The role of autophagy in intestinal T cell homeostasis

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Acknowledgements

Unexpectedly, this part of the thesis turned out to be the most difficult one to write. I guess that official and pompous acknowledgements is just not my thing and I really hope that people who helped me to get where I am now know well how grateful I am, without the need for reading about it in the acknowledgement section. Still though, this is Oxford after all so we need to follow conventions!

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Abstract

The role of autophagy in intestinal T cell homeostasis

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A polymorphism in the essential autophagy gene *ATG16L1* is associated with susceptibility to inflammatory bowel disease. However, the role of autophagy in maintenance of intestinal immune homeostasis remains unclear. Using targeted deletion of *Atg16l1* in T cells, we demonstrate critical requirement for autophagy in generation of appropriate adaptive immune cell responses within the mucosa. Selective deletion of *Atg16l1* in T cells resulted in spontaneous intestinal inflammation, characterized by accumulation of Th2 cells and aberrant antibody responses towards dietary and microbiota antigens. $Foxp3^+$ Treg cells were dependent on autophagy for their survival and function within the intestinal lamina propria, where they acted to limit Th2 cell accumulation. In addition, we demonstrate a novel role for autophagy in limiting Th2 cell survival in a cell-intrinsic manner. The distinct requirements for autophagy in the survival of different intestinal $CD4^+$ T cell subsets reveals a novel role for autophagy in mucosal T cell homeostasis, with potential implications for understanding and treatment of chronic inflammatory disorders.

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Abbreviations

ADP	Adenosine diphosphate
AIRE	Autoimmune regulator
AMBRA	Autophagy/beclin-1 regulator 1
AMP	Antimicrobial peptides or Adenosine monophosphate
AMPK	AMP-activated protein kinase
ANOVA	Analysis of variance
APC	Antigen presenting cell
APRIL	A proliferation inducing ligand
ATF	Activating transcription factor
Atg	Autophagy related gene
ATP	Adenosine triphosphate
Bcl	B cell lymphoma
Blimp-1	B lymphocyte induced maturation protein 1
BM	Bone marrow
BSA	Bovine serum albumin
CCR	Chemokine (CC) motif receptor
CD	Crohn's disease
CFU	Colony forming unit
cLP	Colon lamina propria
CMA	Chaperone-mediated autophagy
CSR	Class switch recombination
CT	Cholera toxin
CTL	Cytotoxic T lymphocyte
CTLA4	Cytotoxic T-lymphocyte antigen 4
CXCR	Chemokine (CXC) motif receptor
CX_3CR	Chemokine (CXXXC) motif receptor
DAMP	Danger associated molecular pattern
DC	Dendritic cell
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DSS	Dextran sulfate sodium
ECAR	Extracellular acidification rate
EDTA	Ethylene diamine tetraacetic acid
eIF	Eukaryotic initiation factor
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
FA	Fatty acid
FACS	Fluorescence-activated cell sorting
FAO	Fatty acid oxidation
FAS	Fatty acid synthesis
Fc	Fragment constant
FCS	Fetal calf serum
Foxo	Forkhead Box O
Foxp3	Forkhead box P3
FSC	Forward scatter
GABA	γ-aminobutyric acid
GALT	Gut-associated lymphoid tissue
Gata3	GATA binding protein 3
GC	Germinal centers
GF	Germ-free

GFP	Green fluorescent protein
GI	Gastrointestinal
GITR	Glucocorticoid-induced tumour necrosis factor receptor
GM-CSF	Granulocyte macrophage colony-stimulating factor
GPR	G protein–coupled receptor
GWAS	Genome-wide association study
H&E	Haematoxylin and eosin
H.h.	*Helicobacter hepaticus*
Hh + αIL-10R	*H.hepaticus* infection and αIL-10R mAb treatment
HIF	Hypoxia-inducible factor
IBD	Inflammatory bowel disease
ICOS	Inducible T-cell co-stimulator
IEC	Intestinal epithelial cell
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
ILC	Innate lymphoid cell
iNKT cell	Invariant natural killer T cell
IRE	Inositol requiring enzyme
IRGM	Immunity-related GTPase family M protein
i.p.	Intraperitoneal
i.v.	Intravenous
IPEX	Immunodysregulation polyendocrinopathy enteropathy X-linked
IRF4	Interferon regulatory factor 4
JNK	c-Jun N-terminal kinase
KLRG1	Killer cell lectin like receptor G1
LC3	L chain 3
LIR	LC3-interacting region
LP	Lamina propria
LPS	Lipopolysaccharide
LRRK2	Leucine-rich repeat kinase 2
mAb	Monoclonal antibody
Mcl-1	Myeloid cell leukaemia 1
MAIT	Mucosal-associated invariant T cells
MCPT-1	Mast cell protease 1
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
mLN	Mesenteric lymph node
min	Minute
MP	Mononuclear phagocytes
mRNA	Messenger ribonucleic acid
mTORC1	Mechanistic target of rapamycin complex 1
mTORC2	Mechanistic target of rapamycin complex 2
MyD88	Myeloid differentiation primary response gene (88)
NFAT	Nuclear factor of activated T cells
NFκB	Nuclear factor κB
NLR	NOD-like receptor
NLRC	NLR family CARD domain-containing protein
NLRP	Nucleotide-binding domain and leucine rich repeat containing protein
NOD	Nucleotide-binding oligomerization domain
OCR	Oxygen consumption rate
OD	Optical density
OXPHOS	Oxidative phosphorylation

OX40	Tumour necrosis factor superfamily member 4
PAMP	Pathogen associated molecular pattern
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PD-1	Programmed cell death 1
PERK	Protein kinase RNA (PKR)- like ER kinase
PI3K	Phosphatidylinositol 3-kinase
PMA	Phorbol myristate acetate
PP	Peyer's patch
PPAR	Peroxisome proliferator-activated receptor
PRR	Pathogen recognition receptor
pTreg	peripherally induced regulatory T cell
RA	Retinoic acid
Rag	Recombination activating gene
Raptor	Regulatory-associated protein of mTOR
Rictor	Rapamycin insensitive companion of mTOR
RLR	RIG-I-like receptor
RNA	Ribonucleic acid
ROR	Retinoic acid receptor-like orphan receptor
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute Medium
RT	Room temperature
SCFA	Short chain fatty acid
SDS	Sodium dodecyl sulfate
S.E.M	Standard error of the mean
SFB	Segmented filamentous bacteria
SHM	Somatic hypermutation
SI	Small intestine
sIgA	Secretory immunoglobulin A
SMURF1	SMAD specific E3 ubiquitin protein ligase 1
SNARE	Soluble NSF attachment protein receptors
SPF	Specified pathogen free
SRC	Spare respiratory capacity
SREBP	Sterol regulatory element-binding proteins
STAT	Signal transducer and activator of transcription
S6K	S6 kinase
T-bet	T-box transcription factor expressing in T cells
TCR	T cell antigen receptor
TEC	Thymic epithelial cells
TFEB	Transcription factor EB
TGF	Transforming growth factor
Th	T helper
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TSC	Tuberous sclerosis complex
TSLP	Thymic stromal lymphopoietin
tTreg	Thymic Treg cells
UBL	Ubiquitin – like
UC	Ulcerative colitis
Ulk	Unc-51-like kinase
UPR	Unfolded protein response
Vps	Vacuolar protein sorting
WIPI	WD-repeat domain phosphatidylinositide-interacting

WT	Wild type
XBP1	X-box-binding protein 1
4E–BP	eIF4E binding protein

Data presented in the thesis are all from the authors' own experiments, other than some of the data presented in figure 14, 17, 24, 34, 35, which were performed collaboratively, as described in the figure legends.

Chapter 1. Introduction

1.1 Autophagy pathway and its regulation

1.1.1 Introduction

Degradation and recycling of cellular components is critical for all eukaryotic cells in order to maintain cellular homeostasis. As such, cells posses two main systems for the degradation of intracellular constituents: the ubiquitin-proteasomal pathway and the lysosome-autophagy pathway. It is generally considered that proteasomal degradation targets abnormally folded and small short-lived proteins, while autophagy pathway degrades large cytoplasmic components, including organelles, long-lived proteins and protein aggregates, as well as intracellular pathogens, by sequestering these constituents in double-membrane vesicles and delivering this cargo for lysosomal degradation (Mizushima and Klionsky, 2007). The crucial role of autophagy is highlighted by the fact that it is an evolutionary conserved process occurring throughout the eukaryotic phylogenetic tree, with the core autophagy machinery proteins showing great homology between yeast and mammalian cells (Reggiori and Klionsky, 2002; Feng *et al.*, 2014b).

The term autophagy encompasses three major types of lysosomal degradation pathways which differ in the manner of cargo delivery into the lysosomal lumen: microautophagy; chaperone-mediated autophagy; and macroautophagy. In microautophagy, cargo that is in close proximity to the lysosome becomes sequestrated by the direct invagination of the lysosomal membrane, which then pinches off into the lysosomal lumen. Microautophagy has been mostly studied in yeast cells and its importance in mammalian cells remains unclear (Mijaljica *et al.*, 2011). Chaperone-mediated autophagy (CMA) involves the delivery of soluble proteins directly across the lysosomal membrane after binding to a complex composed of the chaperones Hsc70 and Hsp90, and several co-chaperones associated

with the lysosomal receptor LAMP-2A (Cuervo and Wong, 2014). CMA is a selective process, as the proteins that are targeted for the degradation through CMA are individually selected based on Hsc70 recognition of a peptide motif KFERQ (Cuervo and Wong, 2014). CMA has been implicated in neurodegenerative diseases, but a recent report has also highlighted its role in regulating $CD4^+$ T cell activation (Valdor *et al.*, 2014).

During macroautophagy, sequestration of the cytosolic cargo involves "de novo" formation of an isolation membrane that surrounds the cytosolic material to be degraded, forming an intermediate vesicle called an autophagosome. The autophagosome subsequently fuses with the lysosome leading to the formation of the digestive compartment - the autolysosome. Lysosomal enzymes degrade the content of the vesicle, which facilitates the permease-mediated release of the recycled molecules via the lysosomal membrane (Mizushima, 2007; Feng *et al.*, 2014b; Hurley and Schulman, 2014). Despite intense investigation, the membrane source for autophagosome biogenesis remains unclear, and several potential sources have been suggested, including mitochondrial, Golgi, ER and plasma membranes (Mari *et al.*, 2011; Tooze, 2013). Recent evidence places the endoplasmic reticulum-mitochondrial interface as the most likely source (Hailey *et al.*, 2010; Hamasaki *et al.*, 2013). The importance of macroautophagy is highlighted by increased sensitivity of autophagy deficient cells to nutrient withdrawal and by the observation that mice lacking essential autophagy genes are unable to survive the neonatal starvation period and die shortly after birth (Kuma *et al.*, 2004; Saitoh *et al.*, 2008; Kroemer *et al.*, 2010). While macroautophagy is the primary cell response to the stress of nutrient deprivation, in recent years more complex and cell type specific functions have emerged, including roles in innate and adaptive immune responses (Levine *et al.*, 2011; Deretic *et al.*, 2013). Macroautophagy (herein referred to as autophagy) and its role in intestinal $CD4^+$ T cells is the major focus of this thesis.

1.1.2 Targets of autophagy

Although originally described as a non-selective pathway for bulk degradation, autophagy can also act as a highly selective process. While metabolic stress triggers mostly a non-selective type of autophagy where a portion of the cytoplasm is targeted for degradation, intercellular pathogens or damaged organelles are targeted in a selective fashion. This is achieved by the use of cargo-specific autophagy adaptors. These adaptors are able to recognize ubiquitinated substrates and target them to the autophagosome, a process that also requires adaptor binding to the protein LC3 (microtubule-associated protein L chain 3) or GABARAP (γ-aminobutyric acid receptor-associated proteins), through a specific amino acid sequence with the common W-X-X-L motif (where X=any amino acid) called LC3-interacting region (LIR) (Johansen and Lamark, 2011; Rogov *et al.*, 2014). To date, there is evidence for the targeted sequestration and selective autophagy of a diverse array of cytosolic cargos, including; aggregate-prone or misfolded proteins (aggrephagy) (Ravikumar *et al.*, 2002; Szeto *et al.*, 2006; Pankiv *et al.*, 2007; Johansen and Lamark, 2011); protein complexes in signalling cascades (Paul *et al.*, 2012; Shi *et al.*, 2012; Chuang *et al.*, 2013); peroxisomes (pexophagy) (Iwata *et al.*, 2006; Kim *et al.*, 2008); mitochondria (mitophagy) (Kissova *et al.*, 2004; Kim *et al.*, 2007; Schweers *et al.*, 2007; Tal *et al.*, 2007; Twig *et al.*, 2008; Okamoto *et al.*, 2009; Youle and Narendra, 2011); surplus ER (reticulophagy) (Bernales *et al.*, 2006; Cebollero *et al.*, 2012); ribosomes (ribophagy) (Kraft *et al.*, 2008; Cebollero *et al.*, 2012); ferritin (ferritinophagy) (Kidane *et al.*, 2006; Asano *et al.*, 2011); bacteria and viruses (xenophagy) (Levine *et al.*, 2011); lipid droplets (lipophagy) (Singh *et al.*, 2009); and glycogen (glycophagy) (Kotoulas *et al.*, 2004; Rabinowitz and White, 2010).

Well-known examples of autophagy adaptors include p62 (also called sequestosome 1) and NBR1 (neighbour of Brca1 gene), (Johansen and Lamark, 2011; Shaid *et al.*, 2013). These adaptors are also selective targets of autophagy, as they get degraded

together with the substrate that they target to the autophagosome. The adaptor p62 has the ability to bind to both mono- and poly- ubiquitinated proteins and has been implicated in the clearance of protein aggregates, while NBR1 plays a selective role in targeting midbody derivatives (Vadlamudi *et al.*, 1996; Kuo *et al.*, 2011; Shaid *et al.*, 2013). An example of a selective adaptor involved in mitophagy - Nix (Bnip3L) - has also been described (Schweers *et al.*, 2007; Novak *et al.*, 2010). Selective recognition of cytosolic pathogens involves the action of p62, NDP52 (nuclear dot protein 52 kDa) and optineurin, which all have the ability to target ubiquitinated bacteria for autophagic destruction (Rogov *et al.*, 2014). NDP52 was also shown to target inactive components of the miRNA processing pathway, thereby directly linking autophagy to miRNA biogenesis (Gibbings *et al.*, 2012). Interestingly, a recent study identified nuclear receptor coactivator 4 (NCOA4) as a new cargo-specific adaptor that mediates ferritinophagy, revealing new aspects of autophagy involvement in intracellular iron homeostasis (Mancias *et al.*, 2014). Altogether, the diversity of autophagy targets highlights the complex role of this pathway in regulating many aspects of cellular physiology during steady state and stress responses.

1.1.3 Mechanism of autophagy

Formation of the double membrane autophagosome structure is the key step in autophagy. An understanding of how this process is mediated can be largely attributed to a series of genetic studies in yeast, during which the core autophagy machinery was defined, with homologues in mammalian cells subsequently identified (Tsukada and Ohsumi, 1993; Mizushima, 2007; Yang and Klionsky, 2010). There are nearly 40 autophagy related (Atg) proteins that facilitate crucial steps of autophagosome formation and degradation (Mizushima *et al.*, 2011). The membrane dynamics involved in autophagosome formation and maturation are complicated and

still not completely understood. Autophagy is initiated by the formation of the isolation membrane, also called a phagophore, at the phagophore assembly site (PAS). In yeast, a single PSA is present at all times, whereas in mammalian cells the nucleation seems to occur at multiple PAS structures (Feng *et al.*, 2014b). The core autophagy machinery in mammalian cells can be categorised into five functional groups (**Figure 1**):

(1) The primary initiation complex; Ulk1/2 (unc-51-like kinase-1 or 2) – Atg13 – FIP200 - Atg101, which is under direct control of mTORC1 (mechanistic target of rapamycin complex 1) and AMPK (AMP-activated protein kinase). Activation of this complex requires a series of phosphorylation and dephosphorylation events of its components. Once activated, the Ulk1/2 complex translocates to the site of autophagosome formation and activates the second functional complex, the class III phosphatidylinositol 3-kinase (PI3K) complex (Reggiori *et al.*, 2005; Hara and Mizushima, 2009; Jung *et al.*, 2009; Mercer *et al.*, 2009).

(2) The PI3K complex - consisting of Vps34 (vacuolar protein sorting 34), Vps15 (vacuolar protein sorting 15), Beclin1, AMBRA1 (autophagy/beclin-1 regulator 1), and Atg14 or UVRAG (ultraviolet irradiation resistance-associated gene) - mediates the nucleation step of the phagophore. Once activated, this complex produces phosphatidylinositol-3-phosphate (PI3P) and therefore is involved in the recruitment of PI3P-binding effector proteins to the phagophore membrane, including WIPI1/2 (WD-repeat domain phosphatidylinositide-interacting-1 or 2). The PI3K complex is negatively regulated by the anti-apoptotic members of the Bcl-2 protein family. (Proikas-Cezanne *et al.*, 2004; Xie and Klionsky, 2007; Polson *et al.*, 2010).

(3) Atg9, the only known transmembrane Atg protein, and its cycling system, involving Atg2 and WIPI1/2, that shuttle between endosomes, autophagosomes and the Golgi apparatus, and are thought to provide membrane for the growing autophagosome (He *et al.*, 2008; Weidberg *et al.*, 2011).

Once initiated, elongation of the isolation membrane requires the subsequent action of two ubiquitin – like (UBL) conjugation systems:

(4) The Atg5–Atg12/Atg16l1 complex, which is assembled through the action of Atg7 (E1-like enzyme) and Atg10 (E2-like enzyme), and, once formed, locates to the growing autophagosome membrane (Mizushima *et al.*, 1998a; Mizushima *et al.*, 1998b; Mizushima *et al.*, 2003; Yang and Klionsky, 2009; Shaid *et al.*, 2013);

(5) The LC3 complex, containing LC3A/B/C, GABARAP, GABARAPL1/2, Atg7 (E1-like enzyme), Atg3 (E2-like enzyme) and the cysteine protease Atg4, which cleaves LC3 at the C-terminus to expose glycine, allowing the conjugation of the membrane lipid phosphatidylethanolamine (PE) to the soluble form of LC3, named LC3-I, and subsequent incorporation of LC3-PE (also called LC3-II) into the inner and outer membranes of autophagosomes (Kabeya *et al.*, 2000; Kabeya *et al.*, 2004; Yang and Klionsky, 2009; Weidberg *et al.*, 2010). The Atg5–Atg12/Atg16l1 complex acts as an E3-like enzyme during LC3 lipidation (Hanada *et al.*, 2007; Shaid *et al.*, 2013). LC3-II that is bound to the isolation membrane is thought to play a scaffolding role in membrane growth and is needed for autophagosome closure, but is also important for the binding of autophagy adaptors and thus in mediating selective types of autophagy (Geng and Klionsky, 2008). Upon closure of the autophagosome, the Atg5–Atg12/Atg16l1 complex and LC3-II located on the outer membrane both dissociate, whereas LC3-II that is incorporated to the inner membrane remains associated with the autophagosome and will eventually be degraded after fusion with the lysosome (Kabeya *et al.*, 2000). The mature autophagosome migrates into close proximity to the lysosome through the action of the dynein motor complex and microtubules (Yoshimori and Noda, 2008). Fusion with the lysosome requires the action of the HOPS complex (homotypic fusion and protein sorting) and three SNARE proteins (soluble NSF attachment protein receptors): syntaxin 17, SNAP-29 (synaptosomal associated protein 29 kDa) and VAMP7/8 (vesicle-associated membrane protein 7 or 8) (Itakura *et al.*, 2012; Takats *et al.*, 2013; Jiang *et al.*, 2014; Takats *et al.*, 2014).

Fusion is regulated by the Rab GTPase Rab7 (Kirisako *et al.*, 1999; Gutierrez *et al.*, 2004; Jager *et al.*, 2004) and recent evidence suggests that glycosylation of SNARE protein SNAP-29 is also involved in controlling autophagosome fusion (Guo *et al.*, 2014). Upon formation of the autolysosome compartment, lysosomal acidic hydrolases degrade the inner membrane and the luminal content, including carbohydrates, lipids, proteins and nucleic acids. Ultimately, this provides building blocks for anabolic processes and fuel for ATP synthesis. Eventually the autolysosome fissions to release lysosomes and autophagy is terminated (Mehrpour *et al.*, 2010)

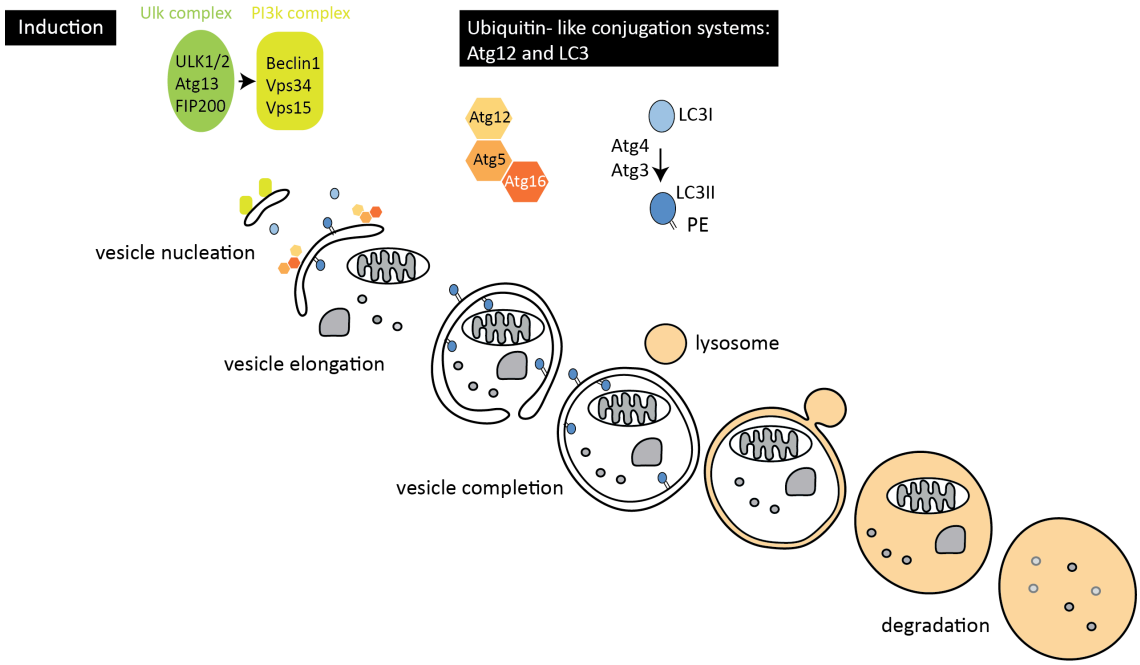


Figure 1 Schematic representation of macroautophagy pathway.

1.1.4 Cytoplasmic and nuclear regulation of autophagy

Autophagy is modulated in response to adverse micronenvironmental conditions, including nutrient depletion, hypoxia, growth factor withdrawal, inflammatory cytokines and infection. As such, a network of regulatory pathways governs its activity. For a long time autophagy was believed to be predominantly regulated at the post-transcriptional level by signalling mediators. Recently however, mechanisms of transcriptional, translational and epigenetic regulation of the autophagy pathway have emerged. It is now thought that cytosolic regulation generally serves as a more rapid, short-term response, whereas transcriptional modulation provides long-term regulation, although some nuclear events can also have a rapid effect on autophagy (Mehrpour *et al.*, 2010; Fullgrabe *et al.*, 2014; Galluzzi *et al.*, 2014).

The primary role of autophagy is to respond to cellular metabolic perturbations. Many signals that modulate autophagy levels do so by converging on the mechanistic target of rapamycin (mTOR). mTOR is a conserved serine/threonine kinase that integrates signals from various stimuli including amino acids, growth factors, energy, glucose and oxygen levels (Laplante and Sabatini, 2012). In mammals, mTOR forms two functionally distinct complexes, a rapamycin sensitive mTORC1 and a less rapamycin sensitive mTORC2. mTORC1 can be distinguish by the presence of the specific scaffolding protein Raptor (regulatory-associated protein of mTOR), while mTORC2 is characterised by the presence of the scaffolding protein Rictor (rapamycin-insensitive companion of mTOR) (Laplante and Sabatini, 2012). Both of these complexes take part in regulating autophagy, although the role for mTORC1 is more direct (Mehrpour *et al.*, 2010).

As mTORC1 can be activated by diverse stimuli, the modes of its activation can differ. In fact, how mTORC1 mechanistically integrates such a wide spectrum of signals is still not completely understood. On the one hand, mTORC1 integrates

systemic signals, by detecting the presence of extracellular growth factors and cytokines; on the other hand, it has the ability to respond to a variety of local cellular signals, including amino acid, glucose or oxygen levels. Ultimately, this leads to coordinated anabolic responses and cell growth (Dibble and Manning, 2013). Indeed, mTORC1 activation leads to increased protein synthesis through its downstream targets: the eukaryotic initiation factor 4E (eIF4E)-binding proteins (4E-BP) and the ribosomal S6 kinases (S6K). mTORC1 also induces lipid synthesis, mainly through activation of the transcription factors SREBP1/2 (sterol regulatory element-binding proteins 1 or 2), which induce the expression of enzymes involved in *de novo* sterol and fatty acid synthesis (Porstmann *et al.*, 2008; Duvel *et al.*, 2010). However, regulation of lipogenesis by mTORC1 might not be straightforward, as hyperactivation of mTORC1 by genetic deletion of the inhibitory protein TSC1 (tuberous sclerosis 1) results in impaired lipid synthesis, suggesting that regulatory feedback loops operate between mTORC1, Akt (also known as protein kinase B; PKB) and possibly mTORC2 (as described below) to control lipogenesis (Lamming and Sabatini, 2013). Other downstream effects of mTORC1 activation include upregulation of the pentose phosphate pathway, increased nucleic acid synthesis, and inhibition of autophagy (Ben-Sahra *et al.*, 2013; Robitaille *et al.*, 2013; Shimobayashi and Hall, 2014).

