* transgenic animals, the gender of embryos have been determined by genotyping as detailed in the genotyping section of materials and methods. In this experimental set up only female embryos were used as thymic donors.

Deoxyguanosine treatment. E15.5-E16.5 thymic lobes were placed on 0.45µm membrane filters (Milipore) supported by Gelfoam (Pharmacia&Upjohn) and were incubated for 5 days with 10% FCS (vol/vol) IMDM supplemented with 1.35 mM 2-deoxyguanosine, glutamine, β-mercaptoetanol, penicillin and streptavidin n 37°C incubator before transplantation.

Bone marrow chimeras. For mixed bone marrow chimeras CD90 depleted *TCR-HA rag2*$^{-/-}$ CD45.1$^{+/+}$ and *DO11.10 rag2*$^{-/-}$ CD45.1$^{+/-}$ bone marrow was mixed at a ratio of 1:1 and a total of 1x10^{7} cells were injected into the lateral tail vein of irradiated BALB/c recipients (2 × 450 rad). Animals were transplanted with *Atg5*$^{-/-}$ or *Atg5*$^{+/+}$ (WT) lobes 4 weeks after bone marrow reconstitution. Grafts were analyzed 6 weeks after transplantation.

Fetal liver chimeras. Fetal livers were harvested from E15.5-E16.5 embryos. Single cell suspensions were filtered through nylon mash filters (BD Bioscience) and directly used as donor cells for reconstitution experiments. A total of 5.5 × 10^{6} fetal liver cells were injected into the lateral tail veins of irradiated (2 × 550 rad) CD45.1 C57BL/6 recipients. Chimeras were analyzed 12 weeks after reconstitution.

Semiquantitative RT-PCR. mTEC were isolated from 4-5 transplanted thymi as described above and total RNA was isolated using the High Pure RNA isolation kit (Roche). RNA was reverse transcribed using the iScript RT kit (Biorad). PCR reactions were carried out in a final volume of 25µl using the Advantage 2 Polymerase mix (Clontech) and Advantage 2 PCR buffer SA (Clontech) according to the manufacturers recommendations. dNTPs (Fermentas) were used at a final concentration of 200µM and primers were used each at 400nM final concentration.

Primer list:

Primer	Sequence	Amplicon size
Aire forward:	5'- GAGTCACAGCACCTTCCTCTT - 3'	(438bp)
Aire reverse:	5'- GGGACAGCCGTCACAACA - 3'	(438bp)
Spt1 forward:	5'- TGAAACTCAGGCAGATAG - 3'	(383bp)
Spt1 reverse:	5'- GAGGGAGAATAGTCAGGAT - 3'	(383bp)
Tff3 forward:	5'- CTGGCTAATGCTGTTGGTG- 3'	(377bp)
Tff3 reverse:	5'- TGTTGGCTGTGAGGTCTTT - 3'	(377bp)
Csn2 forward:	5'- TCATCCTCGCCTGCCTTGT - 3'	(401bp)
Csn2 reverse:	5'- GCGGAGCACAGTTTCAGAGTT - 3'	(401bp)
Actb forward:	5'- GGTGGGAATGGGTCAGA - 3'	(380bp)
Actb reverse:	5'- GAGCATAGCCCTCGTAGAT- 3'	(380bp)

All amplicons span at least one intron. Cycling conditions were as follows: 94°C for 3min; 31 × (*Actb*), 34 × (*Aire*), 36 × (*Csn2/Tff3*), 38 × (*Spt1*) [94°C for 20s, 56°C for 30s, 72°C for 30s]; 72°C for 5min. Samples were run on a 1.5 % (w/v) Agarose / EtBr gel in TAE buffer.

Histopathology. Organs were harvested from donor animals and immersion-fixed with either 4% paraformaldehyde (PFA) overnight at 4°C or in an IHC Zink Fixative (Becton Dickinson) for 48 hours at room temperature. Samples were embedded in paraffin blocks. Sections (3-5 µm thick) were stained with hematoxylin and eosin (H & E) and automatically scanned using the Zeiss MIRAX SCAN system. Subsequently, sections were analyzed "blind" using the Zeiss MIRAX VIEWER software (Carl Zeiss AG, Germany).