Growth factors activate mTORC1 through upstream signalling cascades involving class I PI3K and Akt. Activated Akt phosphorylates and inhibits the TSC1-TSC2 complex leading to activation of the small GTPase Rheb (Ras homolog enriched in brain) that in turn activates mTORC1. Moreover, Akt can also activate mTORC1 directly, independently of TSC and Rheb (Shimobayashi and Hall, 2014).

How mTORC1 detects intracellular amino acids is an area of intense investigation. Amino acids can activate mTORC1 on the lysosomal membrane from within the lumen and this involves the RAS-related GTP-binding protein family of small GTPases and a Ragulator complex, that together mediate the translocation of mTORC1 from the

cytoplasm to the surface of the lysosome, where mTORC1 becomes activated by Rheb (Dibble and Manning, 2013). This process also involves vacuolar ATPases (Zoncu *et al.*, 2011) and a series of recent studies revealed possible amino acid sensors for this lysosome-based activation of mTORC1 (Thomas *et al.*, 2014; Jewell *et al.*, 2015; Rebsamen *et al.*, 2015; Wang *et al.*, 2015b).

The autophagy pathway is regulated by mTORC1 in several ways. In the presence of nutrients, including amino acids, mTORC1 is active and suppresses autophagy through inhibitory phosphorylation of Ulk1, Atg13, AMBRA and Atg14 (Ganley *et al.*, 2009; Hosokawa *et al.*, 2009; Jung *et al.*, 2009; Kim *et al.*, 2011; Nazio *et al.*, 2013; Yuan *et al.*, 2013). mTORC1 also indirectly regulates autophagy by inhibiting TFEB (transcription factor EB), a transcription factor that induces several lysosomal and autophagy-specific genes; mTORC1 mediated phosphorylation of TFEB leads to its sequestration in the cytoplasm (Martina *et al.*, 2012; Roczniak-Ferguson *et al.*, 2012; Settembre *et al.*, 2012). During starvation, mTORC1 is inhibited, which activates autophagy. There is some evidence suggesting that, once activated, autophagy can further inhibit mTORC1 activity by promoting a negative feedback loop involving S6K, or through Ulk1-mediated inhibitory phosphorylation of Raptor (Mehrpour *et al.*, 2010; Dunlop *et al.*, 2011; Jung *et al.*, 2011). Conversely, autophagy activity eventually leads to increased nutrient levels, which in turn reactivates mTORC1 to terminate autophagy. This reactivation of mTORC1 that occurs during prolonged starvation is critical for the restoration of lysosomal homeostasis after prolonged autophagy (Yu *et al.*, 2010).

In comparison to mTORC1, the regulatory network for mTORC2 is less well understood, but recent finings are beginning to reveal new connections. Growth factors can activate mTORC2 through PI3K and known downstream targets include the kinases Akt, SGK1 (serum and glucocorticoid-regulated kinase 1) and PKC (protein kinase C). It was recently shown that mTORC2 could participate in regulating protein synthesis, as activated mTORC2 associates with actively translating ribosomes and

mediates co-translational phosphorylation and stabilization of the substrates. It was also shown that mTORC2 becomes activated after associating with ribosomes (Oh *et al.*, 2010; Zinzalla *et al.*, 2011; Shimobayashi and Hall, 2014). New evidence also places mTORC2 at the center of lipid metabolism regulation, as active mTORC2 increases lipid biosynthesis, acting through SREBP1c and the nuclear receptor PPARγ (peroxisome proliferator-activated receptor γ) (Hagiwara *et al.*, 2012; Yuan *et al.*, 2012), and might also play a role in inhibiting lipolysis (Cybulski *et al.*, 2009; Kumar *et al.*, 2010; Lamming and Sabatini, 2013). So far, there is no evidence that mTORC2 interacts with components of the autophagy machinery directly. However, mTORC2 can modulate autophagy indirectly through phosphorylation of Akt, which in turn activates mTORC1 and inhibits autophagy. Additionally, activation of Akt results in nuclear exclusion and transcriptional inactivation of Foxo3 (Forkhead box O3), a transcription factor recently found to promote autophagy by increasing the expression of several autophagy genes (including *Ulk2*, *Beclin 1*, *Vps34*, *Atg12*, *Atg4b*, *Lc3*, and *Gabarapl1)* and by modulating glutamine metabolism (Mammucari *et al.*, 2007; van der Vos *et al.*, 2012; Warr *et al.*, 2013; Fullgrabe *et al.*, 2014).

Another key sensor that coordinates cellular metabolic responses is AMPK, which, next to mTOR, can be considered a signalling hub for autophagy modulation. AMPK is a serine/threonine kinase that functions as a heterotrimer, consisting of a catalytic subunit (α), a scaffolding subunit (ß) and a regulatory subunit (γ). AMPK senses decreased energy levels by detecting changes in the ATP:ADP:AMP ratio in the cytoplasm, as binding of AMP and ADP to the γ subunit introduces allosteric changes and prevents inactivating dephosphorylation of the α subunit (Hardie *et al.*, 2012). Additionally, three upstream kinases that can activate AMPK by phosphorylation of the α subunit have been identified; LKB1 (liver kinase B1), CAMKKß ($Ca2^+$/calmodulin-activated protein kinase kinase-ß) and TAK1 (TGF (transforming growth factor-ß activated kinase-1) (Blagih *et al.*, 2012; Hardie *et al.*, 2012). In response to decreased intracellular ATP levels, AMPK initiates metabolic

reprograming towards catabolic reactions including glycolysis and lipid oxidation, increases glucose uptake, stimulates autophagy and mitochondrial biogenesis (Mihaylova and Shaw, 2011).

AMPK activates autophagy directly and indirectly. In the direct regulation, AMPK phosphorylates Ulk1/2 at multiple sites (Bach *et al.*, 2011; Egan *et al.*, 2011; Kim *et al.*, 2011). Of note, inhibitory phosphorylation of Ulk1 on Ser758 by active mTORC1 prevents association with AMPK and thereby inhibits autophagy (Kim *et al.*, 2011). AMPK can also phosphorylate various components of the Vps34 complexes that do not contain pro-autophagic adaptors, leading to the inhibition of its non-autophagic functions in Golgi-endosome trafficking (Kim *et al.*, 2013b). In addition to direct interactions, AMPK can indirectly activate autophagy by inhibiting mTORC1 through at least two mechanisms: by phosphorylation of the TSC complex, or by inhibitory phosphorylation of the Raptor subunit (Inoki *et al.*, 2003; Gwinn *et al.*, 2008). Importantly though, AMPK activity is not indispensable for autophagy induction, as starvation still induces autophagy in AMPK-null cells, suggesting a fine tuning role for this kinase in autophagy modulation (Kim *et al.*, 2011). Of note, there is some evidence that autophagy can downregulate AMPK activity, as Ulk1/2 were shown to inhibit AMPK through phosphorylation (Loffler *et al.*, 2011).

The unfolded protein response (UPR), a major ER stress pathway, is another described inducer of autophagy. There are three classical pathways of UPR that are mediated by three distinct ER membrane-embedded sensors: ATF6 (activating transcription factor-6), IRE1 (inositol requiring enzyme 1) and PERK (protein kinase RNA-like ER kinase) (Senft and Ronai, 2015). During steady state these proteins are inactivated by the chaperone BiP (also called GRP78), but during the UPR they are released from BiP inhibition and induce specialized transcription programmes mediated through cleaved ATF6 (for ATF6), spliced X-box-binding protein 1 (XBP1, for IRE1) and ATF4 (for PERK). The UPR also leads to PERK-mediated global inhibition of translation through the phosphorylation of the translation initiation factor eIF2α

(Senft and Ronai, 2015). Autophagy intersects with the UPR in several ways. For instance, the UPR can inhibit Akt – mTORC1 activity, and thereby promote autophagy (Qin *et al.*, 2010). In addition, activation of the PERK-eIF2α-ATF4 pathway leads to the transcriptional upregulation of several autophagy-related genes, including genes encoding LC3, Atg5 and Atg12 (Kroemer *et al.*, 2010; Rouschop *et al.*, 2010; B'Chir *et al.*, 2013). IRE1, which can act as both a serine/threonine kinase and an endoribonuclease, can activate autophagy by acting together with JNK (c-Jun N-terminal kinase) to promote Beclin1 activity (Deegan *et al.*, 2013). However, it appears that IRE1 may also act as a negative regulator of autophagy, as deletion of IRE1 or XBP1 in mice enhances autophagy (Vidal *et al.*, 2012; Deegan *et al.*, 2013). Interestingly, the UPR and autophagy seem to exhibit compensatory activity when one of these degradative pathways is defective and this has been demonstrated in several experimental systems (Ogata *et al.*, 2006; Yang *et al.*, 2010; Adolph *et al.*, 2013).

As already mentioned, transcriptional regulation is now appreciated to be one of the main regulatory mechanisms of autophagy. The transcription factor TFEB, which belongs to the basic helix–loop–helix leucine zipper family, is a master regulator of lysosomal and autophagy gene expression (Settembre *et al.*, 2011). TFEB controls the gene network called CLEAR (coordinated lysosomal expression and regulation), which contains the majority of genes encoding lysosomal proteins (Settembre *et al.*, 2013b). TFEB also regulates the expression of several genes encoding proteins belonging to the core autophagy machinery, including *Lc3*, *p62* and *Atg9* (Settembre *et al.*, 2011; Fullgrabe *et al.*, 2014). Upon autophagy induction, TFEB is rapidly recruited from the cytosol to the nucleus, and this is at least partially mediated by inhibition of mTORC1 (see above). Furthermore, TFEB appears to control lipid catabolism in response to starvation by controlling lysosomal lipase content and by inducing expression of PPARα (peroxisome proliferator-activated receptor-α) and

PGC1α (PPARγ co-activator 1α), which are important regulators of lipid metabolism (O'Rourke and Ruvkun, 2013; Settembre *et al.*, 2013a). In addition, two recent studies revealed that the nuclear receptor FXR (farnesoid X receptor), known for its role in regulating bile-acid metabolism, strongly suppressed autophagy induction and autophagy-associated lipid catabolism in liver cells (Lee *et al.*, 2014; Seok *et al.*, 2014). Acting in opposition to TFEB, the DNA binding protein ZKSCAN3 (zinc-finger protein with KRAB and SCAN domains 3) represses an extensive set of autophagy genes, including *Lc3* and *WIPI2* (Chauhan *et al.*, 2013). During starvation ZKSCAN3 accumulates in the cytoplasm and its activity is inhibited. Thus, TFEB and ZKSCAN3 seem to provide a switch mechanism during starvation-induced autophagy (Fullgrabe *et al.*, 2014).

Some of the other important regulators of autophagy include anti-apoptotic protein family members (Bcl-2, Bcl-X_L, and Mcl-1), which inhibit autophagy through interaction with Beclin1 (Kroemer *et al.*, 2010). The key tumour suppressor p53 is able to activate or inhibit autophagy, depending on its cellular localization (Pietrocola *et al.*, 2013; Fullgrabe *et al.*, 2014). The transcription factor NF-κB (nuclear factor-κB) can promote autophagy through increasing expression of some of the autophagy genes (including *Beclin1*) and NF-κB is in turn regulated by the autophagy pathway, although NF-κB can also downregulate autophagy, for instance after TNF stimulation (Kroemer *et al.*, 2010; Criollo *et al.*, 2012; Paul *et al.*, 2012; Pietrocola *et al.*, 2013). Autophagy is also induced during oxidative stress through the activity of the transcription factor HIF-1α (hypoxia-inducible factor 1α) (Zhang *et al.*, 2008; Bellot *et al.*, 2009). Additionally, activation of NAD-dependent class II histone deacetylases of the sirtuins family was shown to induce autophagy by interacting with several autophagy involved proteins, including Atg5, Atg7, LC3 and Foxo3a (Galluzzi *et al.*, 2014). Moreover, regulation through histone modifications is emerging as a new regulator of autophagy activity (Fullgrabe *et al.*, 2013). In

summary, multiple intersecting pathways modulate autophagy on many levels, reflecting the importance of this complex homeostatic pathway in the cellular adaptations to environmental factors.

1.1.5 Autophagy in the immune system – overview

Initially described as a 'self-eating' process that enables nutrient recycling during starvation, autophagy has now been connected to multiple cellular responses, among which immunological responses are playing a prominent role. The first links between autophagy and host immunity came from the observations that autophagy can target intracellular bacteria for degradation. Beyond this, autophagy plays a role in antigen processing, thymic selection, lymphocyte homeostasis and regulation of immunoglobulin and cytokine production. In the following section, the role of autophagy in the context of the intestinal immune system will be described.

1.2 Mechanisms enforcing intestinal immune homeostasis

1.2.1 General introduction

Large structural and functional differences are found between the small and large intestine, reflecting differences in their physiological functions. The small intestine (SI) is divided into three main segments: duodenum, jejunum and ileum. The large intestine starts with the caecum and continues through the proximal colon, the transverse (middle) colon, the distal colon and the rectum, terminating at the anus (Mowat and Agace, 2014). SI and colon differ in size and anatomical structure; the marked length of the SI and the characteristic finger-like projections known as villi increase the surface area of the SI, facilitating efficient nutrient absorption. In contrast, the large intestine, where water absorption and polysaccharide

fermentation by commensal microbes occurs, is much shorter than the SI and its surface is flat (Mowat and Agace, 2014). The anatomical and functional compartmentalisation of the gastrointestinal tract also dictates the major stimuli for the immune system: food-derived antigens predominate in the SI, while in the colon microbiota derived antigens dominate (Izcue *et al.*, 2009; Mowat and Agace, 2014).

The gastrointestinal tract contains a vast network of non-lymphoid and secondary lymphoid tissues that host numerous populations of leukocytes, many of which are intestine-specific subpopulations (Mayer, 2005). The gut associated lymphoid tissue (GALT) comprises the intestinal epithelium and lamina propria compartments (LP), as well as various secondary lymphoid structures, including the mesenteric lymph nodes (mLN), the Peyer's patches (PP) of the small intestine and isolated lymphoid follicles and cryptopatches that are distributed throughout the intestine (Mowat, 2003). The intestinal mucosa, comprising the epithelium, the underlying lamina propria and the muscularis mucosa, is the site where majority of immunological processes take place.

The gastrointestinal tract presents a unique challenge to the immune system, as it has to constantly monitor the vast surface for the presence of pathogens while at the same time maintaining tolerance to beneficial or innocuous antigens. In the intestinal mucosa both innate and adaptive components of the immune system participate in directing appropriate immune responses towards diverse challenges. Innate cells provide a broad immediate defence and initiate and instruct adaptive immunity, while cells of the adaptive immune system mediate specific responses and ensure immunity upon subsequent reencounter. The immune system has evolved to prevent invasion of the host by microbial species. However, the mammalian gastrointestinal tract is a preferential site for colonization of the host by commensals, a diverse community consisting of fungal, viral and bacterial species. The presence of a commensal microbiota is vital for optimal digestion and nutrient acquisition, as well as resistance to pathogenic infection, but commensal microbes also contribute to the development, maturation and activation of the host immune

system by influencing both innate and adaptive immune responses (Kabat *et al.*, 2014). A mutualistic dialog between the microbiota and intestinal immune system ensures coexistence through multiple mechanisms that we are just beginning to understand (Hill and Artis, 2010; Kabat *et al.*, 2014). Besides microbial communities, the intestinal immune system constantly encounters a vast dietary antigenic load, and the induction of the state of immune unresponsiveness towards these antigens (oral tolerance) is one of the main tasks for the mucosal immune system (Pabst and Mowat, 2012). As such, the intestinal immune system often employs different rules than the systemic immune system to ensure the right balance between tolerance and immunity, crucial for mucosal homeostasis. Disruption of this equilibrium can lead to the chronic pathologies of the gastrointestinal tract, such as inflammatory bowel diseases (IBD) or food allergies (Kaser *et al.*, 2010; Maloy and Powrie, 2011; Pabst and Mowat, 2012).

1.2.2 Intestinal epithelial cells

Intestinal epithelial cells (IEC) form a single barrier layer on the surface of the intestinal mucosa. The primary function of these cells is the nutrient absorption from the lumen. However, although not *bona fide* immune cells, their interactions with the intestinal microbiota and host leukocytes influence the immune response and play a crucial role in maintaining homeostasis (Pott and Hornef, 2012). The IEC monolayer is composed of several specialized cell types: stem cells, Paneth cells, goblet cells, neuroendocrine cells, and enteroabsorptive cells (van der Flier and Clevers, 2009). Multi-potent $Lgr5^{+}$ stem cells are located at the bottom of the intestinal crypts and by division these cells give rise to either transient amplifying cells or stem cells. The transient amplifying cells rapidly proliferate and differentiate and thereby ensure the renewal of the epithelial layer every 4-5 days (van der Flier and Clevers, 2009). Paneth cells that localize to the base of small intestinal crypts

are specialized secretory cells that produce large amounts of antimicrobial products including lysozyme, α-defensins and Reg3γ (Reg3α in humans) (Bevins and Salzman, 2011). An antimicrobial peptides (AMP) α-defensins play a crucial role not only in the defence against enteric pathogens but also in shaping the host microbiota, as mice lacking MMP7, an enzyme required for the maturation of α-defensins, exhibited significant changes in microbiota composition (Salzman *et al.*, 2010). In addition, AMP have modulatory functions in chemotaxis, TLR signalling and wound healing (Gallo and Hooper, 2012). Paneth cells also participate in maintaining crypt stem cell activity through production of EGF, TGF-α, Wnt3 and the Notch ligand Dll4 (Sato *et al.*, 2011).

Goblet cells are another class of secretory cells that produce heavily glycosylated mucins which, after secretion to the lumen, form a mucus gel layer (Pott and Hornef, 2012). This serves as a protective physical barrier and as a matrix loaded with secretory IgA and AMP, which fortify the mucosal barrier (Hill and Artis, 2010). Recent studies have suggested that mucus also has an additional role in promoting tolerogenic responses toward food and commensal antigens (McDole *et al.*, 2012; Shan *et al.*, 2013). For instance, glycosylated MUC2, the main component of the mucus layer, in complex with galectin-3 was shown to bind Dectin-1 and prime dendritic cells (DC) to induce Treg cells via enhanced production of TGF-β_1, IL-10 and retinoic acid (RA) (Shan *et al.*, 2013). The observation that mice with defects in MUC2 production develop spontaneous colitis emphasizes that mucus is essential for intestinal homeostasis (Van der Sluis *et al.*, 2006; Heazlewood *et al.*, 2008).

IEC are actively engaged in the dialogue between the microbiota and the immune system. Sensing of bacterial metabolites and structural components by IEC fortifies barrier integrity and protects from pathogen invasion (Goto and Kiyono, 2012). For example, recent studies underlined the crucial role of inflammasome signalling in the epithelium in regulating microbiota composition and for protection against infectious colitis (Elinav *et al.*, 2011; Nordlander *et al.*, 2014; Song-Zhao *et al.*, 2014). In

addition, the metabolite acetate, produced by commensal bacteria belonging to the genus *Bifidobacterium*, protected against mortality during enterohaemorrhagic *Escherichia coli* infection by promoting anti-apoptotic responses in IEC (Fukuda *et al.*, 2011). IEC also influence the recruitment, activation and differentiation of leukocytes by producing a variety of other modulatory factors in response to commensal microbiota, including TSLP (thymic stromal lymphopoietin), TGF-β_1, RA, IL-25 and IL-18 (Hill and Artis, 2010; Harrison *et al.*, 2015).

1.2.2.1 Autophagy in IEC

The IEC monolayer is in close proximity to microbiota communities within the gastrointestinal tract and is an entry site for mucosal pathogens. Recent studies that assessed the impact of autophagy deficiency on bacterial handling by IEC found that autophagy was essential for protection against intracellular bacteria, including *Salmonella typhimurium* and *Shigella flexneri*, by acting to limit bacterial replication and subsequent dissemination (Travassos *et al.*, 2010; Benjamin *et al.*, 2013; Conway *et al.*, 2013b; Lassen *et al.*, 2014).

Most studies of autophagy in IEC have concentrated on the functional role of ATG16L1 and in trying to understand how Crohn's disease-associated polymorphisms in *ATG16L1* may impact on epithelial homeostasis. It has been reported that Paneth cells from patients with Crohn's disease homozygous for the *ATG16L1* T300A variant allele, or from mice with a hypomorphic mutation of *Atg16l1*, exhibit abnormal granule structure and reduced AMP secretion (Cadwell *et al.*, 2008; Cadwell *et al.*, 2010). However, *Atg16l1* hypomorphic mice did not exhibit signs of spontaneous intestinal inflammation, although they showed increased susceptibility to DSS-induced colitis. However, this increased susceptibility of *Atg16l1* hypomorphic mice, as well as their Paneth cell abnormalities, were only observed when the mice harboured a commensal microbiota that contained a persistent enteric norovirus

(MNV) (Cadwell *et al.*, 2008; Cadwell *et al.*, 2010). These studies suggested that decreased autophagy levels drive Paneth cell abnormalities only when additional triggering factors are present. A recent study showed that ER stress could also be such a trigger (Adolph *et al.*, 2013). Analyses of mice with an IEC-specific deletion of the UPR response element *Xbp1* demonstrated that defects in the UPR pathway in Paneth cells were partially compensated by increased autophagy. However, when autophagy was also impaired, through IEC-specific deletion of the essential autophagy genes *Atg16l1* or *Atg7*, ER stress could not be resolved and this double defect led to the development of severe intestinal inflammation (Adolph *et al.*, 2013). Interestingly, Paneth cells from patients with the *ATG16L1 T300A* variant allele showed increased ER stress markers (Deuring *et al.*, 2014).

Consistent with the notion that manifestation of defective autophagy in Paneth cells could depend on additional environmental or genetic factors, two recent independent studies reported contradictory results when mice with a 'knock-in' of the risk-associated *Atg16l1* gene variant were analysed. Murthy *et al.* reported no changes in the morphology of Paneth cells in the knock-in mice (Murthy *et al.*, 2014), whereas Lassen *et al.* observed Paneth cell abnormalities (Lassen *et al.*, 2014). One possible explanation for these differences could be the distinct microbiota composition of the examined mice, unfortunately though these studies did not report the norovirus status of their experimental mice. Beyond Paneth cells, autophagy could also play an important functional role in other secretory IEC types, as *Atg5*-deficient colonic goblet cells were reported to show impaired mucus secretion (Patel *et al.*, 2013).

1.2.3 Intestinal mononuclear phagocytes (MP)

Mucosal mononuclear phagocytes (MP) compromise dendritic cells (DC) and macrophages. These cells are key players in intestinal homeostasis, as they provide a

crucial link between innate and adaptive immunity, and in maintaining functional compartmentalisation of systemic and mucosal immune system. Intestinal MP are a heterogeneous population; expression of CX_3CR1 (CX3C chemokine receptor 1) and CD103 (αE integrin) can be used to identify two major intestinal MP populations, which appear to promote intestinal tolerance in different ways (Coombes and Powrie, 2008; Varol *et al.*, 2010). Under homeostatic conditions, $CD103^+$ DC uptake intestinal antigens and migrate from the intestinal LP to the mLN, where they initiate T cell responses, promoting intestinal tropism through induction of homing receptors CCR9 and $\alpha4\beta7$, and preferentially induce tolerogenic Treg cells through production of TGF-β_1 and RA (Iwata *et al.*, 2004; Johansson-Lindbom *et al.*, 2005; Jaensson *et al.*, 2008). The ability to convert latent TGF-β_1 into its active form is important for this tolerogenic $CD103^+$ DC function (Paidassi *et al.*, 2011; Worthington *et al.*, 2011). Additionally, this $CD103^+$ DC subset also acts on B cells, promoting differentiation of naive B cells into IgA^+ plasma cells within the intestine lamina propria (Uematsu *et al.*, 2008). $CD103^+$ DC have also been identified as a crucial subset in promoting oral tolerance against food antigens (Pabst and Mowat, 2012), although CX_3CR1^+ MP may also promote oral tolerance through the transfer of soluble food antigens to $CD103^+$ DC via gap junctions, to facilitate Treg cell induction by $CD103^+$ DC (Mazzini *et al.*, 2014). CX_3CR1^+ MP (a population comprising both DC and macrophages), sample luminal contents through extended dendrites but appear to be non-migratory and have poor abilities for the naïve T cell priming (Rescigno *et al.*, 2001; Niess *et al.*, 2005; Schulz *et al.*, 2009b). However, CX_3CR1^+ MP may be involved in the secondary expansion of Tregs in the LP that had been primed initially in gut-draining lymph nodes (Hadis *et al.*, 2011).

During infection or inflammation, intestinal MP adopt a different phenotype, characterized by the production of proinflammatory cytokines and chemokines that coordinate host protective immune responses, but excessive activation of mucosal myeloid cells has also been associated with chronic inflammatory conditions (Laffont

et al., 2010; Zigmond *et al.*, 2012; Cerovic *et al.*, 2014). Commensal bacteria are able to directly modulate intestinal MP functions to regulate effector T cell responses in the LP. For instance, Atarashi *et al.* showed that ATP produced by commensal bacteria activates CX_3CR1^+ MP and leads to the induction of Th17 cells (Atarashi *et al.*, 2008) and microbiota derived signals were shown to induce IL-1ß production from mucosal MP that is essential for the induction of Th17 cells in the steady state gut (Shaw *et al.*, 2012).

1.2.3.1 Autophagy in mononuclear phagocytes (MP)

As professional phagocytic cells, DC and macrophages are particularly well equipped to handle bacteria and autophagy is now appreciated to play an important role in intracellular bacterial killing. Autophagy can be activated in MP upon pattern recognition receptor (PRR) triggering, including activation of TLR (Toll-like receptors) (Lee *et al.*, 2007a; Sanjuan *et al.*, 2007; Xu *et al.*, 2007; Delgado *et al.*, 2008; Shi and Kehrl, 2010) and NLR (NOD-like receptors) (Cooney *et al.*, 2010; Travassos *et al.*, 2010). In particular, activation of DC and macrophages with the NOD2 ligand MDP (muramyldipeptide) induces autophagy and this activation is reduced in DC with the *ATG16L1* T300A allele variant (Cooney *et al.*, 2010; Travassos *et al.*, 2010). Activation of NOD1 and NOD2 in the cytoplasm directs the autophagy machinery by recruiting ATG16L1 to the site of bacterial entry, although this interaction was not affected in cells with homozygous expression of the T300A variant (Travassos *et al.*, 2010). Sequestration of cytosolic bacteria by xenophagy requires the coordinated action of specialized autophagy adaptors that recognize ubiquitin-tagged or galectin-tagged pathogens for degradation (Thurston *et al.*, 2012).

The importance of autophagy in defence against cytosolic pathogens is highlighted by the fact that several pathogens have developed sophisticated adaptations to inhibit specific stages of autophagy (Levine *et al.*, 2011; Huang and Brumell, 2014). An

important role for autophagy in pathogen handling in DC and macrophages has been observed in *Salmonella*, *Shigella* and *Listeria* infections (Patel and Stappenbeck, 2013; Baxt and Xavier, 2015). The role of autophagy in MHC II (major histocompatibility complex class II) antigen presentation is well documented; autophagy can enhanced MHC II expression on MP and is directly engaged in delivering cytoplasmic antigens, including bacterial antigens, into MHC II compartments (Dengjel *et al.*, 2005; Schmid *et al.*, 2007; Lee *et al.*, 2010a).

In addition to PRR triggering, inflammatory cytokines can also influence autophagy in MP. For instance, autophagy is induced by IFN-γ and other Th1 type cytokines that are secreted during bacterial infection, while Th2 type cytokines inhibit autophagy (Levine *et al.*, 2011). Conversely, autophagy can also influence cytokine signalling in myeloid cells. In particular, autophagy was shown to downregulate secretion of the inflammasome-associated cytokines IL-1ß and IL-18 by murine and human macrophages (Saitoh *et al.*, 2008; Plantinga *et al.*, 2011). The link between autophagy defects and excessive inflammasome activation may be due to reactive oxygen species (ROS) production in response to mitochondrial stress (Nakahira *et al.*, 2011; Zhou *et al.*, 2011), as well as defects in targeting of assembled inflammasomes for autophagosomal degradation (Shi *et al.*, 2012). In bone marrow chimeric mice with an Atg16l1-deficient hematopoietic compartment, increased production of inflammasome cytokines was associated with increased susceptibility to DSS-induced colitis (Saitoh *et al.*, 2008). Apart from the crosstalk between autophagy and inflammasome signalling, autophagy also regulates type I IFN responses. In plasmacytoid DC, autophagy was essential for IFN-α production in response to viral dsRNA and this was attributed to the role of autophagy in delivering viral replication intermediates to endosomal TLR7 (Lee *et al.*, 2007a). Conversely, in some instances autophagy appears to negatively regulate virus-sensing pathways by limiting signalling through RLR (RIG-1-like receptors) (Tal *et al.*, 2009; Lei *et al.*, 2012). Recently, autophagy was shown to enhance NFκB signalling in F4/80^{hi} macrophages

by selective degradation of the negative regulator A20 and this contributed to enhanced protection against *Candida albicans* infection (Kanayama *et al.*, 2015). This is interesting, as autophagy was previously shown to limit NFκB-signalling in activated T cells through selective degradation of Bcl-10 (Paul *et al.*, 2012), suggesting that the impact of autophagy on proinflammatory signalling pathways could be cell-type specific.

Taken together, these studies suggest that the autophagy pathway intersects with other pathogen sensing and cellular stress responses to promote immune defence in MP populations. However, detailed analysis of the role of autophagy in the intestinal subsets of DC and macrophages is lacking. Since mucosal MP show some unique features, including hyporesponsiveness to PRR stimulation (Cerovic *et al.*, 2014), it would be interesting to investigate the potential contribution of autophagy to these specific intestinal adaptations.

1.2.4 B cells and antibody responses

B cells are abundant within the GALT. Early stages of B cell development primarily occur in the bone marrow where production of antibodies begins with V(D)J recombination to generate IgM and IgD expressing immature B cells (Stavnezer *et al.*, 2008). Somatic hypermutation (SHM) and class switch recombination (CSR) are elicited following antigen recognition to increase antibody affinity and to generate different antibody isotypes, including, IgE, IgG and IgA. Intestinal B cell development shows some unique features as LP resident B cells were shown to undergo V(D)J recombination and B cell receptor (BCR) editing (Wesemann *et al.*, 2013). Importantly, early B cell development in the gut was promoted by commensals, suggesting involvement of microbiota-derived antigens in driving BCR editing (Wesemann *et al.*, 2013), which might have implications for immunoglobulin diversification at mucosal sites and for tolerance against commensal antigens.

IgA constitutes the major antibody isotype produced in mammals and secretory IgA (sIgA) is the most abundant immunoglobulin in mucosal secretions (Macpherson *et al.*, 2011). As such, it is estimated that around 80% of the total antibody production takes place in the intestinal mucosa, making the gut the largest antibody-producing organ of the body (Brandtzaeg, 2009). sIgA is a dimeric antibody that binds to the polymeric Ig receptor (pIgR) on the basolateral surface of IEC and is subsequently translocated across the epithelium and released into the lumen, where it interacts with various intestinal antigens, including self, dietary and commensal antigens. This limits the access of commensal bacteria and soluble antigens to the intestinal epithelium and LP, and it appears that bacteria with high colitogenic potential can be distinguished on the basis of their high IgA coating (Pabst, 2012; Palm *et al.*, 2014). Of note, pentameric IgM antibodies are also actively secreted into the intestinal lumen via the same pIgR. IgM antibodies have a similar function in shielding IEC from antigenic exposure and are particularly important in newborns (Brandtzaeg, 2009).

Class switch recombination to IgA occurs mainly in the mLN and PP through both T cell-dependent and T cell-independent mechanisms, and studies in GF mice established that commensal microbiota are strong inducers of IgA production (Macpherson *et al.*, 2011; Pabst, 2012; Bunker *et al.*, 2015). In the T cell-independent pathway microbiota induce production of IgA through the modulation of IEC and MP, that in turn secrete BAFF (B cell-activating factor), APRIL (a proliferation-inducing ligand) and TGF-β_1, cytokines that promote IgA switching (Macpherson *et al.*, 2011). Production of IgA in response to microbiota possesses some unique features, as it requires a high dose of bacteria to be induced, but at the same time is very persistent. In addition, it is flexible, as IgA specificity can rapidly change and adapt in response to alterations in microbiota composition (Hapfelmeier *et al.*, 2010; Lindner *et al.*, 2015). Moreover, it appears that gut residing plasma cells can also acquire unique "innate like" properties that are dependent on

microbiota stimulation, as IgA^+ plasma cells can secrete TNFα and inducible nitric oxide synthase (iNOS) (Fritz *et al.*, 2012).

Presentation of commensal antigens by $CD103^+$ DCs in the mLN induces an IgA response (Kinnebrew and Pamer, 2012). Additionally, follicular dendritic cells (FDC), which are crucial organizers of B cell follicles, promote class switching to IgA in germinal centers of PP after being stimulated synergistically through retinoic acid receptor and by commensal bacteria through TLRs (Suzuki *et al.*, 2010). Sensing microbiota through innate signalling pathways is important in several aspects of the B cell response. For example, MyD88 signalling is required for protection from microbial dissemination after DSS-induced tissue damage, through influencing IgM, but not IgA production (Kirkland *et al.*, 2012). Similarly, in *Myd88*-deficient mice, protection from bacteraemia caused by commensal bacteria is provided by systemic T cell-dependent IgG production directed against microbiota antigens (Slack *et al.*, 2009). The lack of systemic antibody responses towards commensal microbiota is considered a sign of well-maintained sequestration of commensals and homeostasis (Macpherson *et al.*, 2011), but it proves to be beneficial in the absence of innate immune protection (Lochner *et al.*, 2011).

Although the important role for mucosal secretion of sIgA and pentameric IgM has been well established, there is evidence that active transport across the epithelial layer takes place also for IgG and IgE. In case of IgG antibodies, the neonatal Fc receptor (FcRn) is responsible for transcytosis across the IEC and moreover, this transport is bidirectional, as IgG are able to bind antigen in the lumen and immune complexes may then be retrieved and released into LP, where they are proposed to provide antigen for dendritic cell sampling (Yoshida *et al.*, 2004; Pyzik *et al.*, 2015).

IgE responses are an important effector arm of type 2 responses as they mediate first line of defence in parasitic infections and have also been implicated in protection

from toxins and venoms (Marichal *et al.*, 2013; Wu and Zarrin, 2014; Tsai *et al.*, 2015). IgE also mediates type I hypersensitivity, which encompasses local, as well as potentially life threatening systemic anaphylaxis. Inappropriate IgE-mediated immune responses towards innocuous antigens can lead to the development of allergic diseases (Wu and Zarrin, 2014). The high affinity IgE receptor FcεR1 is expressed predominantly on mast cells and basophils. Unlike other immunoglobulin classes, IgE effector function is related almost entirely to its capacity to bind to its high affinity receptor before antigen recognition. In a classic immediate hypersensitivity reaction antigen-mediated crosslinking of IgE that is bound to the FcεR1 leads to rapid mast cell degranulation and the release of proteases and vasoactive peptides, as well as triggering synthesis of lipid mediators and cytokines. The latter factors produce the "late phase" symptoms, which include recruitment and activation of inflammatory leukocytes (Gould and Sutton, 2008).

Presumably because of the rapid and potent effects of IgE crosslinking, the amount of this immunoglobulin is tightly controlled. IgE has the shortest half-life and is the least abundant immunoglobulin, being present in the ng/ml concentration range in serum, which is around 1000-fold lower than other antibody isotypes (Wu and Zarrin, 2014). Mucosal sites, including the intestine, support isotope switching to IgE and indeed IgE is relatively abundant in the intestine (Coeffier *et al.*, 2005; Gould and Sutton, 2008), with increased production being described in patients with food allergies (Belut *et al.*, 1980; Berin and Sampson, 2013).

IgE transcytosis across the intestinal epithelial barrier involves the low affinity IgE-specific receptor CD23/FcRII, and, similarly to IgG, also appears to be bidirectional, potentially resulting in antigen retrieval from the lumen to the intestinal LP (Yang *et al.*, 2000; Bevilacqua *et al.*, 2004; Baker *et al.*, 2010). Active transport of IgE across the intestinal IEC to the lumen might have a particularly important role during helminth infections, as the concentration of IgE in the lumen after parasitic infection rapidly increases (Negrao-Correa *et al.*, 1996). However, the transport of antigen

complexes from the lumen is thought to facilitate the rapid intestinal physiological changes that occur during allergic reactions to food antigens (Gould and Sutton, 2008). Consistent with this notion, IEC expression of the IgE receptor CD23/FcRII is increased in individuals with food allergies and IBD (Kaiserlian *et al.*, 1993).

Recent data demonstrated that IgE in the gut acts beyond driving immediate hypersensitivity reactions and mediates long lasting immunomodulatory functions by enhancing the induction of pro-allergic Th2 cells and inhibiting Treg cell induction (Burton *et al.*, 2014). Of note, increases in allergen-specific IgA and IgG_4 (or its equivalent IgG_1 in mice (Mestas and Hughes, 2004)) during the course of food allergy may constitute a protective mechanism, acting by blocking the interaction between the allergen and IgE (Berin and Sampson, 2013; Valenta *et al.*, 2015).

1.2.4.1 Autophagy in B cells

In contrast to what has been described for T lymphocytes, autophagy seems largely dispensable for the development and maintenance of mature B lymphocytes in the periphery. Studies using mice with B cell specific deletion of Atg5 or Atg7 (Cre expressed under the control of CD19 promoter: $Atg5^{\Delta CD19}$ or $Atg7^{\Delta CD19}$ mice) indicated that the numbers of mature B cells and the ratios of marginal-zone B cells to follicular B cells are not affected when autophagy is lacking (Conway *et al.*, 2013a; Pengo *et al.*, 2013; Chen *et al.*, 2014). Interestingly, while B-2 and B-1b populations were not affected by Atg5 or Atg7 deficiency, the B-1a B cell population in the peritoneal cavity was markedly reduced. It remains unclear why the development of this population of peripheral B-1a cells is uniquely sensitive to autophagy deficiency (Conway *et al.*, 2013a; Pengo *et al.*, 2013; Chen *et al.*, 2014).

Studies on antibody responses, plasma cell and memory B cell formation provided evidence of a role for autophagy in regulating these processes. After examining the capacity of autophagy-deficient B cells to produce immunoglobulins, Conway *et al.*

reported decreased primary antibody responses to antigen immunization or following infection with *Heligmosomoides polygyrus*, along with defective plasma cell differentiation (Conway *et al.*, 2013a). However, in contrast, a recent report observed no defects in primary antibody responses from autophagy-deficient B cells after antigen immunization (Chen *et al.*, 2014). Moreover, an extensive analysis of autophagy deficient plasma cells revealed that although autophagy did not affect differentiation or proliferation of plasma cells, it was necessary to promote their long-term survival (Pengo *et al.*, 2013). Surprisingly, immunoglobulin production was in fact increased in Atg5-deficient plasma cells, a phenomenon that authors attributed to a dysregulated ER stress pathway. They detected signs of elevated ER stress in autophagy-deficient plasma cells and showed that this led to increase Blimp-1 expression, which in turn resulted in increased IgH expression and immunoglobulin production (Pengo *et al.*, 2013). However, after *in vivo* challenge with a pneumococcal vaccine, antibody levels were reduced, as a result of decreased survival of autophagy-deficient plasma cells (Pengo *et al.*, 2013). As the choice of the antigen and adjuvant for immunization had a significant impact on the *in vivo* antibody responses in the $Atg5^{\Delta CD19}$ mice, it is plausible that some of the discrepant observations on antibody production by autophagy-deficient B cells were due to the different immunization regimes (Pengo *et al.*, 2013). It is noteworthy that a requirement for autophagy appears to be shared by distinct types of professional secretory cells, as Paneth cells, goblet cells and plasma cells are all sensitive to perturbations in autophagy and ER stress pathways.

Other investigators focused on analysing memory B cell responses after influenza virus infection and showed that although autophagy was dispensable for initial memory B cell formation, the survival of memory B cells and secondary antibody responses after re-challenge were heavily dependent on autophagy, and this defect could be partially rescued by treatment with a ROS scavenger (Chen *et al.*, 2014; Chen *et al.*, 2015). These results parallel studies on memory $CD8^+$ T cell responses

during viral infections where autophagy was also implicated in the later stages of memory formation (Puleston *et al.*, 2014; Xu *et al.*, 2014).

Overall, it appears that autophagy, while largely dispensable for mature B lymphocyte development, is necessary to maintain secondary, long-lasting antibody responses. However, one study reported decreased numbers of B cells in the intestinal LP and PP in $Atg5^{\Delta CD19}$ mice, indicating that intestinal B cells might have a higher dependence on autophagy compared to other peripheral B cells, although this has not been further investigated (Conway *et al.*, 2013a). Taking into account the marked differences displayed by the intestinal B cell compartment, it would be of interest to address the potential role of autophagy in homeostasis of mucosal B cells and IgA^+ plasma cells.

1.2.5 Innate lymphoid cells

Innate lymphoid cells (ILC) are a recently recognized type of innate cells that are found at enriched frequencies in mucosal tissues. ILC arise from a common lymphoid precursor and share functional characteristics with $CD4^+$ T cells, however they do not express a rearranged antigen receptor (Spits *et al.*, 2013). These cells can be broadly classified into three subgroups named ILC1, ILC2 and ILC3, characterised by expression of the lineage-specifying transcription factors, T-bet (ILC1), Gata3 (ILC2) or Rorγt (ILC3), resembling Th1, Th2 or Th17 cells, respectively (Spits *et al.*, 2013).

ILC provide early innate protection from mucosal pathogens and play a role in regulating microbiota composition and in tissue remodelling (Sonnenberg and Artis, 2015). ILC2 are an early source of IL-5 and IL-13 that they secrete in response to IL-25 and IL-33 released by IEC (Sonnenberg and Artis, 2015). These cells can mediate rapid innate type-2 immune responses and contribute to anti-helminth protection in the gut by inducing eosinophilia and mucus secretion (Moro *et al.*, 2010), but they can also drive eosinophilic lung inflammation (Lambrecht and Hammad, 2015).

ILC3 are the major innate source of IL-22 in the intestinal LP and depletion of ILCs in *Rag*$^{-/-}$ (Recombination activating gene)-mice led to the systemic dissemination of potentially pathogenic commensals such as *Alcaligenes xylosoxidans*, which was prevented by administering IL-22 (Sonnenberg *et al.*, 2012). IL-22 signals drive secretion of protective AMP by IEC, including Reg3γ (Zheng *et al.*, 2008; Pickert *et al.*, 2009; Sonnenberg *et al.*, 2011). IL-22 production by ILC3 can be indirectly regulated by commensal bacteria: for instance, systemic administration of flagellin triggers TLR5 LP MP to produce IL-23 that stimulates IL-22 production by RORγt^{+} ILC, leading to Reg3γ production by IEC (Kinnebrew *et al.*, 2010; Van Maele *et al.*, 2010; Kinnebrew *et al.*, 2012). Dietary compounds, such as phytochemicals derived from the catabolism of cruciferous vegetables, may also influence ILC3 function via the Ahr (aryl hydrocarbon receptor) (Li *et al.*, 2011a). Indeed, Ahr$^{-/-}$ mice display reduced frequencies of NKp46^{+} ILC, a subset of the ILC3 family, and are impaired in IL-22 production (Lee *et al.*, 2012; Qiu *et al.*, 2012). While ILC3 are clearly important in protecting against commensal dissemination in some settings, excessive ILC activity can drive intestinal inflammation in chronic models of colitis (Buonocore *et al.*, 2010) and similar pro-inflammatory populations of ILC have been identified in IBD patients (Geremia *et al.*, 2011). While it would be interesting to establish if and how autophagy affects the development and functions of ILC and thereby mucosal homeostasis, thus far this has not been experimentally addressed.

1.2.6 T cells

The intestinal LP and epithelium together contain the largest population of T lymphocytes in the body (Mowat and Agace, 2014). Gut T cells are highly heterogeneous and many populations are unique to the mucosa. T cells found at the basement membrane between enterocytes are classified as intraepithelial lymphocytes (IEL). IEL are particularly enriched in the small intestine. Two major

subtypes can be distinguished in mice: conventional IEL, which express CD4 or the CD8αβ heterodimer as well as an αβTCR; and unconventional IEL, which express the CD8αα homodimer and either a γδTCR or an αβTCR (Mowat and Agace, 2014). Overall γδ T cells constitute a large proportion of IEL (approximately 60%), while $CD4^+$ T cells are greatly under-represented (Cheroutre *et al.*, 2011). Intestinal IEL regulate epithelial growth and homeostasis, for example through secretion of TGF-β_1 (Cheroutre *et al.*, 2011), but are also essential in protection against pathogens, as γδ T cells are an important source of IL-17A (Martin *et al.*, 2009).

The intestinal LP harbours a significant population of $CD4^+$ T cells, which predominantly express TCRαβ. $CD8\alpha\beta^+$ cells are also present, although in lower frequencies (Ullrich *et al.*, 1990; Shale *et al.*, 2013). LP $CD4^+$ T cells are the main focus of the work presented in this thesis and these cells will be described in more detail in the following section. In addition, small subsets of T cells that express an invariant TCR are also present in the intestinal mucosa (Mowat and Agace, 2014). These subsets include mucosal-associated invariant T (MAIT) cells and invariant natural killer T (iNKT) cells. MAIT cells express a semi-invariant TCR that recognizes bacteria-derived vitamin B metabolites presented by the MHC class I-related protein (MR1) (Ussher *et al.*, 2014). MAIT cells contribute to protection against enteric bacteria, as they rapidly produce cytokines and exert cytolytic activity upon activation (Ussher *et al.*, 2014). iNKT cells, which in mice constitute approximately 0.5% of small intestinal LP lymphocytes, express an invariant form of the αβ TCR that is able to recognize lipid antigens presented by the CD1d molecule (Matsuda *et al.*, 2008). As well as cytolytic activity, iNKT cells can also rapidly produce a spectrum of effector cytokines at an early stage in immune responses, including IFN-γ, IL-4, IL-10, IL-13 and IL-17A, allowing these cells to participate in a range of immune responses, including antimicrobial defence (Matsuda *et al.*, 2008).

1.3 $CD4^+$ T cells

1.3.1 Selection and differentiation of $CD4^+$ T cells

Generation of $CD4^+$ T cells occurs in the thymus and requires continuous trafficking of bone marrow derived lymphoid progenitor cells (Koch and Radtke, 2011). The aim of the thymic selection is the generation of T cells that have MHC restricted TCR and do not display pathological self-reactivity. The earliest thymic progenitors are double negative (DN) for CD4 and CD8 expression and in this stage reside in the thymic cortex where they undergo Rag-dependent rearrangement of the TCRß locus. Assembly of the surface pre-TCR complex allows the progression through ß-selection and upregulation of the co-receptors CD4 and CD8 to give rise to double positive (DP) cells in which TCRß locus rearrangement is ceased (Schatz and Ji, 2011). DP thymocytes undergo rearrangement of the TCRα locus, resulting in the expression of a functional αßTCR complex. Somatic recombination of TCR gene loci facilitates generation of a diverse TCR repertoire with potentially useful antigen specificity, however several thymic mechanisms ensure selection and adjustment of the TCR repertoire. Thymocytes with the capacity of interacting with self peptides presented on MHC I or MHC II molecules expressed on thymic cortical epithelial cells (cTEC) are positively selected towards the CD8 or CD4 lineages, respectively, whereas thymocytes that fail to proceed through positive selection die by neglect (Stritesky *et al.*, 2012).

Negative selection ensures deletion of thymocytes with potentially pathogenic specificity. During negative selection thymocytes cease TCR rearrangement and migrate to the medulla where they interact with peptides expressed by thymic medullary epithelial cells (mTEC) and thymic antigen presenting cells (APC) (Derbinski *et al.*, 2001). Expression of the transcription factor AIRE endows mTEC with the capacity to present peptides derived from peripheral tissue antigens that would not otherwise be present in the thymus (Mathis and Benoist, 2009). At this

stage, the affinity of peptide-MHC–TCR binding dictates the fate of the thymocytes. Intermediate affinity to self-peptide-MHC complexes promotes selection, whereas high affinity leads to the removal of such self-reactive clones by induction of apoptosis (clonal deletion). Foxp3 (forkhead box P3)$^+$ Treg cells are thought to originate from clones whose TCR receptor exhibits the affinity at the threshold between intermediate and high, and this enables generation of Treg cells with relatively high self-reactivity, that are subsequently able to enforce dominant peripheral tolerance (Stritesky *et al.*, 2012; Xing and Hogquist, 2012).

Interestingly, autophagy has been implicated in shaping the thymic repertoire. Both cTEC and mTEC show high levels of autophagy (Nedjic *et al.*, 2008; Kasai *et al.*, 2009) and the presentation of certain self-antigens on cTEC was promoted by autophagy, implying a role in positive selection (Nedjic *et al.*, 2008). During negative selection, it appears that autophagy in mTEC is dispensable for abundant antigens, as it can be compensated by presentation by thymic APC, but may be more important when antigen is present at lower doses (Aichinger *et al.*, 2013). However, the physiological relevance of autophagy-associated antigen presentation during negative selection remains controversial, as autoimmunity development depended on the experimental model used; T cell deficient mice which received an Atg5-deficient thymus developed autoimmunity (Nedjic *et al.*, 2008), but this was not the case in mice where *Atg7* was selectively deleted in thymic epithelium using a Cre-loxP approach (Sukseree *et al.*, 2012).

T cells that successfully complete positive and negative selection leave the thymus and recirculate through the bloodstream and the peripheral lymphoid tissues, where they persist by receiving homeostatic signals, which include pro-survival signalling through the IL-7R (Tan *et al.*, 2001). Recirculating naïve T cells can be activated in secondary lymphoid organs by antigen-driven TCR signalling and co-stimulatory signals provided by activated APC (T cell priming) (Chen and Flies, 2013). Within the intestinal mucosa the majority of the CD4$^+$ T cells display an activated/memory

phenotype (Shale *et al.*, 2013). Once an antigen-specific T cell response is initiated, it goes through a phase of clonal expansion and differentiation, and following antigen/pathogen eradication the T cells undergo a contraction phase, and the establishment of immune memory.

Activated $CD4^+$ T cells differentiate into subsets of T helper cells, which have traditionally been classified according to the expression of the lineage-specifying transcription factors (so-called master transcriptional regulators) and effector cytokine profiles. As such, Th1, Th2, Th17, Tfh and Treg cells can be distinguished. Metabolic signals and the surrounding cytokine milieu greatly affect the differentiation process (Zhu *et al.*, 2010). Although the $CD4^+$ T cell subsets paradigm provides a useful framework for defining different functional $CD4^+$ T cell responses, recent data suggest that this paradigm oversimplifies the dynamic interactions within the transcriptional network that orchestrates $CD4^+$ T cell differentiation, and which in turn provides a degree of functional and phenotypic plasticity within $CD4^+$ T cell subsets (O'Shea and Paul, 2010; Wang *et al.*, 2015a). Nevertheless, the functional specialization of $CD4^+$ T cells generates effective immune responses that are tailored to meet particular infectious or inflammatory insults.