Supplementary Methods- Genotyping Primers

Primers	Sequence	Description
DO11.10 5´	CAGGAGGGATCCAGTGCCAGC	sense DO11.10 TCR
DO11.10 3´	TGGCTCTACAGTGAGTTTGGT	antisense DO11.10 TCR
TCR-HA 5´	ACA AGG TGG CAG TAA CAG GA	sense TCR-HA
TCR-HA 3´	ACA GTC AGT CTG GTT CCT GA	antisense TCR-HA
LK28	CGAGAGGAAGCATGTCTAAC	sense Dep TCR (beta chain)
LK29	ACCGCGGTCATCCAACACAG	antisense Dep TCR (beta chain)
LK44	AGTCGCGAGATGGGCTCC	Sep TCR beta chain sense
LK45	CGCGGTCATCCAACACAG	Sep TCR beta chain antisense
AND 3´	TGAGCCGAAGGTGTAGTCGGAGTTTGCATT	AND TCR antisense
AND 5´	GACTTGGAGATTGCCAACCCATATCTAAGT	AND TCR sense
HY 1	GCTTTGAGGCGGAGTTTAGG	HY TCR forward
HY 2	GCTCACTGTCAGCTTTGTCC	HY TCR reverse
JN40	AAGGTGGAGAGAGACAAAGGATTC	OT-I TCR forward
JN41	TTGAGAGCTGTCTCC	OT-I TCR reverse
JN46	CGAGGATCCTTTAACTGGTACACAGCACG	P14 TCR forward
JN47	CTGACCTGCAGTTATGAGGACAGCAC	P14 TCR reverse
LK39	GAATATGAAGGCACACCCCTGAAATG	ATG5 exon3-1
LK40	ACAACGTCGAGCACAGCTGCGCAAGG	ATG5 check2
LK41	GTACTGCATAATGGTTTAACTCTTGC	ATG5 short2
LK35	TCCTGCTGGAGTTCGTGACCG	GFP1 for GFP-LC3
LK36	TTGCGAATTCTCAGCCGTCTTCATCTCTCTCGC	LC3 rc3
LK37	TGAGCGAGCTCATCAAGATAATCAGGT	mLC3 ex3GT
LK38	GTTAGCATTGAGCTGCAAGCGCCGTCT	mLC3 ex4AG
RAG2 3'	TTGGGAGGACACTCACTTGCCAGT	RAG2 3' primer
RAG2 5'	GCAACATGTTATCCAGTAGCCGGT	RAG2 5' primer
RAG2 int	GTATGCAGCCGCCGCATTGCATCA	RAG2 internal primer (ko)
MA329	TTGTCTAGAGAGCATGGAGGGCCATGTCAA	SRY forward –male specific
MA330	CCACTCCTCTGTGACACTTTAGCCCTCCGA	SRY reverse-male specific

Supplementary Methods- PCR protocols

TD 54 × 30

94 °C	3:00 min	1× cycle
94 °C	0:45 min	
60 °C	0:45 min	2× cycles
72 °C	1:00 min	
94 °C	0:45 min	
58 °C	0:45 min	2× cycles
72 °C	1:00 min	
94 °C	0:45 min	
56 °C	0:45 min	2× cycles
72 °C	1:00 min	
94 °C	0:45 min	
54 °C	0:45 min	30× cycles
72 °C	1:00 min	
72 °C	5:00 min	1× cycle

HY-TCR

95°C	15 min	1x cycle
95°C	45 sec	
60°C	1 min	30x cycles
72°C	1 min	
72°C	5 min	1x cycle

OT-I TCR

94° C	3 min	
94 °C	30 sec	
52° C	30sec	38x cycles
72 °C	30sec	
72° C	2 min	
15° C	for ever	

Atg5 program

94°C 3 min

94°C 30 sec
60°C 30 sec 36x cycles
72°C 30 sec

72° 10 min

Tail digestion program

55°C 5 hours

95°C 5 min

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Nedjic J. *et al.* Autophagy and antigen presentation, Current Opinion in Immunology, in revision, review article

Nedjic J. E *et al.* Autophagy and T cell education in the thymus: Eat yourself to know yourself, Extra Views, Cell Cycle, 2008 Dec; 7 (23)

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