1.3.2 Metabolic responses to TCR activation

Activation of $CD4^+$ T cells initiates multiple changes in the transcriptional and translational programme, which go hand in hand with dynamic metabolic changes, matching bioenergetic and biosynthetic demands. Naïve T cells are maintained in a metabolic state that favours energy production over biosynthesis and rely on mitochondrial oxidative pathways for maximal energy generation, fuelled predominantly by lipid and amino acid oxidation (MacIver *et al.*, 2013). Activation of $CD4^+$ T cell drives a rapid proliferative response, which drastically increases the

demand for energy and building blocks for biosynthesis (MacIver *et al.*, 2013). During the initial growth phase (up to 24h after activation) lipid oxidation is downregulated, and glycolytic, pentose-phosphate and glutaminolytic pathways increase (Wang and Green, 2012). This initial metabolic shift is orchestrated by the transcription factors c-Myc, HIF-1α (hypoxia inducible factor-1α) and the nuclear receptor ERRα (estrogen-related receptor α), leading to an increase in amino acids, nucleic acids and lipid synthesis (Michalek *et al.*, 2011b; Wang *et al.*, 2011a; Buck *et al.*, 2015). As activated $CD4^+$ T cells begin to proliferate and differentiate, metabolic programs support the commitment into separate lineages, with major roles for mTOR and AMPK in tailoring the metabolic adaptations of particular $CD4^+$ T cell subsets (Michalek *et al.*, 2011a; Pollizzi and Powell, 2014). Towards the end of an immune response, some of the $CD4^+$ T cells become memory cells and revert back to lipid oxidation, maintaining increased capacity for efficient energy generation (Buck *et al.*, 2015).

1.3.3 Th1 cells

The original description of two distinct subsets of $CD4^+$ T cells, which were named Th1 and Th2, dates from the work of Mosmann & Coffman, almost 30 years ago (Mosmann , 1986). Th1 and Th2 cells can be generated from cells with identical TCR specificity under appropriate polarising conditions *in vitro* (Hsieh *et al.*, 1992; Seder *et al.*, 1992). The strength of TCR signalling during differentiation can dictate the fate of naïve CD4 T cells; generally, stronger signalling leads to Th1 differentiation while very weak signalling is thought to favour Th2 differentiation (Constant and Bottomly, 1997; Tao *et al.*, 1997).

Th1 cells are characterised by production of the signature cytokine IFN-γ, but can also secrete TNF-α, GM-CSF and lymphotoxin and they are considered good producers of IL-2 (Zhu *et al.*, 2010). Th1 cells typically express the chemokine receptors CXCR3

and CCR5 (Bonecchi *et al.*, 1998; Zhu *et al.*, 2010). T-bet (T-box expressed in T cells) is the master transcription factor that orchestrates Th1 cell development (Szabo *et al.*, 2000). Activation of naïve $CD4^+$ T cells in the presence of IFN-γ activates T-bet and IRF1 (IFN-γ-inducible transcription factor 1) in a STAT1 (signal transducer and activator of transcription 1)-dependent manner (Lighvani *et al.*, 2001; Kano *et al.*, 2008). As Th1 differentiation proceeds, the *Ifny* gene locus becomes epigenetically activated by T-bet, which leads to reinforcement of Th1 commitment through an autocrine feedback loop (Lighvani *et al.*, 2001; Zhang *et al.*, 2001b). In addition, T-bet acts in conjunction with IRF1 to up-regulate expression of the IL-12 receptor (Mullen *et al.*, 2001; Afkarian *et al.*, 2002; Kano *et al.*, 2008). IL-12 can then further enhance and maintain Th1 differentiation through the activity of STAT4 (Mullen *et al.*, 2002; Yang *et al.*, 2007; Schulz *et al.*, 2009a). Metabolic sensors that favour differentiation towards Th1 lineage include signalling through mTORC1, which results in a strong engagement of glycolysis (MacIver *et al.*, 2013). During Th1 lineage commitment the Th2 programme is actively supressed as T-bet represses expression of Gata3, which is a master transcription regulator for Th2 cells (Szabo *et al.*, 2000). Direct interactions between T-bet and Gata3 inhibit Gata3 target gene binding (Hwang *et al.*, 2005). As such, T-bet-deficient mice spontaneously develop Th2 cell mediated asthma (Finotto *et al.*, 2002; Finotto *et al.*, 2005). Through the production of IFN-γ, Th1 cells are potent activators of macrophages and thus play a major role in the defence against intracellular pathogens like *Leishmania* (Scott *et al.*, 1989) and *Toxoplasma* (Suzuki *et al.*, 1988; Suzuki and Remington, 1990). However, aberrant Th1 responses have been implicated in several chronic inflammatory disorders including type I diabetes (Trembleau *et al.*, 1995), multiple sclerosis (Bettelli *et al.*, 2004) and IBD (Powrie *et al.*, 1994).

1.3.4 Th2 cells and type 2 responses

Th2 cells are characterised by the production of IL-4, IL-5, IL-9, IL-13 and amphiregulin. IL-4 plays a crucial role in directing Th2 polarisation, although IL-4 independent Th2 differentiation has also been reported (Jankovic *et al.*, 2000). Other factors that promote Th2 differentiation include the alarmins TSLP, IL-33 and IL-25, which are released by epithelial cells in response to tissue injury and prime DC and basophils to promote Th2 responses (Kitajima *et al.*, 2011; Wynn, 2015). Th2 cell lineage commitment is orchestrated by the transcription factor Gata3, which is induced in response to IL-4 driven activation of STAT6 (Zhang *et al.*, 1997a; Zheng and Flavell, 1997). Gata3 positively regulates its own expression by binding to its regulatory elements and also binds to *Il5* and *Il13* promoters to induce their expression (Tripathi and Lahesmaa, 2014). Thus, Gata3 deficiency in T cells abrogates Th2 differentiation and Th2 cytokine production (Pai *et al.*, 2004; Zhu *et al.*, 2004). Similarly, STAT6 deficiency also impairs Th2 differentiation (Kaplan *et al.*, 1996; Shimoda *et al.*, 1996; Takeda *et al.*, 1996). Furthermore, STAT5 activation driven by IL-2 also contributes to Th2 lineage commitment, as STAT5 and Gata3 bind to different cis-regulatory sites of the *Il4* locus to enhance IL-4 production and maintain IL-4Rα expression (Zhu *et al.*, 2003; Liao *et al.*, 2008). Other transcription factors implicated in Th2 lineage specification include c-MAF (Ho *et al.*, 1996), IRF4 (Lohoff *et al.*, 2002; Rengarajan *et al.*, 2002), TCF-1 (Yu *et al.*, 2009b), Gfi-1 (Zhu *et al.*, 2006; Shinnakasu *et al.*, 2008), JunB (Li *et al.*, 1999) and several NFAT family members (Zhu *et al.*, 2010). Th2 cell associated transcription factors act to restrain development of other $CD4^+$ T cell effector subsets. For instance, Gata3 directly interacts with Runx3 to limit IFN-γ production (Yagi *et al.*, 2010) and suppresses expression of IL-12Rβ2 (Ouyang *et al.*, 1998) and STAT4 (Usui *et al.*, 2003).

Th2 cell commitment was shown to be favoured when antigen is present in a very high or very low concentration *in vitro,* and this effect is proposed to be mediated by

the strength of ERK signalling downstream of TCR activation, as weak and transient ERK activation enhances IL-4 production (Hosken *et al.*, 1995; Jorritsma *et al.*, 2003; Yamane and Paul, 2013). In addition, NFκB signalling promotes Th2 development, as *Nfkb1*$^{-/-}$ CD4^{+} T cells have impaired Gata3 expression and decreased ability to polarise into Th2 cells (Das *et al.*, 2001; Kimura *et al.*, 2005). Notch signalling was also shown to regulate commitment into the Th2 lineage, as interaction of Jagged1 with Notch directs Th2 differentiation (Amsen *et al.*, 2004). Although mTORC1 signalling is needed for Th2 cell lineage specification, Th2 cells are considered to be more reliant on mTORC2 in comparison to other T helper subsets (Delgoffe *et al.*, 2011; Chapman and Chi, 2014).

When the Th1/Th2 paradigm was originally described Th2 responses were considered to represent a regulatory arm of adaptive immunity that counteracted the detrimental inflammatory consequences of Th1-mediated immunity. Indeed, Th2 responses can ameliorate Th1 or Th17 driven inflammation (Lubberts *et al.*, 2000a; Anthony *et al.*, 2011) and are important in promoting tissue repair pathways (Gause *et al.*, 2013). However, multiple other effector functions have been assigned to Th2 cells and type 2 responses, and this includes defence against large helminth parasites (Grencis, 2015), resistance against toxins and venoms (Tsai *et al.*, 2015), and regulation of glucose homeostasis, adiposity and thermogenesis (Cheng and Locksley, 2015; Odegaard and Chawla, 2015). Type 2 responses are characterised by a strong innate component, as they engage ILC2, eosinophils, basophils, mast cells and type 2 macrophages (IL-4/IL-13-'alternatively activated' macrophages). Effector cytokines produced by Th2 cells orchestrate a diverse range of type 2 responses. For instance, IL-4 mediates the IgE switch in B cells, IL-5 drives generation and activation of eosinophils, IL-9 was shown to activate mast cells and ILC2, amphiregulin promotes epithelial repair, while IL-13 drives mucus production, smooth muscle hyperreactivity, goblet cell metaplasia and type 2 macrophage differentiation (Pulendran and Artis, 2012; Wynn, 2015). However, the host protective functions of

type 2 immunity are mirrored by detrimental effects when they are persistent or dysregulated. As such, type 2 responses can induce fibrosis, promote allergic diseases, including asthma and food allergies, and antagonize antitumor defence by mobilizing immunosuppressive macrophages and by inhibiting Th1 and cytotoxic T cell responses (Wynn, 2015).

1.3.5 T follicular helper (Tfh) cells

While some antigens can induce B cell antibody production in a T cell-independent manner (mainly large polyvalent antigens such as lipids and polysaccharides), T cell help is required for B cells to produce antibodies in response to protein antigens (Linterman *et al.*, 2012). T follicular helper (Tfh) cells are becoming recognized as a separated lineage of $CD4^+$ T cells that specialize in the provision of help for B cell responses. Tfh assistance is essential to induce maturation, isotype switching and terminal differentiation of B cells (Linterman *et al.*, 2012), events that occur mainly within germinal centers (GC). As such, Tfh cells are essential for the production of most types of antibodies, although their role in IgE responses remains unclear (Kemeny, 2012). Tfh cells secrete IL-21 that facilitates GC formation and express high levels of the T cell–inhibitory receptor PD-1, the chemokine receptor CXCR5, and the co-stimulatory receptors CD40L and ICOS, all of which play a role in Tfh migration and function (Crotty, 2011).

The transcriptional repressor Bcl-6 (B cell lymphoma 6) orchestrates Tfh lineage commitment and is both necessary and sufficient to drive Tfh differentiation (Johnston *et al.*, 2009; Nurieva *et al.*, 2009; Yu *et al.*, 2009a). Bcl-6 was shown to repress T-bet, Gata3 and Rorγt (retinoic acid receptor-like orphan receptor γt), thereby blocking the Th1, Th2 or Th17 transcriptional programmes. Blimp-1 (B-lymphocyte induced maturation protein-1) is an inhibitor of Bcl-6 and the balance between expression of Blc-6 and Blimp-1 is important in determining Tfh

differentiation, as they are mutually repressive (Johnston *et al.*, 2009). Additionally, two transcription factors, LEF-1 and TCF-1, were recently shown to play an important role in directing Tfh differentiation, acting upstream of Bcl-6 (Choi *et al.*, 2015). Tfh cell lineage specification was shown to be promoted by the presence of IL-6 and IL-21 (Crotty, 2011). Not much is known about specific metabolic requirements of Tfh cells, however recent evidence suggest that these cells have reduced mTORC1 activity and are less glycolytic compared to Th1 cells (Ray *et al.*, 2015).

While Tfh differentiation can be induced early upon naïve T cell activation, there is evidence that Tfh cells can also originate from other $CD4^+$ T cell subsets. For instance, it appears that Th2 cells can give rise to Tfh cells during parasitic infections (Glatman Zaretsky *et al.*, 2009). With regards to the intestinal mucosa, $Foxp3^+$ Treg cells were proposed to differentiate into Tfh cells in the PP (Tsuji *et al.*, 2009). However, this finding was recently challenged by fate-mapping studies, which demonstrated that a large proportion of Tfh cells in PP were derived from Th17 cells (and not from Treg cells) and that these Th17-derived Tfh cells were crucial for the induction of T cell-dependent intestinal IgA responses (Hirota *et al.*, 2013). The relationships and plasticity between Treg, Th17 and Tfh cells in the intestinal mucosa requires further investigation, but given that different species of microbiota are potent inducers of Treg or Th17 cells (Kabat *et al.*, 2014), this might explain how they are able to induce T cell-dependent intestinal IgA production.

1.3.6 Th17 cells

Th17 cells are characterised by production of the signature cytokines IL-17A, IL-17F and IL-22 (Aggarwal *et al.*, 2003; Murphy *et al.*, 2003; Harrington *et al.*, 2005; Park *et al.*, 2005; Liang *et al.*, 2006) and expression of IL-23R and the chemokine receptor CCR6 (Hirota *et al.*, 2007; Awasthi *et al.*, 2009). The transcription factor Rorγt is considered to be the master regulator of Th17 cells (Ivanov *et al.*, 2006). A natural

ligand for Rorγt - an intermediate of cholesterol biosynthesis pathway - has recently been identified (Hu *et al.*, 2015; Santori *et al.*, 2015). While Rorγt expression is necessary for Th17 differentiation, it is not sufficient, and interactions with multiple other transcription factors are involved, including STAT3, IRF4, Runx1, Aiolos and Ahr (Ciofani *et al.*, 2012; Gaffen *et al.*, 2014). The differentiation into Th17 cells requires TGF-β_1 in the presence of pro-inflammatory cytokines, including IL-1β, and STAT3-activating cytokines, like IL-6 or IL-21 (Korn *et al.*, 2009), whereas IL-23 plays an essential role in sustaining Th17 differentiation and promoting their survival and acquisition of pathogenic effector potential (Langrish *et al.*, 2005; McGeachy *et al.*, 2009; Ahern *et al.*, 2010). Similarly to Th1 cells, Th17 cells require mTORC1 activation during differentiation, but are additionally dependent on HIF-1α activity and are thought to heavily rely on glycolysis, as blocking glycolysis with 2-DG (2-Deoxy-d-glucose) inhibits Th17 cell differentiation (Dang *et al.*, 2011; Shi *et al.*, 2011). Recently, fatty acid synthesis (FAS) was also shown to dictate the balance between Th17 and Treg differentiation, where *de novo* FAS promoted Th17 over Treg cell differentiation (Berod *et al.*, 2014). Th17 cells are enriched at mucosal sites where they play a key role in protection against a variety of extracellular pathogens, including fungal and bacterial infections (Ye *et al.*, 2001; Huang *et al.*, 2004; Ishigame *et al.*, 2009; Korn *et al.*, 2009). However, Th17 cells have also been implicated in several chronic inflammatory and autoimmune disorders, including intestinal inflammation (Kullberg *et al.*, 2006; Korn *et al.*, 2009; Ahern *et al.*, 2010).

1.3.7 Regulatory T cells (Treg cells)

In comparison to the systemic immune compartment, the intestinal mucosa is significantly enriched in regulatory T cells and their non-redundant role in controlling intestinal inflammation is well documented (Izcue *et al.*, 2009; Harrison and Powrie, 2013). The majority of these cells are Foxp3$^+$ Treg cells, which are the main focus of

this thesis. Foxp3 is a master transcriptional regulator of Treg cells, necessary for their development and maintenance (Josefowicz *et al.*, 2012a). In mice and humans mutations in the *FOXP3* gene lead to spontaneous and lethal autoimmunity that manifests in neonates. Patients with loss of function mutations in the *FOXP3* gene develop IPEX syndrome (immunodeficiency, polyendocrinopathy, and enteropathy, X-linked syndrome), characterised by auto-reactive T cell activation and skewing towards Th2 responses (Chatila *et al.*, 2000; Bennett *et al.*, 2001; Wildin *et al.*, 2001). The clinical symptoms of IPEX can manifest in many organs, but almost always affect the intestine. In addition, many patients with IPEX display high serum IgE levels and allergic symptoms.

Treg cells can be generated in the thymus during development (tTreg cells), or in the periphery from conventional naive $CD4^+$ T cells (pTreg cells). Studies in TCR-transgenic mouse models established that differentiation of tTreg cells requires a particular strength of TCR signalling following recognition of self-peptides, which corresponds to a TCR avidity between the one that dictates positive selection and the one that leads to negative selection (Hsieh *et al.*, 2012). Additionally, TCR repertoire analysis revealed that tTreg cells possess a largely distinct repertoire compared to effector $CD4^+$ T cells with only a limited overlap, emphasising their elevated affinity for self-peptides (Hsieh *et al.*, 2006; Pacholczyk *et al.*, 2006; Wong *et al.*, 2007). With regards to signal transduction downstream of TCR engagement, the balance between the NFκB and AKT/mTOR signalling cascades is thought to control tTreg cell differentiation. Signalling through Akt/mTOR seems to be largely inhibitory for Treg cell lineage specification (Haxhinasto *et al.*, 2008; Sauer *et al.*, 2008; Delgoffe *et al.*, 2009). Although the explanation for this observation is still not well defined, one possibility is that inhibition of mTORC1 promotes activation of the Foxo1 and Foxo3 transcription factors, which enhance the expression of *Foxp3* gene (Harada *et al.*, 2010; Kerdiles *et al.*, 2010; Ouyang *et al.*, 2010; Ouyang *et al.*, 2012). In contrast, activation of NFκB pathway is necessary and sufficient for tTreg cell

generation. Enforced activation of the NFκB pathway can bypass the requirement for TCR antigen recognition in tTreg cell development (Long *et al.*, 2009) and deletion or mutation of components of the NFκB signalling cascade, including PKCθ, CARMA1, TAK1 and IKKß, led to a marked decrease in tTreg cell numbers (Feuerer *et al.*, 2009). In particular, c-Rel acts as a pioneer transcription factor that promotes opening of the *Foxp3* locus (Isomura *et al.*, 2009; Ruan *et al.*, 2009). Activation of STAT5 by IL-2 is also essential for Treg cell differentiation, as it was shown to induce Foxp3 expression (Hsieh *et al.*, 2012).

Similar rules of Treg cell generation seem to apply for the differentiation of pTreg cells, however there is a strong additional influence of environmental factors. *In vitro*, induction of Treg cells can be achieved by activating naïve $CD4^+$ T cells in the presence of IL-2 and TGF-$ß_1$. Induction of pTreg cells from naive $CD4^+$ T cells *in vivo* occurs when antigen is presented under sub-immunogenic or non-inflammatory conditions, during chronic inflammation, and in the setting of a tolerogenic microenvironment, which includes the intestinal LP (Bilate and Lafaille, 2012). Indeed, the gastrointestinal tract is a preferential site of pTreg cell conversion, however whether pTreg and tTreg have overlapping or distinct functions in maintaining gut homeostasis is an ongoing question (Shale *et al.*, 2013).

The stability and composition of the Treg cell population is controlled by three conserved non-coding sequences (CNS) situated within *Foxp3* locus (Zheng *et al.*, 2010). Generation of mice where distinct CNS sites of *Foxp3* locus were genetically manipulated revealed that the CNS1 region, containing a TGF-$ß_1$ - NFAT response element, was necessary for pTreg generation, as CNS1-deficient mice had no defects in tTreg cells, but showed defective pTreg cell induction, which resulted in enhanced Th2 responses at the mucosa (Zheng *et al.*, 2010; Josefowicz *et al.*, 2012b). Conversely, CNS3 deficiency leads to marked decrease in the population of tTreg cells (Zheng *et al.*, 2010) and CNS3 was shown to be a binding site for c-Rel (Zheng *et al.*, 2010). This data suggested that pTreg cells are particularly important in

controlling allergic responses at mucosal surfaces. Furthermore, it has been proposed that LP tTreg and pTreg cells might contribute distinct TCR repertoires to the intestinal Treg cell pool. This was based on the observation that pTreg cells in the intestinal LP bear a unique subset of TCR that recognize commensal microbiota (Lathrop *et al.*, 2011). The TCR repertoire in these pTreg cells was distinct from that expressed by naïve and effector $CD4^+$ cells in the LP, suggesting that exposure to commensal antigens may preferentially direct naïve T cells to differentiate into pTreg cells (Lathrop *et al.*, 2011). However, a recent report challenged this concept (Cebula *et al.*, 2013). In this study it was shown that vast majority of TCR on the colonic Treg cells were shared with the thymic tTreg population and that the TCR repertoire of thymus-derived tTreg cells was influenced by the composition of microbiota, suggesting that thymic tTreg cells are important in maintaining intestinal tolerance toward commensals (Cebula *et al.*, 2013). Although differences in methodological approach might have contributed to the conflicting results regarding the ontogeny of microbiota-specific intestinal Treg cells, the current evidence suggests that the microbiota might shape the population of intestinal Treg cells in at least two ways; by providing factors that promote the induction of pTreg cells (as discussed below) and by providing antigenic peptides that stimulate particular clones of thymus derived tTreg cells.

The mechanisms used by Treg cells to supress deleterious inflammatory responses have been extensively studied. They include production of regulatory cytokines (IL-10, TGF-$ß_1$, IL-35), suppression by metabolic disruption, direct modulation of DC function, and cytolysis (Vignali *et al.*, 2008). In the context of intestinal homeostasis, IL-10 and TGF-$ß_1$ are of particular importance in enforcing tolerance and genetic deletion of IL-10, IL-10R or impairment of the pathway results in spontaneous intestinal inflammation (Izcue *et al.*, 2009; Harrison and Powrie, 2013).

In recent years it has become apparent that $Foxp3^+$ Treg cells appear to display functional specialisations that match the effector responses that they antagonize.

This specialisation has been mostly described on the level of transcription modules. For instance, expression of T-bet by Treg cells was shown to be necessary to efficiently inhibit Th1 cells and this was attributed to increased expression of CXCR3 that allowed more efficient homing of T-bet^{+} Treg cells to sites of Th1 driven inflammation (Koch *et al.*, 2009; Hall *et al.*, 2012). Similarly, expression of STAT3, which is important for the Th17 lineage specification, seems to facilitate Treg cell control over Th17 cells (Chaudhry *et al.*, 2009). However, the concept of mirrored expression of effector T cell transcriptional modules by Treg cells may not be straightforward; for instance, expression of IRF4 and Gata3, transcription factors that are typically expressed in Th2 cells, are increased in peripheral and activated Treg cells and appear to play broader roles in Treg cell homeostasis (Cretney *et al.*, 2011; Wang *et al.*, 2011b; Wohlfert *et al.*, 2011).

Treg cells also display specific adaptations that are tailored to the environmental context in which they operate. It was recently proposed that circulating Treg cells could be therefore divided into central, effector and tissue-resident Treg cell populations (Liston and Gray, 2014). The existence of memory Treg cells has also been postulated (Rosenblum *et al.*, 2011; Rowe *et al.*, 2012; Sanchez *et al.*, 2012). Circulating or naïve Treg cells constitute the majority of Treg cells in the blood and secondary lymphoid organs. They express CD62 and CCR7, which facilitate homing to secondary lymphoid tissue, have high-levels of anti-apoptotic molecules (including Bcl-2 and Mcl-1), yet they maintain suppressive function. Effector or activated Treg cells are highly proliferative, display a CD62L^{low} CD44^{hi} KLRG1^{+} CD103^{+} phenotype and are thought to have been recently activated by antigen (Liston and Gray, 2014). Finally, tissue-resident Treg cells are long-term residents within various non-lymphoid organs, to which they adapt through transcriptional and metabolic reprogramming (Burzyn *et al.*, 2013a). Potentially, each organ might have its own specific Treg cell population and tissue-resident Treg cells have been described in skin, muscle, adipose tissue, placenta and the intestine (Burzyn *et al.*, 2013a). These

Treg cells can support tissue functions beyond their classic immunoregulatory role; for instance, muscle resident Treg cells have been shown to facilitate tissue repair (Burzyn *et al.*, 2013b).

Gut resident Treg cells are reported to express chemokines and receptors that facilitate gut homing, such as ß7 family integrins and CCR9, expression of which is promoted by RA (Iwata *et al.*, 2004; Denning *et al.*, 2005), and GPR15 (G protein-coupled receptor 15) (Kim *et al.*, 2013c). Additionally, gut-resident Treg cells can be characterised by expression of the high affinity IL-2 receptor and the SCFA (short chain fatty acid) receptor GPR43 (Smith *et al.*, 2013). It is important to note that due to difficulties in distinguishing between effector and tissue-resident Treg cell populations the degree of plasticity between them remains unclear. Overall, Treg cells are thought to metabolically resemble memory T cells, in that they preferentially rely on lipid instead of glucose metabolism for energy generation, however it remains largely unexplored whether particular subpopulations of Treg cells are metabolically distinct (MacIver *et al.*, 2013).

1.3.8 Modulation of T cells by intestinal microbiota

Much recent interest has focused on how commensal microbiota modulate the balance among T cell subsets and growing evidence indicates that intestinal microbiota can influence the composition and function of both innate and adaptive T cell populations, particularly those populations that are enriched in the intestinal mucosa. In terms of innate T cell populations, commensal microbiota modulate the abundance of γδ T cells in the intestine, as GF mice (or mice treated with antibiotics) have fewer IL-17 producing γδ T cells (Duan *et al.*, 2010). In addition, the antimicrobial response of γδ T cells was triggered by a distinct subset of commensal bacteria that penetrated the epithelial barrier (Ismail *et al.*, 2011). The influence of commensals also extends to iNKT cells, as exposure to commensal

microbiota during the neonatal period limits the accumulation of iNKT cells at mucosal sites (Olszak *et al.*, 2012). Lack of commensal exposure during early life resulted in adult mice exhibiting increased mortality to oxazolone-induced colitis and increased susceptibility to asthma (Olszak *et al.*, 2012). Mechanistically, the inhibition of colonic iNKT cell development by commensal microbiota is at least in part mediated by the interaction of iNKT cells with commensal-derived inhibitory sphingolipids (An *et al.*, 2014).

Other investigators have examined the effects of commensal microbiota on local $CD4^+$ T cell subsets, in particular on Th17 and Treg cells. GF mice show reduced frequencies of intestinal Th17 cells, which can be restored upon colonisation with the Segmented Filamentous Bacteria (SFB), gram-positive bacteria belonging to the *Clostridiales* genus (Gaboriau-Routhiau *et al.*, 2009; Ivanov *et al.*, 2009). How SFB promotes effector T cell polarisation and accumulation is still not completely understood but it was proposed that SFB colonisation increases levels of the acute-phase protein serum amyloid A (SAA), which conditioned intestinal MP to promote Th17 cells (Ivanov *et al.*, 2009). More recently, presentation of SFB antigens on MHC II molecules by intestinal DC was implicated in SFB-specific Th17 cells differentiation (Goto *et al.*, 2014; Yang *et al.*, 2014). Of note, it remains unknown whether equivalent single bacteria species able to promote Th17 cells exist in humans. Additionally, it was shown that Th17 cells could directly detect microbial associated molecules through TLR2 and that this potentiated Th17 responses (Reynolds *et al.*, 2010).

How commensals influence $Foxp3^+$ Treg cell induction in the gut has remained poorly understood, but recent findings have shed new light on this process. The capsular polysaccharide A (PSA) of the gram-negative anaerobic commensal *Bacteroides fragilis* was shown to promote IL-10 producing $Foxp3^+$ Treg cells (Round and Mazmanian, 2010) through direct interaction of PSA with TLR2 on T lymphocytes (Round *et al.*, 2011). Furthermore, metabolites such as SCFA are emerging as key

homeostatic signals provided by commensal microbiota to regulate local Treg cells. Recent reports present compelling evidence that SCFA, such as butyrate and acetate, can act directly on mucosal Treg cells to promote their expansion. Mechanistically, butyrate appeared to promote pTreg cell induction by inhibiting histone deacetylases (HDAC), because butyrate-treated naive $CD4^+$ T cells exhibited increased acetylation of the *Foxp3* locus, including the key CNS1 enhancer region that is essential for pTreg differentiation (Smith *et al.*, 2013, Atarashi *et al.*, 2013, Arpaia *et al.*, 2013).

Collectively, these findings illustrate that the microbiota have profound effects on the development and activation of both innate and adaptive T cell populations in the gut. Notably, influence of the commensal microbiota extends beyond the gut, as recent studies indicated that the development of systemic T cell populations and susceptibility to autoimmune phenotypes is strongly influenced by commensals (Kamada *et al.*, 2013).

1.3.9 Autophagy in T cells

The first indication of the importance of the autophagy pathway for T lymphocyte homeostasis *in vivo* came from the study of $Atg5^{-/-}$ fetal liver chimeric mice where decreased numbers of thymic and splenic autophagy deficient $CD4^+$ and $CD8^+$ T cells were reported (Pua *et al.*, 2007). Several genetic models were subsequently employed to investigate a specific role for autophagy in T cell development. These studies mainly focused on investigating T cells within the thymus and in the secondary lymphoid organs, including spleen and lymph nodes. T cell-specific deletion of *Atg3, Atg5, Atg7, Vps34* or *Beclin1* consistently showed decreased frequencies and numbers of $CD4^+$ and $CD8^+$ T cells in the secondary lymphoid organs, whereas thymic development was largely unperturbed (Pua *et al.*, 2009; Jia and He, 2011; Kovacs *et al.*, 2012; Willinger and Flavell, 2012; Parekh *et al.*, 2013; Puleston *et al.*, 2014). The exception was NKT cells, which seem to require autophagy during

thymic selection (Parekh *et al.*, 2013; Salio *et al.*, 2014). Studies where activation marker expression was analysed revealed increased proportions of effector/memory phenotype ($CD62^{low}$ $CD44^{hi}$) T cells among peripheral autophagy-deficient T cells, which was interpreted to be a result of decreased survival of naïve T cells (Pua *et al.*, 2009; Jia and He, 2011). However this phenotype might also occur as a result of lymphopaenia-induced proliferation (Puleston *et al.*, 2014).

The role of autophagy in activated T cells has been predominantly studied *in vitro*. Autophagy appeared to be activated following TCR triggering with agonistic anti-CD3 and anti-CD28 antibodies in $CD4^+$ T cells (Li *et al.*, 2006; Hubbard *et al.*, 2010). Consistent with these reports, defects in proliferation were observed in autophagy-deficient T cells upon TCR crosslinking *in vitro* (Pua *et al.*, 2007; Pua *et al.*, 2009; Parekh *et al.*, 2013). Autophagy-deficient T cells also showed increased apoptosis during prolonged *in vitro* culture (Pua *et al.*, 2009), or after activation (Kovacs *et al.*, 2012). Some of the studies also observed decreased production of effector cytokines by *in vitro* activated autophagy-deficient T cells, including IL-2, IL-17A and IFN (Hubbard *et al.*, 2010; Parekh *et al.*, 2013), however another study reported increased IL-2 production by *Atg7*-deficient $CD4^+$ T cells after TCR crosslinking (Jia *et al.*, 2011).

While decreased numbers of peripheral $CD4^+$ and $CD8^+$ T cells in the absence of T cell-specific autophagy was consistently observed, the mechanistic explanation for this finding differs between reports. The majority of these studies pointed towards defective organelle turnover as the main cause of decreased fitness of autophagy-deficient T cells. In particular, increased mitochondrial mass in naïve autophagy-deficient T cells, which resulted in detrimental increased ROS production, was suggested to eventually lead to increased apoptosis (Pua *et al.*, 2009; Stephenson *et al.*, 2009; Willinger and Flavell, 2012; Parekh *et al.*, 2013). Increases in ER mass and changes in intracellular calcium signalling were also implicated in this impaired fitness and survival (Jia *et al.*, 2011). On the other hand, *Beclin-1*-deficient T cells,

while showing similar decreases in peripheral T cell populations, did not have increased mitochondria mass (Kovacs *et al.*, 2012). Moreover, although increased mitochondrial content and ROS production correlated with decreased survival of autophagy-defective T cells, whether the increased ROS production was directly responsible for this defective survival remains unclear, as it was not formally proven in any of these studies. It was also proposed that imbalanced expression or accumulation of apoptosis-related proteins might contribute to the defective proliferation and survival of autophagy-deficient T cells. However, these results are difficult to interpret as increased levels of both pro-apoptotic (Pua *et al.*, 2009; Kovacs *et al.*, 2012; Parekh *et al.*, 2013) and anti-apoptotic proteins (Bcl-2, Mcl-1) (Pua *et al.*, 2009; Kovacs *et al.*, 2012; Parekh *et al.*, 2013) were reported in autophagy-deficient T cells. Analysis of mice with T cell-specific deletion of *Vps34* suggested that decreased levels of IL-7Rα expression on T cells might be involved, although this was not attributed to the cell intrinsic effects of autophagy deficiency (McLeod *et al.*, 2011; Willinger and Flavell, 2012). Treatment with the pan-caspase inhibitor zVAD could partially rescue the apoptotic phenotype of autophagy-deficient T cells (Pua *et al.*, 2009; Kovacs *et al.*, 2012), however it did not rescue the defects in T cell proliferation (Parekh *et al.*, 2013). Furthermore, it is worth pointing out that in addition to its role in T cell survival and proliferation, autophagy was also reported to promote cell death in activated T cells under some circumstances (Li *et al.*, 2006; Bell *et al.*, 2008; Feng *et al.*, 2008).

Interestingly, autophagy has been directly linked to regulation of signalling cascades downstream of the TCR. For example, in activated, but not in naïve T cells, autophagy provides a negative feedback loop that regulates the NFκB signalling pathway (Paul *et al.*, 2012). Here, autophagy was shown to selectively target Bcl-10 for degradation in a p62-dependent manner, limiting NFkB-dependent effector responses, including IL-2 secretion (Paul *et al.*, 2012). NFκB signalling plays an important role in many aspects of activated T cell physiology, including entry into

cell cycle (Vallabhapurapu and Karin, 2009), but the strength of NFκB signalling can also influence differentiation into distinct Th cell subsets, therefore, by regulating the NFκB pathway, autophagy might contribute to these processes in activated T cells.

Recent reports identified a role of autophagy in the formation of memory $CD8^+$ T cells during viral infections (Puleston *et al.*, 2014; Xu *et al.*, 2014; Schlie *et al.*, 2015). These studies, which focused on *in vivo* responses of autophagy-deficient $CD8^+$ T cells to influenza or lymphocytic choriomeningitis virus infections, revealed new aspects of autophagic regulation of T cells. Although distinct genetic approaches were used to generate mice with a selective autophagy deficiency in T cells, all observed that autophagy was dispensable during the early highly proliferative expansion phase of antigen activated $CD8^+$ T cells during viral infection (Puleston *et al.*, 2014; Xu *et al.*, 2014; Schlie *et al.*, 2015). In addition, autophagy-deficient T cells did not show defects in effector cytokine production and were capable of controlling virus titers during the early phases of infection (Xu *et al.*, 2014). However, activation of the autophagy pathway was shown to be crucial during the transition phase between late effector to memory T cells and mice with autophagy-deficient $CD8^+$ T cells did not mount proper memory $CD8^+$ T cell responses during secondary challenge (Puleston *et al.*, 2014; Xu *et al.*, 2014; Schlie *et al.*, 2015). While the mechanism behind the requirement for autophagy in memory $CD8^+$ T cell responses remains to be elucidated, comparison of metabolic profiles between WT and *Atg7*-deficient memory $CD8^+$ T cells suggested that metabolic adaptation might be involved (Xu *et al.*, 2014). In addition, $CD8^+$ T cells from aged mice (Puleston *et al.*, 2014) and senescent human $CD8^+$ T cells exhibited low levels of autophagy, which in human senescent cells was associated with high activity of p38 kinase (Henson *et al.*, 2014). Increasing autophagy levels was shown to boost memory $CD8^+$ T cell responses after influenza vaccination in aged mice (Puleston *et al.*, 2014).

Overall, while it is clear that autophagy plays a crucial role in the maintenance of peripheral $CD4^+$ and $CD8^+$ T cells and memory $CD8^+$ T cells, it is still not completely understood how autophagy influences different aspects of T cell physiology in naïve and activated T cells. Methodological difficulties of monitoring autophagy, differences in the genetic models used, as well as differences between *in vivo* and *in vitro* stimuli might underlie some of the discrepancies observed. However, autophagy most likely performs many distinct functions in T cells and these roles could be highly context-dependent and might differ between distinct subsets of T cells.

1.4 Inflammatory bowel disease (IBD)

The term IBD describes a spectrum of chronic incurable inflammatory disorders affecting the gastrointestinal tract, but often with extra-intestinal manifestations, with the two most common forms being Crohn's disease (CD) and ulcerative colitis (UC) (Kaser *et al.*, 2010). While the exact aetiology of these disorders remains unknown, experimental and clinical data demonstrate that IBD is a consequence of a loss of intestinal homeostasis, a state that, as discussed above, involves complex interactions between intestinal tissue cells, the immune system and intestinal microbiota (Maloy and Powrie, 2011). IBD is a complex multifactorial disease that emerges on a background of many genetic and environmental factors (Kaser *et al.*, 2010; Maloy and Powrie, 2011). In recent years there has been tremendous progress in understanding the genetics of IBD susceptibility (discussed below). However the identification of susceptibility polymorphisms provides only correlative evidence for the involvement of specific genes or pathways, and an understanding of the functional consequences of the majority of genetic polymorphisms is still lacking. Environmental triggers that provoke the onset of intestinal inflammation in genetically susceptible individuals are also largely undefined.

Inflammatory lesions in patients with CD can occur throughout the gastrointestinal tract, often discontinuously, but most commonly affect ileum and colon. The inflammation in CD is often transmural, with dense cellular infiltrates and granulomas (Baumgart and Sandborn, 2007). Tissue pathology in UC differs significantly, as it is typically restricted to the colon and shows an uninterrupted pattern that affects only the mucosal surface, with crypt abscesses and goblet cell depletion being characteristic features (Baumgart and Sandborn, 2007). CD and UC are often associated with inflammatory diseases outside of the gastrointestinal tract with skin, eyes, joints and liver being most commonly affected (Lees *et al.*, 2011). Patients with IBD also show increased risk of developing colorectal cancer (Ullman and Itzkowitz, 2011). In addition, there seems to be a significant epidemiological overlap between IBD and other chronic inflammatory diseases, including multiple sclerosis, ankylosing spondylitis, psoriasis, asthma and atopic dermatitis (Lees *et al.*, 2011). Current management of this incurable disease involves a combination of immune-suppressive medications, biological agents targeting TNF-α, dietary changes and surgery.

While both CD and UC are chronic inflammatory disorders, the adaptive immune responses in these diseases show marked differences. Inflammation in CD is associated with a strong Th1 and Th17 component, highlighted by increased expression of T-bet and IFN-γ (Fuss *et al.*, 1996), IL-12 (Monteleone *et al.*, 1997; Fuss *et al.*, 2006), IL-12Rβ2 (Parrello *et al.*, 2000), IL-18 (Monteleone *et al.*, 1999), IL-17A (Annunziato *et al.*, 2007) and IL-22 (Brand *et al.*, 2006). UC was originally considered as a Th2-driven inflammatory disorder, however experimental and clinical evidence do not unequivocally support this hypothesis. Elevated levels of IL-5 and IL-13 have been described in UC patients (Fuss *et al.*, 1996; Heller *et al.*, 2005) and NKT cells were implicated as a soured of IL-13 in the oxazolone-driven mouse model of UC (Heller *et al.*, 2002; Fuss *et al.*, 2004). However, other studies in UC patients did not confirm increased type 2 cytokine expression in intestinal biopsies (Vainer *et al.*,

2000; Biancheri *et al.*, 2014; Geremia *et al.*, 2014), and even reported increased IFN-γ expression in UC patients (Geremia *et al.*, 2014). In addition, IL-13 was shown to have a protective role in the IL-$10^{-/-}$ mouse colitis model (Wilson *et al.*, 2011), whereas in CD IL-13 might contribute to pathological fibrosis formation (Bailey *et al.*, 2012). Taken together, current evidence suggests that the division between type 1 responses in CD and type 2 responses in UC is probably an over-simplification. However, as it is becoming increasingly appreciated that UC and CD patients most likely represent many distinct subphenotypes, it is plausible that some forms of IBD might manifest through increased type 2 responses. Furthermore, dynamic changes between Th1 and Th2 responses might be occurring over the course of the disease (Spencer *et al.*, 1999; Strober and Fuss, 2011).

Compelling evidence that IBD has a strong inheritable component came from early twin studies, which motivated further genetic analysis (Liu and Anderson, 2014). While mutations in NOD2 were linked to CD susceptibility through early linkage studies, the key breakthrough in understanding IBD genetics was enabled by technological progress that led to large-scale genome wide association studies (GWAS), followed by meta-analysis and targeted genotype arrays (Immunochip) (Liu and Anderson, 2014). Since these approaches are largely 'hypothesis-free', they facilitate discovery of previously unsuspected genes and pathways. Currently, 163 loci associated with IBD have been identified, with most loci contributing to both CD and UC susceptibility and each locus containing an average of 5 genes (Jostins *et al.*, 2012), which is far more than any other complex immunological disease to date (Van Limbergen *et al.*, 2014). A considerable proportion of IBD-associated genes are involved in immune cell function, innate and adaptive immune responses, promotion of epithelial barrier integrity and bacteria handling (Van Limbergen *et al.*, 2014). Among these, a single nucleotide polymorphism in *ATG16L1* was identified as a

strongly associated risk locus for CD, suggesting for the first time a role for the autophagy pathway in IBD (Hampe *et al.*, 2007; Rioux *et al.*, 2007).

Through these genetic studies it became apparent that the typical effect size on disease susceptibility of an individual IBD risk locus was relatively low (odds ratio below 1.3) and that the majority of described susceptibility loci are fairly common in the population (Geremia *et al.*, 2014). Furthermore, it has also been highlighted that the majority of identified polymorphisms occur in the noncoding gene regions, suggesting that most of them will affect gene expression, but not the protein product itself. Despite this marked progress in uncovering the genetic landscape of IBD, it is important to point out that known susceptibility polymorphisms are estimated to explain only 13% (CD) or 7% (UC) of variation in disease susceptibility (Jostins *et al.*, 2012). Rare genetic variants, as well as common variants with a very small effect sizes are predicted to account for the majority of the remaining unexplained genetic contribution (Liu and Anderson, 2014). Major challenges that remain include not only uncovering these loci, but, even more so, to functionally integrate this knowledge into an improved biological understanding of the disease.

1.5 Rationale and summary of thesis aims

Although the exact aetiology of IBD remains elusive, it is thought that IBD arises from an intersection of environmental and genetic risk factors (Maloy and Powrie, 2011). In recent years new genetic factors that influence susceptibility to IBD have been identified, revealing novel pathways that might contribute to its pathogenesis (Van Limbergen *et al.*, 2014). Among these, a SNP in the autophagy gene *ATG16l1* was associated with an increased risk of CD (Hampe *et al.*, 2007; Rioux *et al.*, 2007). A recent study showed that the IBD predisposing T300A mutation in the coding region of *ATG16l1* led to increased degradation of ATG16L1 protein and reduced autophagy (Murthy *et al.*, 2014), indicating that decreased autophagy may contribute to IBD

development. Polymorphisms in several other autophagy related genes, including *IRGM, LRRK2* and *SMURF1*, are also linked to IBD susceptibility (Van Limbergen *et al.*, 2014), suggesting that changes in the autophagy pathway alter intestinal homeostasis and can predispose to chronic intestinal inflammation. To identify the mechanisms through which autophagy may regulate intestinal tissue homeostasis, it is essential to understand the functional consequences of alterations in autophagy on both immune and tissue cells present in the gut. To date, several studies have examined the role of autophagy and Atg16l1 in intestinal epithelial cells and myeloid cells for intestinal homeostasis. In these reports, Atg16l1 was important for Paneth cell physiology, as well as bacterial handling and regulation of inflammatory IL-1β secretion by myeloid cells (Cadwell *et al.*, 2008; Kuballa *et al.*, 2008; Saitoh *et al.*, 2008; Plantinga *et al.*, 2011; Adolph *et al.*, 2013). However, the role of Atg16l1 in intestinal adaptive immune responses has not yet been addressed.

$CD4^+$ T cells constitute the largest population of intestinal lymphocytes and are central mediators of host protective and tolerogenic responses in the gut (Shale *et al.*, 2013). In particular, $Foxp3^+$ Treg cells are indispensable in promoting tolerance towards commensal and dietary antigens and for the prevention of aberrant effector T cell responses, including Th1, Th2 and Th17 responses (Izcue *et al.*, 2009; Harrison and Powrie, 2013). An imbalance between effector and regulatory $CD4^+$ T cells can promote chronic intestinal inflammation and accumulation of effector $CD4^+$ T cells in the inflamed mucosa is a cardinal feature of IBD (Abraham and Cho, 2009; Maloy and Powrie, 2011; Shale *et al.*, 2013). Therefore it is important to define factors that regulate aberrant $CD4^+$ T cell responses in the gastrointestinal tract.

Previous studies utilizing mice with T cell specific deletion of essential autophagy genes (*Atg3, Atg5, Atg7, Beclin1*) pointed to a key role of autophagy in T cell homeostasis, as these mice exhibited decreased frequencies and numbers of $CD4^+$ and $CD8^+$ T cells and defects in T cell proliferation *in vitro* (Pua *et al.*, 2009; Stephenson *et al.*, 2009; Jia and He, 2011; Kovacs *et al.*, 2012). In addition, recent

studies highlight the importance of autophagy in the development of memory $CD8^+$ T cells (Puleston *et al.*, 2014; Xu *et al.*, 2014; Schlie *et al.*, 2015).

Considering evidence summarised above, and given that the gastrointestinal tract is a site of continuous immune activation by external antigens and is therefore a challenging environment for the adaptive immune system, we hypothesized that a selective defect in autophagy may affect intestinal T cell homeostasis. We sought to investigate this hypothesis by generating the mice in which *Atg16l1* was specifically ablated in T cells, characterising these mice at steady state and during chronic intestinal inflammation, and by analysing the effects of *Atg16l1* deletion on the development and function of $CD4^+$ T cell subsets.

Chapter 2. Materials and Methods

2.1 Mice

Atg16l1$^{fl/fl}$ mice were generated and provided by the H. Virgin laboratory (Washington University, Saint Louis, MO, USA), as previously described (Hwang *et al.*, 2012). *Atg16l1*$^{fl/fl}$ mice were crossed with B6.Cg-Tg(Cd4-cre)1Cwi/BfluJ (CD4-Cre mice) and B6.129(Cg)-Foxp3$^{tm4(YFP/cre)Ayr}$/J (Foxp3-YFP-Cre mice, Jackson Laboratory, Bar Harbor, Maine, USA) to generate *Atg16l1*$^{\Delta CD4}$ and *Atg16l1*$^{\Delta Foxp3}$ mice, respectively. *Atg16l1*$^{\Delta CD4}$ mice were also crossed with B6.Cg-Gt(ROSA)26Sor$^{tm14(CAG-tdTomato)Hze}$/J (Jackson Laboratory, Bar Harbor, Maine, USA) to introduce the Cre excision reporter. All the above strains, together with B6.SJL-CD45.1 (CD45.1^{+}), B6 Rag1$^{-/-}$ (Jackson Laboratory), and B6 Foxp3^{hCD2} mice (Komatsu *et al.*, 2009) were bred and maintained under specific pathogen-free condition facilities at the University of Oxford. In addition to routine SPF screening, mice were regularly screened for *Helicobacter* species. All experiments were conducted in accordance with the UK Scientific Procedures Act (1986) under a Project License (PPL) authorised by the UK Home Office Animal Procedures Committee and approved by the Sir William Dunn School of Pathology Local Ethical Review Committee. If not stated otherwise, mice were analysed at 8-12 weeks (young mice) or > 5 months of age (aged mice). In the gene expression analysis *Atg16l1*$^{\Delta Foxp3}$ mice and Foxp3-YFP-Cre mice were age- and sex-matched. In all other experiments, mice used were age- and sex-matched littermates that were co-housed throughout the experiment.

2.2 Genotyping

Mice were genotyped by ear tissue samples. Samples were digested overnight at 56°C in lysis buffer (5mM EDTA, 100mM Tris-HCl, 0.2% SDS, 200mM NaCl and 0.2mg/ml

proteinase K (Bioline)). DNA was isolated from the digested supernatants by precipitation with isopropanol. DNA pellets were washed with 70% ethanol and resuspended after drying in 80 µl of MiliQ water. PCR reactions were carried on in total volume of 15µl using Taq polymerase (0.7 U/µl, Invitrogen), PCR buffer (1x, Bioline), MgCl2 solution (2mM, Bioline), dNTP mix (0.2mM, Sigma), primers (0.5 µM) and 1ul of DNA samples.

Primer sequences for genotyping were as follows:

Atg16l1-flox/flox:

Primer F: CTTTCTCAACAGAACCAGCAGTAC
Primer R: GTAGAAAGACTGGTGATGGTAAACC

CD4-Cre and WT control:

Mutant Primer F: AGGTTCGTTCACTCATGGA
Mutant Primer R: TCGACCAGTTTAGTTACCC
Control Primer F: CCTCCGGAGAGCAGCGATTAAAAGTGTCAG
Control Primer R: TAGAGCTTTGCCACATCACAGGTCATTCAG

Foxp3-Cre and WT control:

Mutant Primer F: AGGATGTGAGGGACTACCTCCTGTA
Mutant Primer R: TCCTTCACTCTGATTCTGGCAATTT
Control Primer F: CCTAGCCCCTAGTTCCAACC
Control Primer R: AAGGTTCCAGTGCTGTTGCT

Tomato reporter and WT control:

Mutant Primer F: CTGTTCCTGTACGGCATGG
Mutant Primer R: GGCATTAAAGCAGCGTATCC
Control Primer F: AAGGGAGCTGCAGTGGAGTA
Control Primer R: CCGAAAATCTGTGGGAAGTC

DNA for Atg16l1-flox/flox and CD4-Cre PCR was amplified under following conditions (one PCR reaction):

1. 94°C, 4 min
2. 94°C, 30 sec
3. 60°C, 30 sec
4. 72°C, 1 min
5. 30 cycles from step 2
6. 72°C, 5 min
7. Hold at 4°C

DNA for Foxp3-Cre and WT control PCR was amplified under following conditions (separate PCR reactions):

1. 94°C, 2 min
2. 94°C, 20 sec
3. 65°C, 15 sec (– 0.5°C per cycle)
4. 68°C, 10 sec
5. Repeat steps 2-4 for 10 cycles
6. 94°C, 15 sec
7. 60°C, 15 sec
8. 72°C, 10 sec
9. Repeat steps 6-8 for 28 cycles
10. 72°C, 2 min
11. Hold at 4°C

DNA for Tomato reporter and WT control PCR was amplified under following conditions (one PCR reaction):

1. 94°C, 3 min
2. 94°C, 20 sec
3. 61°C, 30 sec
4. 72°C, 30 sec
5. 35 cycles from step 2

6. 72°C, 2 min
7. Hold at 4°C

2.3 Flow cytometry antibodies

All antibodies used were specific for murine targets unless stated otherwise.

The following antibodies from eBioscience (Hatfield, UK) were used in the flow cytometry experiments: anti-CD16/32 (93), anti-CD4 (GK1.5), anti-CD8α (53.6.7), anti-TCRß (H57-597), anti-CD45 (30-F11), anti-CD45RB (16A), anti-CD44 (1M7), anti-CD62L (MEL-14), anti-CD45.1 (A20), anti-CD45.2 (104), anti-CD103 (2E7), anti-CD69 (H1.2F3), anti-KLRG1 (2F1), anti-CD25 (7D4), anti-hCD2 (RPA-2.10), anti-CTLA4 (UC10-4B9), anti-GR.1 (RB6-8C5), anti-CD11b (M1/70), anti-Siglec F (E50-2440), anti-IL-7Rα (A7R34), anti-ICOS (7E.17G9), anti-Gata3 (TWAJ), anti-Foxp3 (FJK-16s), anti-Ki67 (SolA15), anti-Helios (22F6), anti-IRF4(3E4), anti-Bcl-2 (10C4), anti-PS6 (cupk43k), anti-IL-2 (JES6-5H4), anti-IFN-γ (XMG1.2), anti-IL-17A (eBio17B7), anti-IL-13 (eBio13A), isotype controls: IgG1 (R3-34) and IgG2a (R35-95).

The following antibodies were from BioLegend (San Diego, USA): anti-CD138 (281-2), anti-CD161 (PK136), anti-F4/80 (BMB), anti-CD11b (M1/70).

The following antibodies were from BD Biosciences (San Jose, USA): anti-B220 (RA3 6B2), anti-GL7 (GL7), anti-CD95 (Jo2), anti-CD3 (145-2C11), anti-CD19 (1D3), anti-Ly6C (AL-21), anti-Ly6G (1A8), anti-IgM (R6-60.2), anti-IgG1 (A85-1), anti-Rorγt (Q31-378), isotype controls: IgG1 (R3-34) and IgG2b (A95.1).

Anti-Neuropilin1 polyclonal antibody was from R&D Systems (FAB566A, Minneapolis, USA). Fixable Viability Dye from eBioscience was used to stain dead cells. Annexin V staining was performed using eBioscience kit (88-08006) according to manufacture instructions. Mitochondrial staining was performed with MitoTracker Green (Life technologies) at 50nm according to manufacturer instructions.

2.4 Culture of Helicobacter hepaticus

Helicobacter hepaticus NCI-Frederick Isolate 1A (American Type Culture Collection, Hh1A, strain 51449) was grown on blood agar plates containing selection antibiotics, Trimethoprim (5μg/ml), Vancomycin (10μg/ml) and Polymixin-B (25U/ml) (TVP; Oxoid, UK) at 37°C under a microaerophilic atmosphere (10% H_2, 10% CO_2, N_2 balance) in CampyPak jars (Oxoid). Once established, after 2 days, bacteria were expanded in liquid cultures of Tryptone Soy Broth (TSB) with TVP and 10% Foetal Calf Serum (FCS) (PAA Solutions, UK) in vented Erlenmeyer flasks under a microaerophilic atmosphere, shaking at 170rpm at 37°C. Liquid cultures were split daily and inoculated at OD_{600} 0.05.

2.5 Flow cytometry

2.5.1 Restimulation for cytokine staining

Cells were restimulated for 4 hours in 96 well round bottom plates at 37°C in RPMI media supplemented with 10% FCS, 2mM L-glutamine, 100U/ml Penicillin/Streptomycin (all PAA solutions) with 0.1μg/ml Phorbol 12-myristate 13-acetate (PMA), 1μg/ml Ionomycin and 10μg/ml Brefeldin A (all Sigma Aldrich, UK), or 10μg/ml Monensin (BioLegend, UK). Following restimulation, cells were centrifuged and the supernatant discarded. Cells were washed twice in PBS/0.1% BSA and cell surface stained and fixed as described in section 2.5.2.

2.5.2 Surface staining

Single cells suspensions (~$5x10^5$ - $1x10^6$ cells/well) were used for staining. All staining incubations were performed in 50µl PBS/0.1% BSA in round bottom 96-well plates for 30 minutes (min) at 4°C. Cells were first stained with Fc-block (anti-CD16/32) to prevent any non-specific antibody binding. Cells were then washed with PBS/0.1% BSA then resuspended in PBS/0.1% BSA containing a cocktail of fluorescently labelled antibodies (described in section 2.3). Cells were again washed in PBS/0.1% BSA and analysed or resuspended in Fixation/Permeabilisation buffer (eBioscience, UK) for future analysis or intracellular staining.

2.5.3 Intracellular staining

Fixed cells were washed and resuspended in 200µl Perm Buffer (eBioscience, UK) with addition of rat serum, incubated for 45 min at 4°C protected from light. Cells were then resuspended in 50µl Perm Buffer containing a cocktail of fluorescently labelled antibodies (described in section 2.3) and incubated at 4°C for 30 min in the dark. Cells were washed twice in Perm Buffer and then once in PBS/0.1% BSA before being resuspended in 400µl PBS/0.1% BSA for analysis.

2.5.4 Autophagy LC3 antibody-based assay and glucose uptake assay

Autophagosome formation detection by flow cytometry was performed using FlowCellect Autophagy LC3 Antibody-based Assay Kit (FCCH100171, Merk-Millipore, MA, USA) according to the manufacturer's instructions and following cell surface markers staining, as described in section 2.5.2. The Autophagy LC3 Antibody-based Assay Kit involves a permeabilization step to wash out cytosolic LC3-I, allowing for antibody-based detection of membrane bound LC3-II. For autophagy detection in WT Treg cells B6 $Foxp3^{hCD2}$ were used, as this allowed the detection of $Foxp3^+$ Treg cells on the basis of surface expression of hCD2 marker. For the glucose uptake

assessment fluorescent 2-deoxyglucose analog (2-NDBG, Life technologies) was added to the culture at 100 μM for 30 minutes before proceeding with surface staining. Flow cytometry data were acquired using a Cyan ADP (Beckman Coulter, High Wycombe, UK) and analysed using FlowJo software (Tree Star, Ashland, USA).

2.6 Western blot

$CD4^+$ T cells purified by negative selection were lysed in RIPA buffer (50mM Tris-HCl, 150mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) containing protease inhibitor cocktail (Roche, Basel, Switzerland). Protein levels were normalized by BioRad DC protein assay (Bio-Rad Laboratories, Hercules, CA), resolved by SDS-PAGE and, following transfer onto nitrocellulose membranes, were blotted with anti-LC3 antibody (L7543; Sigma-Aldrich) and anti-tubulin antibody (sc5286, Santa Cruz Biotechnology, Dallas, USA), and secondary HRP conjugated anti-rabbit antibody (7074S, Cell Signalling, Danvers, USA).

2.7 Immunofluorescence microscopy

Colonic and small intestine tissue samples were formalin-fixed, paraffin-embedded and sectioned as per histological analysis. Sections were deparaffinized, rehydrated, and subjected to sodium citrate-based antigen retrieval, then stained with mouse pAb anti-β-catenin (610153, BD Bioscience), rabbit pAb anti-CD3 (ab5690, Abcam, Cambridge, UK) and secondary goat antibodies conjugated to AlexaFluor488 or 555 (Life Technologies, Carlsbad, USA). Slides were mounted with DAPI-containing Vectashield (Vector Laboratories, Burlingame, USA). Images were acquired with an Olympus Fluoview FV1000 confocal microscope and Olympus Fluoview Software (Olympus, Tokyo, Japan).

2.8 Measurement of cytokines and antibody responses

All immunoglobulin isotypes except for IgE were measured by enzyme-linked immunosorbent assay (ELISA) using the SBA Clonotyping System (Southern Biotech, Birmingham, USA). IgE concentration was determined using an anti-mouse IgE ELISA (BioLegend), according to the manufacturer's instructions. For the detection of soy-specific, CBir-specific and *Helicobacter*-specific antibodies ELISAs were performed using plates coated with purified soy antigen (5 µg/ml, courtesy of Dr Amin Moghaddam), CBir peptide (10 µg/ml, courtesy of Dr Emily Thornton) and soluble *Helicobacter* antigen (sHel antigen, 10 µg/ml) respectively. Soluble Helicobacter (sHel) antigen was prepared as previously described (Kullberg *et al.*, 1998). For the detection of OVA-specific IgE, a sandwich ELISA was performed with biotinylated-OVA used for detection (courtesy of Dr Amin Moghaddam). IL-2 and MCPT-1 concentrations were measured by ELISA (eBioscience).

2.9 Cell isolation, enrichment and sorting

2.9.1 Isolation of thymus, spleen and lymph node cells

Suspensions of single cells were prepared from individual lymphoid organs by passage through 70 - 100µM cell strainers (Falcon, BD Biosciences, UK), or 70µM pore nylon mesh (Plastok Associates, UK). Cells were washed in PBS/0.1% BSA followed by centrifugation at 1500 rpm for 5 min. Red blood cell lysis was performed by addition of ACK lysis buffer (10mM $KHCO_3$, 150mM NH_4Cl, 0.1mM Na_2.EDTA, pH 7.2-7.4) for 3 min at room temperature (RT). Cells were washed, filtered through 70µM cell strainers and resuspended in ice-cold PBS/0.1% BSA.

2.9.2 Isolation of colonic lamina propria leukocytes (cLPL)

Large intestines (colon and caecum) were cut open longitudinally and the luminal contents removed. Whole colons were cut into small pieces (~2cm) and placed in ice-cold PBS/0.1% BSA. Supernatants were aspirated and replaced with ~20ml pre-warmed (37°C) RPMI/5% FCS supplemented with 5mM EDTA (VWR International, UK). Tubes were placed in a shaking incubator (15 min, 37°C, 180 rpm) to remove intestinal epithelial cells (IECs). This washing process was performed a total of 2 times. Next, in order to neutralise the EDTA activity, ~20ml of RPMI/5% FCS containing 15mM Hepes (PAA, UK) was added to each sample for 10 min at room temperature. Supernatant was discarded and replaced with pre-warmed RPMI 1640/10% FCS containing 0.4mg/ml type VIII collagenase (Sigma Aldrich, UK) and 40µg/ml DNase I (Roche, UK). Tubes were shaken (45 - 60 min, 37°C, 180 rpm) in order to aid tissue digestion. Supernatants were collected and EDTA at 5mM was added to quench collagenase activity. Supernatants were filtered through 70µM nylon mesh, then centrifuged and resuspended in 3ml P30 solution (30% Percoll (GE, Amersham, UK)). Overlaying 3ml P75 with 4ml P40 and then 3ml P30 containing colonic lamina propria leukocytes (cLPL) created a three-layered discontinuous gradient. Gradients were centrifuged (1800 rpm, 10°C, minimal acceleration, no brake, 20 min and enriched leukocytes isolated from the P40/P75 interface. Cells were then washed, centrifuged and resuspended in ice-cold PBS/0.1% BSA.

2.9.3 Isolation of small intestine lamina propria leukocytes (SI LPL)

Small intestines (SI) were dissected and placed into ice-cold PBS/0.5% BSA in a petri dish on ice. Peyer's patches were removed. SI were opened longitudinally and washed out with PBS/BSA, then cut into ~ 2 cm sections and stored in 50ml tubes in 10ml RPMI/10% FCS on ice. Tube contents were emptied into petri dishes and SI sections were placed back in the tubes. 15ml of pre-warmed RPMI/5% FCS/5mM

EDTA/0.154 mg/ml DTT (Sigma) was added to the SI sections and tubes were incubated for 15 min at 37°C shaking (180rpm). Tissue sections were washed 3 times in RPMI/5mM EDTA (ice cold) with vigorous shaking. Tissue sections were then incubated in RPMI/10% FCS for 10 min. RPMI was poured off and the tissue placed back in tube. 10ml of pre-warmed RPMI/5% FCS with collagenase VIII (0.2mg/ml, Sigma) and DNase I (40U/ml, Sigma) was added. Tubes were shaken at 37°C in shaking incubator (180rpm). After digestion, supernatants were filtered through cell strainers into new 50ml tubes and topped up with ice-cold RPMI/5% FCS/5mM EDTA to stop collagenase digestion. Supernatants were then centrifuged and resuspended in 3ml P30 solution (30% Percoll (GE, Amersham, UK)). Overlaying 3ml P75 with 4ml P40 and then 3ml P30 containing small intestine lamina propria leukocytes created a three-layered discontinuous gradient. Gradients were centrifuged (1800 rpm, 10°C, minimal acceleration, no brake, 20 min) and enriched leukocytes isolated from the P40/P75 interface. Cells were then washed, centrifuged and resuspended in ice-cold PBS/0.1% BSA.

2.9.4 $CD4^+$ T cell purification

Single cell suspensions prepared from the spleen and mesenteric lymph nodes (mLP) were incubated with a panel of rat anti-mouse monoclonal antibodies (mAb) against cell surface markers CD8, MHC-II, CD11b and B220, for 20 min on ice. Cells were washed and resuspended in ice-cold PBS/0.1% BSA at $1x10^8$ cells/ml. Sheep anti-rat IgG-coated beads (Dynal, Invitrogen) were washed twice in PBS/0.1% BSA and mixed with the cells at a 1:1 ratio. The cell/bead mixture was incubated with constant rotation for 20 min at 4°C. The suspension was then placed on a magnet (Dynal, Invitrogen) and the enriched cell suspension, depleted of beads, was aspirated to a fresh 14ml tube (Falcon, BD Biosciences, UK). This process was repeated once more for a total of 2 enrichments on the magnet. Cell suspensions were washed with

PBS/0.1% BSA and resuspended in ice-cold PBS/0.1% BSA. Isolated cells were consistently ~80% $CD4^+$ T cells.

For some experiments $CD4^+$ T cells purification was performed using EasySep™ Mouse $CD4^+$ T Cell Isolation Kit and EasySep™ Mouse Naïve $CD4^+$ T Cell Isolation Kit according to manufacturer instructions (Stem Cell, Vancouver, Canada).

2.9.5 Fluorescence-activated cell sorting (FACS)

Isolated spleen and mLN cells were enriched for $CD4^+$ cells as described in section 2.9.4. Enriched $CD4^+$ T cells were surface stained, at a concentration of $1x10^8$/ml, for expression of CD4, CD25, CD62L, CD44, CD45RB using antibodies. Stained cells were washed in ice-cold PBS/0.1% BSA, filtered through cell strainers and resuspended at $5x10^7$ cells/ml. Naive cells were sorted as $CD4^+$ $CD25^-$ $CD45RB^{Hi}$ (*in vivo)* or $CD4^+$ $CD62L^+$ $CD44^-$ $CD25^-$ (*in* vitro). Treg cells were sorted as $CD4^+$ $CD25^+$ when sorted from *Atg16l1*$^{\Delta CD4}$ and *Atg16l1*$^{fl/fl}$ mice, and as $CD4^+$ YFP^+ when sorted from *Atg16l1*$^{\Delta Foxp3}$ and Foxp3-YFP-Cre mice. Cells were sorted using an Astrios, Beckman Coulter MoFlo XDP or AriaIII BD Bioscience. Post-sort flow cytometry analyses confirmed that the purity of sorted populations was >97%.

2.10 Cellular immunological assays

Naive $CD4^+$ T cells were cultured ($3x10^5$ cells/well) in 96 well flat bottom plates coated with anti-CD3 mAb (5 µg/ml) and soluble anti-CD28 mAb (1 µg/ml) and kept in the presence of IL-2 (100 U/ml). For Th0 conditions anti-IL-4 (10 µg/ml) and anti-IFN-γ (10 µg/ml) mAb were included in the culture. Cultures were supplemented with IL-12 (10 ng/ml) and anti-IL-4 mAb (10 µg/ml) for Th1 polarisation; with IL-4 (20 ng/ml), anti-IFN-γ (20 µg/ml) and anti-IL-12 (10 µg/ml) for Th2 polarisation; and with TGF-$ß_1$ (5 ng/ml), anti-IFN-γ, anti-IL-4 mAb and anti-IL-12 (all 10 µg/ml) for

induced Treg polarisation. Sorted Treg cells were activated for 48h with anti-CD3 mAb (5 µg/ml) and soluble anti-CD28 mAb (1 µg/ml) plus IL-2 (100 U/ml) and then cultured with IL-4 (10 ng/ml), IL-13 (10 ng/ml) and IL-2 (100 U/ml) for 5 days. All cytokines were from R&D Systems. Anti-CD3 (145-2C11), anti-CD28 (37.51), anti-IFN-γ (XMG1.2), anti-IL-12 (C17.8) and anti-IL-4 (11B11) mAb were from eBioscience. Cells were cultured in RPMI-1640 Medium, supplemented with 10% FCS, 2mM L-glutamine, 100U/ml Penicillin/Streptomycin (all PAA solutions).

2.11 Metabolic analysis using XF 94 Extracellular Flux Analyser

The real-time extracellular acidification rate (ECAR) and oxygen consumption rate (OCR) were measured by the Mito-stress test with a XF 94 extracellular flux analyser (Seahorse Bioscience, Massachusetts, United States). Cells were sorted or $CD4^+$ purified as described in section 2.9 and immediately analysed or activated with anti-CD3 mAb (5 µg/ml) and soluble anti-CD28 mAb (1 µg/ml) for 48h. Cells were harvested and washed twice in assay medium (RPMI 1640 without sodium bicarbonate, 20 mM glucose, 1% FCS, 2mM pyruvate). Cells were seeded at 0.5×10^6 per well in 100 µl of assay medium in a 94-well XF plate coated with poly-L-lysine (Sigma). Cells were rested for 1h at 37°C without CO^2. During the Mito-stress test sequential addition of oligomycin (30 µM, Seahorse Bioscience), fluoro-carbonyl cyanide phenylhydrazone (FCCP, 20 µM, Seahorse Bioscience), antimycin A (20 µM, Seahorse Bioscience) and rotenone (20 µM, Seahorse Bioscience) was performed.

2.12 Fluidigm Gene Expression analysis

$CD4^+$ T cells and Treg cells were sorted for each population based on surface marker and YFP expression from spleen and cLP of *Atg16l1*$^{\Delta Foxp3}$ and Foxp3-YFP-Cre mice. 200 cells/population were sorted in triplicates from a total of four (spleen) or six (cLP)

mice per group. RNA was reverse transcribed and cDNA was pre-amplified using the CellsDirect OneStep q-RT kit (Invitrogen). The selected autophagy, apoptotic and metabolic genes were amplified and analysed for expression using a dynamic 48x48 array (Biomark Fluidigm) as previously described (Tehranchi *et al.*, 2010). Data were analysed using the $2^{-\Delta Ct}$ method and results were normalized to Actin or HPRT expression.

2.13 Induction of intestinal inflammation

2.13.1 T cell transfer and mixed T cell transfer colitis

Naïve $CD4^+$ $CD25^-$ $CD45RB^{Hi}$ T cells were isolated and FACS-purified following the steps described above from the spleen of *Atg16l1*$^{\Delta CD4}$ or *Atg16l1*$^{fl/fl}$ mice (T cell transfer experiment) or WT $CD45.1^+$, *Atg16l1*$^{\Delta CD4}$ or *Atg16l1*$^{fl/fl}$ mice (mixed T cell transfer experiment). Cells were resuspended in sterile PBS and then injected intraperitoneally (i.p.) into sex matched recipient mice ($4x10^5$ cells/mouse). In mixed T cell transfer experiment cells from WT $CD45.1^+$ and *Atg16l1*$^{\Delta CD4}$ or WT $CD45.1^+$ and *Atg16l1*$^{fl/fl}$ mice were mixed in 1:1 ratio before injection. Mice were monitored weekly to assess clinical signs of disease and weight loss. Mice were euthanized at the development of severe weight loss (>20% of initial mass) or when clinical symptoms of colitis were observed.

2.13.2 Induction of T cell mediated colitis by infection with *Helicobacter hepaticus* and concomitant IL-10R blockade

Experimental T cell-mediated colitis was induced by infection with *Helicobacter hepaticus* and concomitant IL-10R blockade as described (Song-Zhao and Maloy, 2014). Briefly, *H. hepaticus* was cultured as described in section 2.4, mice were

infected with *H. hepaticus* (10^8 CFU per mouse) by oral gavage on three consecutive days and anti-IL-10R mAb (1B1.2) was administrated via i.p. injection (1mg per mouse) on the first and seventh day of the infection. Mice were sacrificed 2 weeks after colitis induction.

2.14 Assessment of intestinal inflammation

Mice were euthanized at indicated time points whereupon tissue sections were isolated and rinsed in PBS/0.1% BSA. Tissue sections were fixed in 4% formyl saline (Sigma Aldrich, UK) for >24 hours before being paraffin wax embedded. Transverse sections of 4μm thickness were cut using a microtome (Leica Biosystems) and mounted onto microscope slides with or without Haematoxylin and Eosin staining (Leica Biosystems). Histological analysis of intestinal inflammation in T cell transfer experiment and T cell mediated colitis by infection with *Helicobacter hepaticus* and concomitant IL-10R blockade was performed as described (Song-Zhao and Maloy, 2014). Briefly, inflammation was graded semi-quantitatively on a scale from 0 to 3, for four criteria; (a) epithelial hyperplasia and goblet cell depletion, (b) lamina propria leukocyte infiltration, (c) area of tissue affected, and (d) markers of severe inflammation, including crypt abscesses, sub- mucosal inflammation, and ulceration. Scores for individual criteria were totalled for an overall inflammation score between 0 and 12.

2.15 Generation of bone marrow chimeric mice

Bone marrow cells were isolated from the tibia and femur of WT ($CD45.1^+$) mice and $Atg16l1^{fl/fl}$ or $Atg16l1^{\Delta CD4}$ ($CD45.2^+$) mice. Bones were briefly washed in 70% EtOH. Femurs and tibias were flushed with ice-cold PBS, cells filtered through 70μM cell strainers and red blood cell lysis performed for *in vitro* experiments. Cells were

resuspended in ice-cold PBS and injected i.v. at 1:1 ratio (a total of $1x10^7$ cells per mouse) into lethally irradiated (2 doses of 550 Rad γ-irradiation from a ^{137}Cs source given ~180 minutes apart) *Rag1*$^{-/-}$ recipients. Mice were monitored daily for 7 days following irradiation and then were allowed to reconstitute for at least 8 weeks before analysis.

2.16 Immunization with ovalbumin

For induction of OVA-specific IgE antibodies two treatment regimes were utilized. For OVA only immunization mice were fed three times by oral gavage with ovalbumin grade VII (5mg per mouse, Sigma-Aldrich, St Louis, USA) with 21d intervals between feeds. For adjuvanted immunization, mice were initially fed with OVA (5mg per mouse) plus cholera toxin (10 μg per mouse, Biologial Compbell), after which they were fed twice with OVA only (5mg per mouse), with 21 days intervals between feeds.

2.17 Adoptive transfer of naïve CD4$^+$ T cells

Naïve CD4$^+$ T cells from WT (CD45.1$^+$) mice were sorted as described in section 2.9.5 and transferred to *Atg16l1*$^{\Delta CD4}$ recipient (CD45.2$^+$) mice via intravenous (i.v.) injection ($4\text{-}5x10^6$ cells per mouse). Analysis of spleen, mLN and cLP CD4$^+$ T and Treg cells was performed three months after transfer.

2.18 Infection with *Trichuris muris*

Mice were orally infected with ~200 *Trichuris muris* eggs. Serum was collected on day 34-post infection and assayed by ELISA for parasite-specific IgG_1. 96-well plates were coated with 5µg/ml *T. muris* excretory/secretory antigen and incubated with serial 2-fold diluted serum. Bound IgG_1 was detected using biotinylated anti-murine IgG_1 (AbD Serotec, Kidlington, UK).

2.19 Statistic

For weight curves and antibody titers, p-values were determined by two-way ANOVA with Bonferroni post-tests. For all other experiments, p-values were determined by nonparametric Mann–Whitney test. Differences were considered statistically significant when $p < 0.05$ (* $p< 0.05$, ** $p< 0.01$, *** $p< 0.001$). Data are shown as mean ± s.e.m. Statistics were calculated using GraphPad Prism 6 software. With the exception of histological assessment of intestinal inflammation, experimenters were not “blinded” to allocation of animals to experimental groups.

Chapter 3. *Atg16l1* is required for peripheral T cell homeostasis

3.1 Introduction

Although multiple studies have examined the role of essential autophagy genes on the homeostasis of $CD8^+$ and $CD4^+$ T cells, an analysis of how autophagy governs intestinal T cell populations has not been undertaken (Baxt and Xavier, 2015). Furthermore, the effect of deletion of IBD-susceptibility gene *Atg16l1* on the T cell homeostasis has not been described. Interestingly, ATG16L1 is very highly expressed in human $CD4^+$ and $CD8^+$ T cells compared to other primarily immune cells (Rioux *et al.*, 2007). To investigate the role of the *Atg16l1* in intestinal T cell homeostasis we employed a mouse model where *Atg16l1* was deleted in T cells using a Cre-loxP approach, with Cre recombinase expressed under the control of the CD4 promoter (*Atg16l1*$^{\Delta CD4}$ mice).

Atg16l1 is composed of a N-terminal region required for non-covalent binding to Atg5, a central coiled-coil domain involved in self-oligomerisation and a C-terminal domain containing seven WD-repeats whose function remains unclear (Mizushima *et al.*, 2003; Zheng *et al.*, 2004). As described in the previous chapter, Atg16l1 plays a role in the elongation of the autophagosome membrane as a part of the Atg5–Atg12-Atg16l1 complex, a function that was recently shown to involve direct binding of Atg16l1 to WIPI2b (Dooley *et al.*, 2014). In addition, the interaction between Atg16l1 and FIP200 has recently been demonstrated to play a role in stabilizing the Ulk1/2 complex at the isolation membrane (Gammoh *et al.*, 2013; Nishimura *et al.*, 2013). Atg16l1 also directly interacts with clathrin, connecting the endocytic pathway to autophagy (Ravikumar *et al.*, 2010). During bacterial infection Atg16l1 was reported to interact directly with Nod2 and this facilitated the recruitment of Atg16l1 to the

membrane site of bacterial entry (Travassos *et al.*, 2010). Moreover, Atg16l1 has also been suggested to have autophagy-independent roles in antiviral defence (Hwang *et al.*, 2012), and in regulating Nod2 signalling (Sorbara *et al.*, 2013).

A CD-associated SNP (*rs2241880*) located in exon 9 of *ATG16L1* is an A to G substitution that changes a threonine at position 300 to alanine (T300A), with the latter allelic variant representing a risk variant (Hampe *et al.*, 2007). At the protein level, the T300A substitution is localized in the unstructured region between the coiled-coil domain and the WD-repeat domain (Mizushima *et al.*, 2003; Zheng *et al.*, 2004; Hampe *et al.*, 2007). Many studies have attempted to reveal the effect of this mutation on the protein function, however until recently it remained unclear, as results were often contradictory. A study using Atg16l1-deficient mouse embryonic fibroblasts into which an Atg16l1 construct with the T300A substitution was introduced, suggested that the polymorphism had no effect on the classic autophagy-related functions of Atg16l1 (Fujita *et al.*, 2009). However, other studies reported that the T300A mutation led to impaired autophagy, leading to defective antibacterial defence in epithelial cell lines (Kuballa *et al.*, 2008) or myeloid cells (Cooney *et al.*, 2010), and increased inflammatory cytokine production (Plantinga *et al.*, 2011). Conversely, a recent study suggested that the T300A polymorphism conferred protection from bacterial infection in epithelial cells (Messer *et al.*, 2013). The discrepancies between these published reports are difficult to explain, but might at least partially be attributed to the cell type in which the T300A polymorphism was examined and the type of stimulus used to trigger autophagy or cell intrinsic immune defence.

However, recent studies have shed new light on the biological impact of the *ATG16L1* mutation. Using human cells homozygously expressing the T300A variant, as well as mice with the corresponding T316A knock-in mutation that recapitulates the CD-associated SNP, Murthy *et al.* identified a caspase cleavage site at amino acids 296–

299 of the ATG16L1 protein and provided evidence that the CD-associated SNP further sensitizes ATG16L1 to caspase 3 cleavage and degradation, ultimately leading to decreased autophagy (Murthy *et al.*, 2014). The enhanced caspase 3 cleavage was observed after subjecting cells to stress conditions, including apoptosis induction, starvation and *Yersinia enterocolitica* infection, but not in unstimulated cells (Murthy *et al.*, 2014). This correlated with decreased xenophagy and elevated IL-1β release during *Y.enterocolitica* infection in human and mouse macrophages carrying the disease-associated SNP (Murthy *et al.*, 2014). These results were supported by a subsequent study where T300A knock-in mice were independently generated, which reported decreased autophagy and compromised xenophagy in epithelial cells with the CD-associated SNP after *Shigella flexineri* infection (Lassen *et al.*, 2014). Together, these studies indicate that homozygous expression of the CD susceptibility variant T300A allele results in defective autophagy during stress conditions. This is in agreement with the fact that mutations in several other autophagy-associated genes, including *IRGM, LRRK2, SMURF1, NDP52*, have been linked to IBD susceptibility (Van Limbergen *et al.*, 2014), strongly suggesting that it is the classical autophagy pathway that connects these genetic alterations to impaired intestinal homeostasis.

As *Atg16l1* is an essential autophagy gene belonging to the core autophagy machinery, we expected that *Atg16l1* deletion in T cells would result in a complete block in the autophagy pathway. While it can be argued that studying the CD-associated T300A variant would be more relevant to human IBD studies, we argue that the system where autophagy was strongly compromised had a higher potential of revealing alterations in functional phenotypes that might otherwise be too subtle to be detected. As discussed above, the difficulty to establish the effects of the T300A mutation on the functions of Atg16l1 indicate that when autophagy is mildly impaired, the manifestation of the phenotype can greatly differ between cell types and environmental or experimental conditions. Taking these findings into account,

we choose the complete *Atg16l1* deletion within the T cell compartment as a model to analyse the relevance of autophagy within this cell type. Further studies are required to address whether the T300A mutation recapitulates the phenotype of the complete deficiency.

The aims of the experiments presented in the following results chapter were to:

(1) verify the genetic model used in the study through assessment of the functional consequences of *Atg16l1* deletion on autophagy levels in T cells isolated from *Atg16l1*$^{\Delta CD4}$ mice;

(2) characterise thymic and peripheral populations of CD8^{+} and CD4^{+} T cells in *Atg16l1*$^{\Delta CD4}$ mice, including colonic and small intestinal T cells, and compare the results with previously published reports where other autophagy genes were deleted in the T cell compartment;

(3) analyse effector responses of *Atg16l1*-deficient CD4^{+} T cells *in vitro;*

(4) analyse effector responses of *Atg16l1*-deficient CD4^{+} T cells *in vivo,* by employing complementary models of experimental intestinal inflammation.

3.2 Autophagy is blocked in T cells from *Atg16l1*$^{\Delta CD4}$ mice

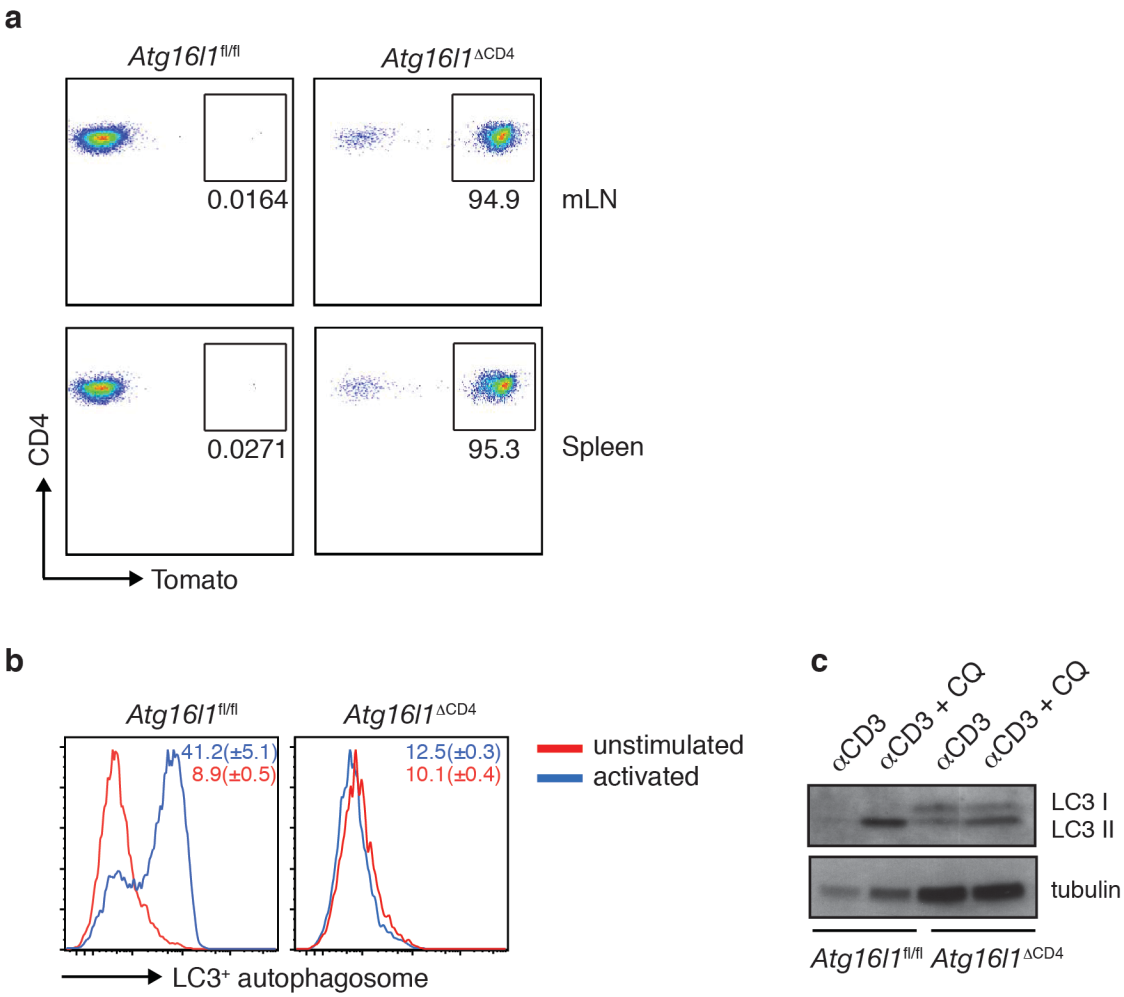
To investigate the role of autophagy in intestinal T cell homoeostasis, mice carrying *lox*P-flanked alleles of *Atg16l1* (*Atg16l1*$^{fl/fl}$) (Hwang *et al.*, 2012) were crossed with CD4-Cre mice (Lee *et al.*, 2001), to generate mice in which the essential autophagy gene *Atg16l1* was selectively ablated in T cells from the double-positive stage of thymic development (denoted as *Atg16l1*$^{\Delta CD4}$). Sex-matched littermate *Atg16l1*$^{fl/fl}$ mice were used as a control group. Animals were kept co-housed throughout the experiments.

To examine the efficiency of Cre-mediated recombination we crossed *Atg16l1*$^{\Delta CD4}$ mice with reporter mice in which Cre-mediated recombination removes a stop codon upstream of a tdTomato gene inserted into a ubiquitously expressed locus (Madisen *et al.*, 2010). We found that recombination was very efficient, as 95% of CD4^{+} T cells in the spleen and mLN expressed the tdTomato reporter (**Figure 2a**). To verify functional deletion of *Atg16l1*, autophagy levels were assayed by analysing autophagosome formation and LC3 lipidation (**Figure 2b,c**). CD4^{+} T cells isolated from control *Atg16l1*$^{fl/fl}$ littermates exhibited increased LC3^{+} autophagosome formation and LC3 lipidation (increased levels of LC3 II relative to LC3 I) after activation with anti-CD3 and anti-CD28 antibodies, whereas these changes were absent in CD4^{+} T cells from *Atg16l1*$^{\Delta CD4}$ mice (**Figure 2b,c**), confirming a block in autophagy. The increase of LC3 II over LC3 I (autophagic flux) was assessed by comparing samples where chloroquine – inhibitor of lysosomal acidification – was used with samples that were not treated with the inhibitor. This method allows to determined whether autophagosome-associated LC3 II accumulates overtime, as chloroquine prevent autophagosome degradation (Mizushima *et al.*, 2010).

Figure 2 Autophagy is blocked in T cells from *Atg16l1*$^{\Delta CD4}$ mice

(**a**) Representative FACS analysis of the expression of the Cre-excision reporter tdTomato on gated CD4$^+$ TCRβ^+ T cells isolated from mLN or spleen of *Atg16l1*$^{\Delta CD4}$ and *Atg16l1*$^{fl/fl}$ littermates. **(b)** FACS analysis of LC3$^+$ autophagosome formation in CD4$^+$ T cells from cLP of *Atg16l1*$^{\Delta CD4}$ and *Atg16l1*$^{fl/fl}$ mice after overnight activation with α-CD3 (5μg/ml) and α-CD28 (1μg/ml). **(c)** Western blot analysis of LC3 lipidation in naïve splenic CD4$^+$ T cells isolated from *Atg16l1*$^{\Delta CD4}$ mice and *Atg16l1*$^{fl/fl}$ mice after 3h activation with α-CD3 (5μg/ml) and α-CD28 (1μg/ml) with or without chloroquine (CQ, inhibitor of lysosomal degradation, 50μM).

Data are from one experiment (a), or are representative of at least three independent experiments (b,c), with at least 3 mice per group. Data are shown as mean ± s.e.m (b). Numbers indicate percentage of cells in gates (a).


a
Atg16l1fl/fl
Atg16l1ΔCD4
0.0164
94.9
mLN
0.0271
95.3
Spleen
CD4
Tomato
b
Atg16l1fl/fl
Atg16l1ΔCD4
41.2(±5.1)
8.9(±0.5)
12.5(±0.3)
10.1(±0.4)
unstimulated
activated
LC3+ autophagosome
c
αCD3
αCD3 + CQ
αCD3
αCD3 + CQ
LC3 I
LC3 II
tubulin
Atg16l1fl/fl
Atg16l1ΔCD4

3.3 *Atg16l1*$^{\Delta CD4}$ mice exhibit peripheral T cell lymphopaenia

To determine how *Atg16l1* governs intestinal and systemic T cell populations, we compared *Atg16l1*$^{\Delta CD4}$ with age-matched *Atg16l1*$^{fl/fl}$ control littermates. We found that T cell development was unperturbed in *Atg16l1*$^{\Delta CD4}$ mice, as numbers of thymocytes were comparable between *Atg16l1*$^{\Delta CD4}$ and *Atg16l1*$^{fl/fl}$ littermates (**Figure 3a**) and frequencies of single positive CD4^{+}, single positive CD8^{+}, double positive (DP) CD4^{+} CD8^{+} and double negative (DN) CD4^{-} CD8^{-} thymocytes were unchanged in *Atg16l1*$^{\Delta CD4}$ mice (**Figure 3b,c**).

In contrast, we found significantly decreased frequencies of CD4^{+} and CD8^{+} T cells in peripheral lymphoid organs (spleen and mLN) of *Atg16l1*$^{\Delta CD4}$ mice compared to *Atg16l1*$^{fl/fl}$ littermates (**Figure 4a,b**). Furthermore, we observed significant decreases in both T cell frequencies and numbers in the intestinal lamina propria of *Atg16l1*$^{\Delta CD4}$ mice compared to *Atg16l1*$^{fl/fl}$ littermates (**Figure 4a-c**), indicating that *Atg16l1* is essential for T cell homeostasis within the intestinal tract. Further analyses revealed that frequencies of CD4^{+} T cells with an activated phenotype (CD62L^{low} CD44^{hi}), but not those with a naive phenotype (CD62L^{hi} CD44^{low}), were significantly decreased in the cLP of *Atg16l1*$^{\Delta CD4}$ mice when compared to *Atg16l1*$^{fl/fl}$ littermates, although this was not observed in the spleen or mLN where the majority of CD4^{+} T cells were naïve (**Figure 4d,e**). While these results indicated that Atg16l1 is essential for both peripheral CD4^{+} and CD8^{+} T cell maintenance, as CD4^{+} T cells are the main drivers and regulators of chronic intestinal inflammation (Shale *et al.*, 2013), we focused subsequent analyses on CD4^{+} T cells.

Figure 3 Thymic T cell development is normal in *Atg16l1*$^{\Delta CD4}$ mice

(a) Total numbers of thymocytes in *Atg16l1*$^{\Delta CD4}$ and *Atg16l1*$^{fl/fl}$ littermates. **(b)** Representative FACS plots and **(c)** frequencies of single positive CD4^{+}, single positive CD8^{+}, double positive (DP) CD4^{+} CD8^{+} and double negative (DN) CD4^{-} CD8^{-} thymocytes in *Atg16l1*$^{\Delta CD4}$ and *Atg16l1*$^{fl/fl}$ littermates.

Data are combined from (a,c), or representative of (b), two independent experiments with at least 4 mice per group. Each dot represents an individual mouse and horizontal bars denote means. Numbers indicate percentage of cells in quadrants. Statistical significance was determined using the Mann Whitney test.

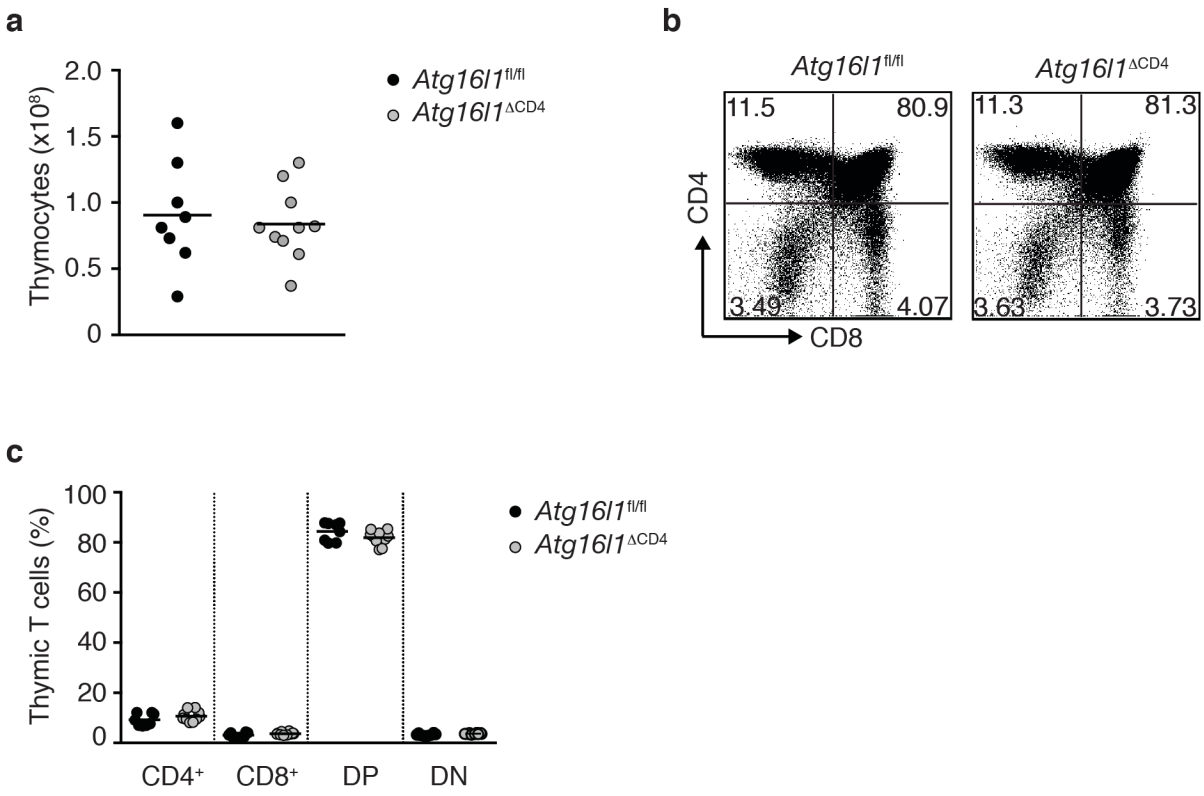
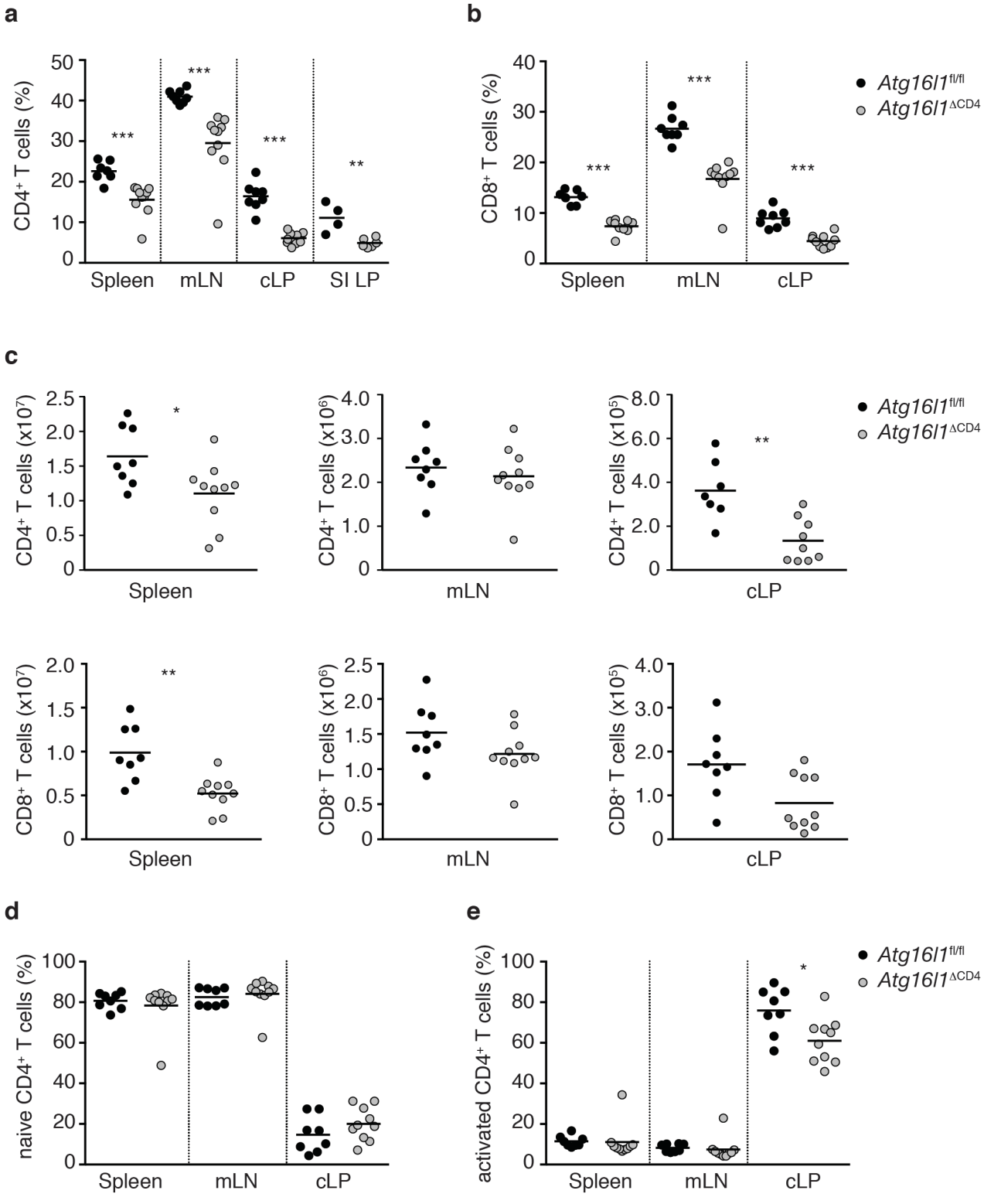

a
Thymocytes (x10^8)
2.0
1.5
1.0
0.5
0
Atg16l1^fl/fl
Atg16l1^ΔCD4
b
Atg16l1^fl/fl
Atg16l1^ΔCD4
11.5
80.9
3.49
4.07
11.3
81.3
3.63
3.73
CD4
CD8
c
Thymic T cells (%)
100
80
60
40
20
0
CD4+
CD8+
DP
DN
Atg16l1^fl/fl
Atg16l1^ΔCD4

Figure 4 *Atg16l1*$^{\Delta CD4}$ mice have peripheral T cell lymphopaenia

(a,b) Frequencies of CD4^{+} T cells **(a)** and CD8^{+} T cells **(b)** as a proportion of live cells in the indicated peripheral lymphoid tissues of *Atg16l1*$^{\Delta CD4}$ and *Atg16l1*$^{fl/fl}$ littermates. **(c)** Total numbers of CD4^{+} T cells in spleen, mLN, and cLP of *Atg16l1*$^{\Delta CD4}$ and *Atg16l1*$^{fl/fl}$ littermates. **(d,e)** Frequencies of naïve CD62L^{hi} CD44^{low} CD4^{+} T cells **(d)** and memory/activated CD62L^{low} CD44^{hi} CD4^{+} T cells **(e),** as a proportion of CD4^{+} T cells in *Atg16l1*$^{\Delta CD4}$ and *Atg16l1*$^{fl/fl}$ littermates.

Data are combined from two (b,c,d,e) or three (a) independent experiments with at least 4 mice per group. Each dot represents an individual mouse and horizontal bars denote means. Statistical significance was determined using the Mann Whitney test, * $p<0.05$; ** $p<0.01$; *** $p<0.001$.


a
b
c
d
e
CD4+ T cells (%)
CD8+ T cells (%)
CD4+ T cells (x10^7)
CD4+ T cells (x10^6)
CD4+ T cells (x10^5)
CD8+ T cells (x10^7)
CD8+ T cells (x10^6)
CD8+ T cells (x10^5)
naive CD4+ T cells (%)
activated CD4+ T cells (%)
Spleen
mLN
cLP
SI LP
Atg16l1fl/fl
Atg16l1ΔCD4

3.4 *Atg16l1*-deficient CD4$^+$ T cells show defective proliferation *in vitro* and impaired reconstitution of *Rag*$^{-/-}$ mice

As *Atg16l1* deficiency resulted in a significant decrease in the numbers of activated CD4$^+$ T cells in the periphery, we further investigated how *Atg16l1* deficiency impacted on CD4$^+$ T cell activation and proliferation *in vitro*. We observed that *Atg16l1*-deficient CD4$^+$ T cells failed to proliferate upon stimulation with agonistic anti-CD3 and anti-CD28 antibodies *in vitro,* whereas control *Atg16l1*$^{fl/fl}$ CD4$^+$ T cells mounted robust proliferative responses (**Figure 5a**). However, modulation of activation markers by *Atg16l1*-deficient CD4$^+$ T cells was comparable to *Atg16l1*$^{fl/fl}$ CD4$^+$ T cells, as stimulation with agonistic anti-CD3 and anti-CD28 mAbs resulted in downregulation of CD62L and upregulation of CD44 and IL-2Rα to a similar extent (**Figure 5b**). In addition, IL-7Rα (CD127) was upregulated to a greater extent by activated *Atg16l1*-deficient CD4$^+$ T cells (**Figure 5b**). Furthermore, CD4$^+$ T cells isolated from *Atg16l1*$^{\Delta CD4}$ and *Atg16l1*$^{fl/fl}$ mice showed similar secretion of IL-2 after 2 days of stimulation with anti-CD3 and anti-CD28 (**Figure 5c**). By day 4 of stimulation IL-2 levels had decreased in control *Atg16l1*$^{fl/fl}$ CD4$^+$ T cell supernatants, while remaining high in supernatants from *Atg16l1*-deficient CD4$^+$ T cell cultures - most likely due to the fact that the latter were not proliferating and therefore not using IL-2 present in the medium (**Figure 5c**). In line with this observation, we found that *ex vivo* IL-2 production in CD4$^+$ T cells isolated from the mLN and cLP of *Atg16l1*$^{\Delta CD4}$ mice was increased when compared to CD4$^+$ T cells from *Atg16l1*$^{fl/fl}$ littermates (**Figure 5d,e**). Overall these results indicated that although *Atg16l1* deficiency ablated CD4$^+$ T cell proliferation *in vitro*, it did not intrinsically impair IL-2 production or surface markers expression upon TCR engagement.

T cells undergo metabolic changes upon antigen-induced activation (Buck *et al.*, 2015) and it has been recently proposed that autophagy plays a role in metabolic regulation of $CD8^+$ T cell differentiation and survival (Xu *et al.*, 2014). We therefore examined if *Atg16l1*-deficiency influenced the metabolic activities of $CD4^+$ T cells, both at basal state and following TCR activation *in vitro*. Increased glucose uptake is a hallmark of T cell activation (Buck *et al.*, 2015), we therefore examined this parameter in $CD4^+$ T cells stimulated with anti-CD3 and anti-CD28 antibodies. We found that $CD4^+$ T cells isolated from *Atg16l1*$^{\Delta CD4}$ and *Atg16l1*$^{fl/fl}$ littermates showed comparable uptake of the fluorescent glucose analog 2-NBDG (**Figure 6a**). However, a more detailed analysis of metabolic parameters in real time using metabolic flux analyser revealed significant metabolic changes in $CD4^+$ T cells with *Atg16l1* deletion. The metabolic flux analyser allows measurement of oxygen consumption rate (OCR), which is an indicator of mitochondrial oxidative phosphorylation (OXPHOS) and extracellular acidification rate (ECAR), an indirect indicator of aerobic glycolysis. Additionally, use of specific inhibitors allows for measuring parameters such as ATP production and spare respiratory capacity (SRC). For instance, the difference in OCR measurement before and after addition of oligomycin (blocks ATP synthesis) gives the indication of ATP turnover in the cells. Subsequent addition of FCCP (uncouples ATP synthesis from the electron transport chain, ETC) gives the measurement of maximal possible respiration, and then treatment with rotenone and antimycin A (block complex I and III of the ETC) allows for calculation of proton leak and non-mitochondrial respiration.

In unstimulated, naïve $CD44^-$ $CD62L^+$ *Atg16l1*-deficient $CD4^+$ T cells OCR was significantly increased compared to control $CD4^+$ T cells, indicating a higher level of mitochondrial respiration (**Figure 6b,c**). In particular, we saw marked increase in the amount of SRC of unstimulated naïve *Atg16l1*-deficient $CD4^+$ T cells, as characterised by the difference between basal and maximal respiration rates measured following

treatment with FCCP (**Figure 6c**). Additionally, basal levels of ECAR were significantly, but mildly increased in naïve *Atg16l1* deficient $CD4^+$ T cells compared to control cells (**Figure 6b**). Increased OCR by *Atg16l1*$^{\Delta CD4}$ $CD4^+$ T cells was also observed immediately following activation with anti-CD3 and anti-CD28 antibodies (**Figure 6d**), whereas ECAR increased similarly in control and *Atg16l1*-deficient $CD4^+$ T cells (**Figure 6e**). However, after prolonged TCR activation *in vitro* we saw a striking change in the metabolic profile of autophagy-deficient $CD4^+$ T cells. While both *Atg16l1*$^{\Delta CD4}$ and *Atg16l1*$^{fl/fl}$ $CD4^+$ T cells showed a comparable increase in ECAR following activation with anti-CD3 and anti-CD28 antibodies for 48h, ATP production associated with mitochondrial respiration was completely absent in *Atg16l1*-deficient $CD4^+$ T cells (**Figure 6f,g**). This was particularly apparent in the metabolic profiling with inhibitors, as treatment with oligomycin, FCCP, antimycin and rotenone had no effect on the level of OCR measurement in *Atg16l1* deficient $CD4^+$ T cells, indicating that the basal measurement was corresponding to non-mitochondrial oxygen consumption (**Figure 6g**). In contrast, control $CD4^+$ T cells had significantly increased OCR after stimulation with anti-CD3 and anti-CD28 antibodies for 48h (compare **Figure 6f,g** to **Figure 6b,c**). These results suggest that *Atg16l1*-deficient $CD4^+$ T cells fail to maintain increased levels of OXPHOS upon activation.

Mitochondria are the main bioenergetic organelle where ATP is produced during OXPHOS, but they also play an important role in regulating signalling pathways in activated T cells, for instance through ROS production (Weinberg *et al.*, 2015). Previous studies indicated that deletion of essential autophagy genes in T cells led to defective turnover of organelles, such as mitochondria, which are removed by mitophagy (Pua *et al.*, 2009; Jia and He, 2011; Jia *et al.*, 2011). In contrast to these studies, preliminary analysis of mitochondrial mass in *Atg16l1*-deficient $CD4^+$ T cells showed minimal increase in mitochondrial content in both unstimulated and activated $CD4^+$ T cells isolated from *Atg16l1*$^{\Delta CD4}$ mice when compared to $CD4^+$ T cells

from *Atg16l1*$^{fl/fl}$ control mice (**Figure 6h**). In summary, *Atg16l1* deletion in CD4$^+$ T cells results in blocked proliferation *in vitro*, even though early changes in activation markers are comparable in *Atg16l1* deficient CD4$^+$ T cells and control CD4$^+$ T cells. However, we established that *Atg16l1*-defcient CD4$^+$ T exhibited alterations in their basal metabolic state and failed to up-regulate their OXPHOS capacity after prolonged TCR activation.

We next wondered if the proliferation block in *Atg16l1*-defcient CD4$^+$ T cells that we saw *in vitro* would also be evident *in vivo*. To assess this, we used an experimental T cell transfer model, where adoptive transfer of naïve CD4$^+$ T cells into lymphopaenic *Rag1*$^{-/-}$ or *Scid* mice results in rapid proliferation of donor CD4$^+$ T cells that ultimately mediate severe systemic and intestinal inflammation in recipient mice, due to unrestrained effector CD4$^+$ T responses towards self and microbiota antigens (Powrie *et al.*, 1993). Thus, we could utilize this model to establish if autophagy-deficient CD4$^+$ T cells would proliferate in an *in vivo* lymphopaenic setting, and if so, if they would be able to drive intestinal inflammation.

We therefore set up two experimental groups of *Rag1*$^{-/-}$ mice reconstituted with naïve CD4$^+$ T cells isolated from either *Atg16l1*$^{\Delta CD4}$ mice or from *Atg16l1*$^{fl/fl}$ control littermates (**Figure 7a**). The weight of experimental mice was regularly monitored to estimate the onset of systemic and intestinal inflammation. As expected, mice transferred with naïve *Atg16l1*$^{fl/fl}$ CD4$^+$ T cells succumbed to severe inflammation, which was manifested by a rapid weight loss between day 20-30 post transfer (**Figure 7b**). In contrast, recipients transferred with *Atg16l1*-deficient naïve CD4$^+$ T cells did not lose weight (**Figure 7b**). Upon examination of spleen, mLN and cLP cell compartments of experimental mice, we found that *Atg16l1*$^{fl/fl}$ CD4$^+$ T cells successfully reconstituted the *Rag1*$^{-/-}$ recipients at all sites, whereas autophagy-deficient CD4$^+$ T cells failed to do so, as we found very few CD4$^+$ T cells in recipients

transferred with naïve *Atg16l1*$^{\Delta CD4}$ CD4^{+} T cells (**Figure 7c**). In line with these observations, the majority of *Rag1*$^{-/-}$ mice reconstituted with *Atg16l1*$^{fl/fl}$ CD4^{+} T cells developed severe colitis, with marked leukocyte infiltration, epithelial hyperplasia and goblet cell depletion, whereas *Rag1*$^{-/-}$ mice that received *Atg16l1*$^{\Delta CD4}$ CD4^{+} T cells remained healthy with no signs of intestinal inflammation (**Figure 7d,e**). Overall, these results indicated that *Atg16l1*-deficient CD4^{+} T cells were unable to reconstitute lymphopaenic hosts.

To establish if the inability in reconstituting the lymphoid niche *in vivo* was due to cell intrinsic defects of autophagy-deficient CD4^{+} T cells, or if it could be rescued by provision of extracellular factors produced by proliferating WT CD4^{+} T cells, we performed mixed T cell transfer. In this experimental setting, recipient *Rag1*$^{-/-}$ mice were transferred with a 1:1 mixture of WT congenic CD45.1^{+} naïve CD4^{+} T cells together with either *Atg16l1*$^{fl/fl}$ or *Atg16l1*$^{\Delta CD4}$ naïve CD4^{+} T cells (CD45.1^{-})(**Figure 8a**). We observed a rapid and severe weight loss in both *Rag1*$^{-/-}$ recipient groups, beginning at around 30 days post transfer (**Figure 8b**). Mice were then sacrificed and composition of cellular compartments in the spleen, mLN and cLP was examined. While *Rag1*$^{-/-}$ recipients transferred with a mixture of congenic CD45.1^{+} and *Atg16l1*$^{fl/fl}$ naïve CD4^{+} T cells showed comparable frequencies of CD4^{+} T cells originating from both donor cell types, in *Rag1*$^{-/-}$ mice transferred with CD45.1^{+} and *Atg16l1*$^{\Delta CD4}$ T cells almost all CD4^{+} T cells present in the spleen, mLN and cLP were of the WT CD45.1^{+} origin (**Figure 8c**). This result confirmed that *Atg16l1*-deficient CD4^{+} T cells fail to reconstitute lymphopaenic hosts and indicated that this was due to cell intrinsic defects.

Figure 5 *Atg16l1*-deficient CD4$^+$ T cells do not proliferate upon activation *in vitro*

(a) CD4$^+$ T cells isolated from *Atg16l1*$^{\Delta CD4}$ or *Atg16l1*$^{fl/fl}$ mice were stained with cell tracer dye and cultured in the presence of plate bound anti-CD3 (5μg/ml) plus soluble anti-CD28 (1μg/ml) for 2 or 4 days after which proliferation was analysed by FACS (gated on CD4$^+$ TCRß$^+$ live cells). **(b)** Expression of surface markers in CD4$^+$ T cells isolated from *Atg16l1*$^{\Delta CD4}$ or *Atg16l1*$^{fl/fl}$ mice was analysed in unstimulated cells or in cells activated with anti-CD3 (5μg/ml) plus anti-CD28 (1μg/ml) for 2 days (gated on CD4$^+$ TCRß$^+$ live cells). (**c**) IL-2 was measured by ELISA in the supernatants of cultures of CD4$^+$ T cells isolated from *Atg16l1*$^{\Delta CD4}$ or *Atg16l1*$^{fl/fl}$ mice following activation with plate bound anti-CD3 (5μg/ml) plus soluble anti-CD28 (1μg/ml) for 2 or 4 days. **(d,e)** Representative FACS plots **(d)** and frequencies **(e)** of IL-2$^+$ CD4$^+$ T cells within cLP and mLN isolated from *Atg16l1*$^{\Delta CD4}$ and *Atg16l1*$^{fl/fl}$ littermates (gated on CD4$^+$ TCRβ^+ live cells). Cells were re-stimulated for the cytokine staining as described in the Material and Methods section.

Data are representative of 2 independent experiments. Numbers indicate percentage of cells in gates (d). Each dot represents an individual mouse and horizontal bars denote means (e). Data are shown as mean ± s.e.m, n=2 (c). Cells used in a,b,c were after CD4$^+$ enrichment, as described in the Material and Methods section. Statistical significance was determined using the Mann Whitney test, * $p<0.05$.

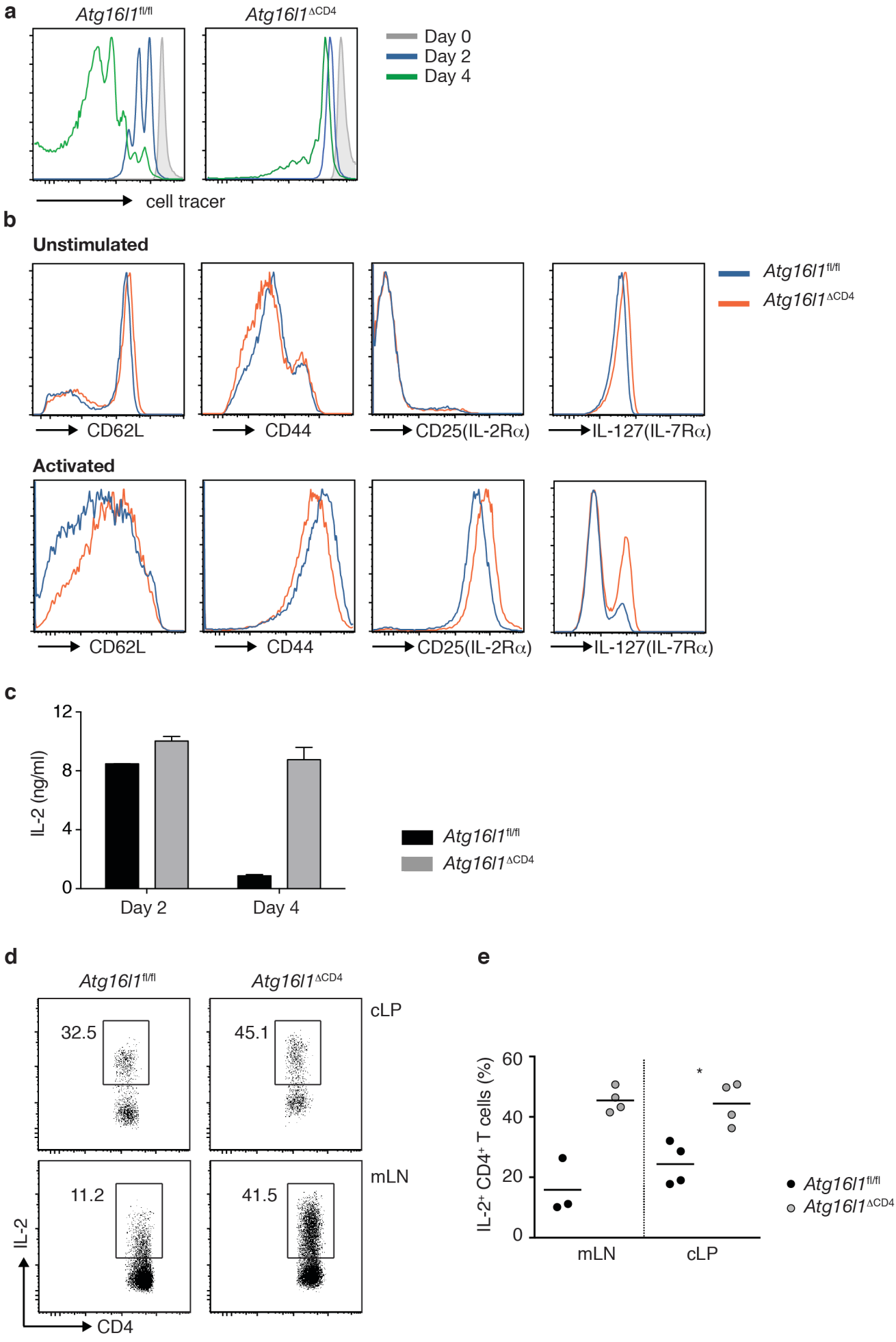

a
Atg16l1fl/fl
Atg16l1ΔCD4
Day 0
Day 2
Day 4
cell tracer
b
Unstimulated
CD62L
CD44
CD25(IL-2Rα)
IL-127(IL-7Rα)
Atg16l1fl/fl
Atg16l1ΔCD4
Activated
CD62L
CD44
CD25(IL-2Rα)
IL-127(IL-7Rα)
c
IL-2 (ng/ml)
12
8
4
0
Day 2
Day 4
Atg16l1fl/fl
Atg16l1ΔCD4
d
Atg16l1fl/fl
Atg16l1ΔCD4
cLP
mLN
32.5
45.1
11.2
41.5
IL-2
CD4
e
IL-2+ CD4+ T cells (%)
60
40
20
0
*
mLN
cLP
Atg16l1fl/fl
Atg16l1ΔCD4

Figure 6 Metabolic changes in *Atg16l1*-deficient CD4$^+$ T cells

(a) CD4$^+$ T cells isolated from the spleen of *Atg16l1*$^{\Delta CD4}$ or *Atg16l1*$^{fl/fl}$ mice were cultured in the presence of anti-CD3 (5µg/ml) plus anti-CD28 (1µg/ml) for 24h after which the fluorescent 2-deoxyglucose analog (2-NDBG, 100 µM) was added to analyse glucose uptake, which was measured by flow cytometry. Numbers indicate mean fluorescence intensity (MFI) of two technical replicates **(b)** Basal level of oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) in naïve (CD44$^-$ CD62L$^+$) unstimulated CD4$^+$ T cells isolated from the spleen of *Atg16l1*$^{\Delta CD4}$ or *Atg16l1*$^{fl/fl}$ mice were measured using metabolic flux analyser (Seahorse Bioscience Extracellular Flux Analyser as described in the Material and Methods section. **(c)** Bio-energetic profiles of unstimulated naïve (CD44$^-$ CD62L$^+$) CD4$^+$ T cells isolated from the spleen of *Atg16l1*$^{\Delta CD4}$ or *Atg16l1*$^{fl/fl}$ mice were measured in real time using metabolic flux analyser. Cells were treated sequentially with oligomycin (oligo, 30 µM), fluoro-carbonyl cyanide phenylhydrazone (FCCP, 20 µM), antimycin A (antimy, 20 µM) and rotenone (roten, 20 µM), as described in the Material and Methods section. **(d,e)** OCR and ECAR levels of CD4$^+$ T cells isolated from the spleen of *Atg16l1*$^{\Delta CD4}$ or *Atg16l1*$^{fl/fl}$ mice measured in real time using metabolic flux analyser at baseline and following activation with anti-CD3 (10µg/ml) plus anti-CD28 (10µg/ml). (**f**) Basal levels of OCR and ECAR in CD4$^+$ T cells isolated from the spleen of *Atg16l1*$^{\Delta CD4}$ or *Atg16l1*$^{fl/fl}$ mice were measured using metabolic flux analyser following activation with anti-CD3 (5µg/ml) plus anti-CD28 (1µg/ml) for 2 days. **(g)** Bio-energetic profiles of CD4$^+$ T cells isolated from the spleen of *Atg16l1*$^{\Delta CD4}$ or *Atg16l1*$^{fl/fl}$ mice and activated *in vivo* as described in (f) were measured in real time using metabolic flux analyser. Cells were treated sequentially with oligomycin (oligo, 30 µM), fluoro-carbonyl cyanide phenylhydrazone (FCCP, 20 µM), antimycin A (antimy, 20 µM) and rotenone (roten, 20 µM), as described in the Material and Methods section. **(h)** CD4$^+$ T cells isolated from the spleen of *Atg16l1*$^{\Delta CD4}$ or *Atg16l1*$^{fl/fl}$ mice were cultured in the presence of anti-CD3 (5µg/ml) plus anti-CD28 (1µg/ml) for 2 days after which mitochondria mass was assessed using Mitotracker Green staining and analysed by flow cytometry. Numbers indicate MFI.

Data are representative of two independent experiments (b,c,h), or are from one experiment (a,d,e,f,g). Data from 3 separate measurements with 7 technical replicates (b,f). Data presented as mean +/- s.e.m (a-g). Each dot represent the mean from individual measurement with at least 4 technical replicates (c-e,g). $CD4^+$ T cells were purified by FACS (b,c) or $CD4^+$ T cell enrich (d-g), as described in the Material and Methods section.

*** $p<0.001$.

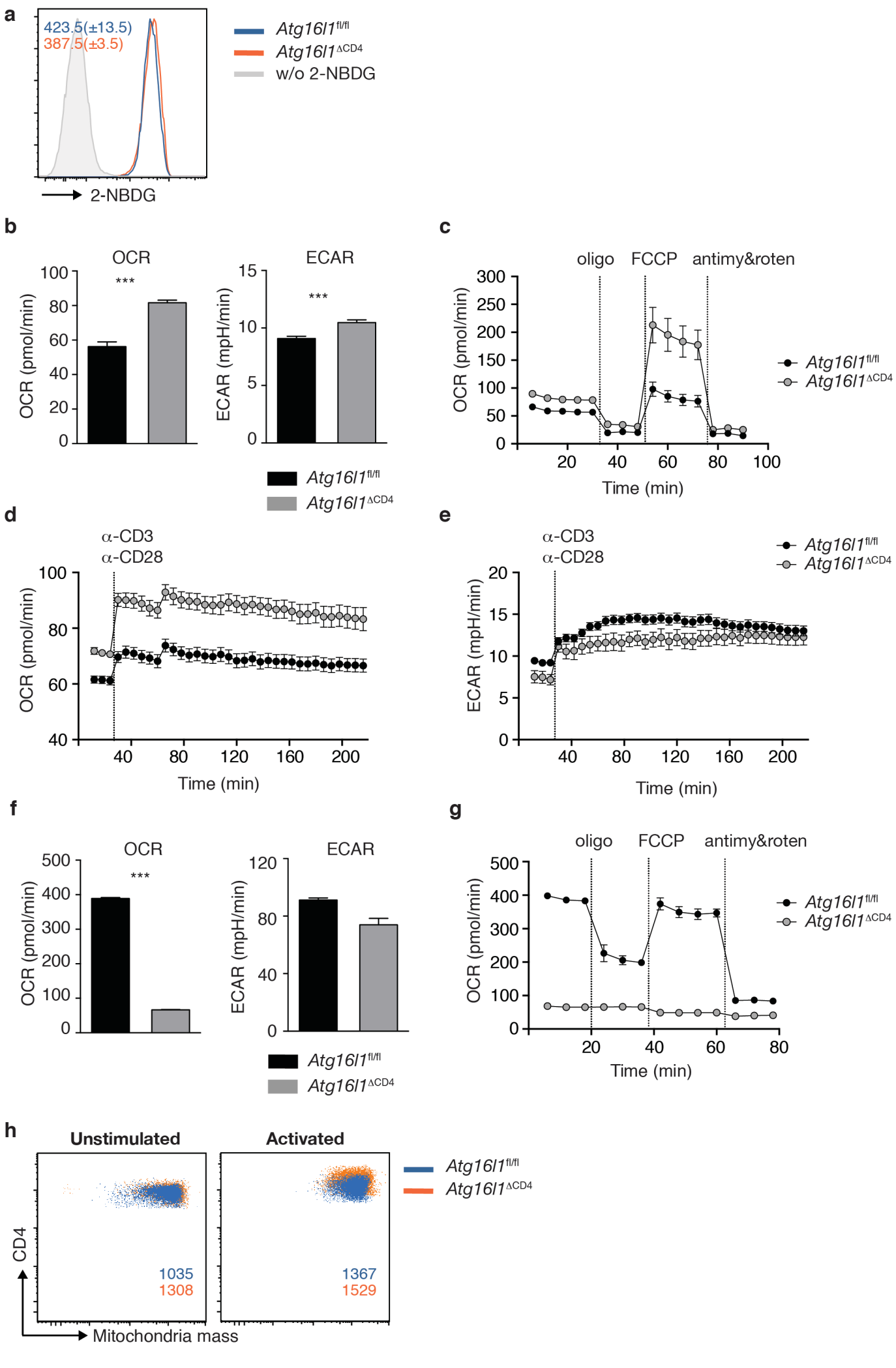

a
423.5(±13.5)
387.5(±3.5)
Atg16l1fl/fl
Atg16l1ΔCD4
w/o 2-NBDG
2-NBDG
b
OCR
ECAR
***
OCR (pmol/min)
ECAR (mpH/min)
Atg16l1fl/fl
Atg16l1ΔCD4
c
oligo
FCCP
antimy&roten
OCR (pmol/min)
Time (min)
Atg16l1fl/fl
Atg16l1ΔCD4
d
α-CD3
α-CD28
OCR (pmol/min)
Time (min)
e
α-CD3
α-CD28
ECAR (mpH/min)
Time (min)
Atg16l1fl/fl
Atg16l1ΔCD4
f
OCR
ECAR
***
OCR (pmol/min)
ECAR (mpH/min)
Atg16l1fl/fl
Atg16l1ΔCD4
g
oligo
FCCP
antimy&roten
OCR (pmol/min)
Time (min)
Atg16l1fl/fl
Atg16l1ΔCD4
h
Unstimulated
Activated
CD4
Mitochondria mass
1035
1308
1367
1529
Atg16l1fl/fl
Atg16l1ΔCD4

Figure 7 *Atg16l1*-deficient CD4$^+$ T cells fail to reconstitute lymphopaenic *Rag*$^{-/-}$ hosts

FACS-purified naïve CD4$^+$ CD25$^-$ CD45RB^{hi} T cells isolated from the spleen of *Atg16l1*$^{\Delta CD4}$ or *Atg16l1*$^{fl/fl}$ mice were injected *i.p.* into *Rag1*$^{-/-}$ mice (4x10^5 cells/mouse). Recipients were sacrificed upon development of clinical signs of disease.

(a) Schematic representation of the experimental design. **(b)** Body weight curves of *Rag1*$^{-/-}$ recipient mice receiving *Atg16l1*$^{\Delta CD4}$ or *Atg16l1*$^{fl/fl}$ naïve CD4$^+$ T cells. **(c)** Frequencies of *Atg16l1*$^{\Delta CD4}$ or *Atg16l1*$^{fl/fl}$ CD4$^+$ T cells as a proportion of live cells in the spleen, mLN and cLP of *Rag1*$^{-/-}$ recipients. **(d)** Representative photomicrographs of haemotoxilin and eosin (H&E) stained sections of mid-colon from *Rag1*$^{-/-}$ recipients receiving *Atg16l1*$^{\Delta CD4}$ or *Atg16l1*$^{fl/fl}$ naïve CD4$^+$ T cells, scale bar 200μm. (**e**) Colon histopathology scores of *Rag1*$^{-/-}$ recipients receiving *Atg16l1*$^{\Delta CD4}$ or *Atg16l1*$^{fl/fl}$ naïve CD4$^+$ T cells.

Data are from one experiment with 6 mice per group. Data shown are means ± s.e.m (b). Each dot represents an individual mouse and horizontal bars denote means (c,e). Statistical significance was determined using two-way analysis of variance (ANOVA) with Bonferroni's correction for multiple comparisons (b) or using the Mann Whitney test (c,e), ** $p<0.01$; *** $p<0.001$.

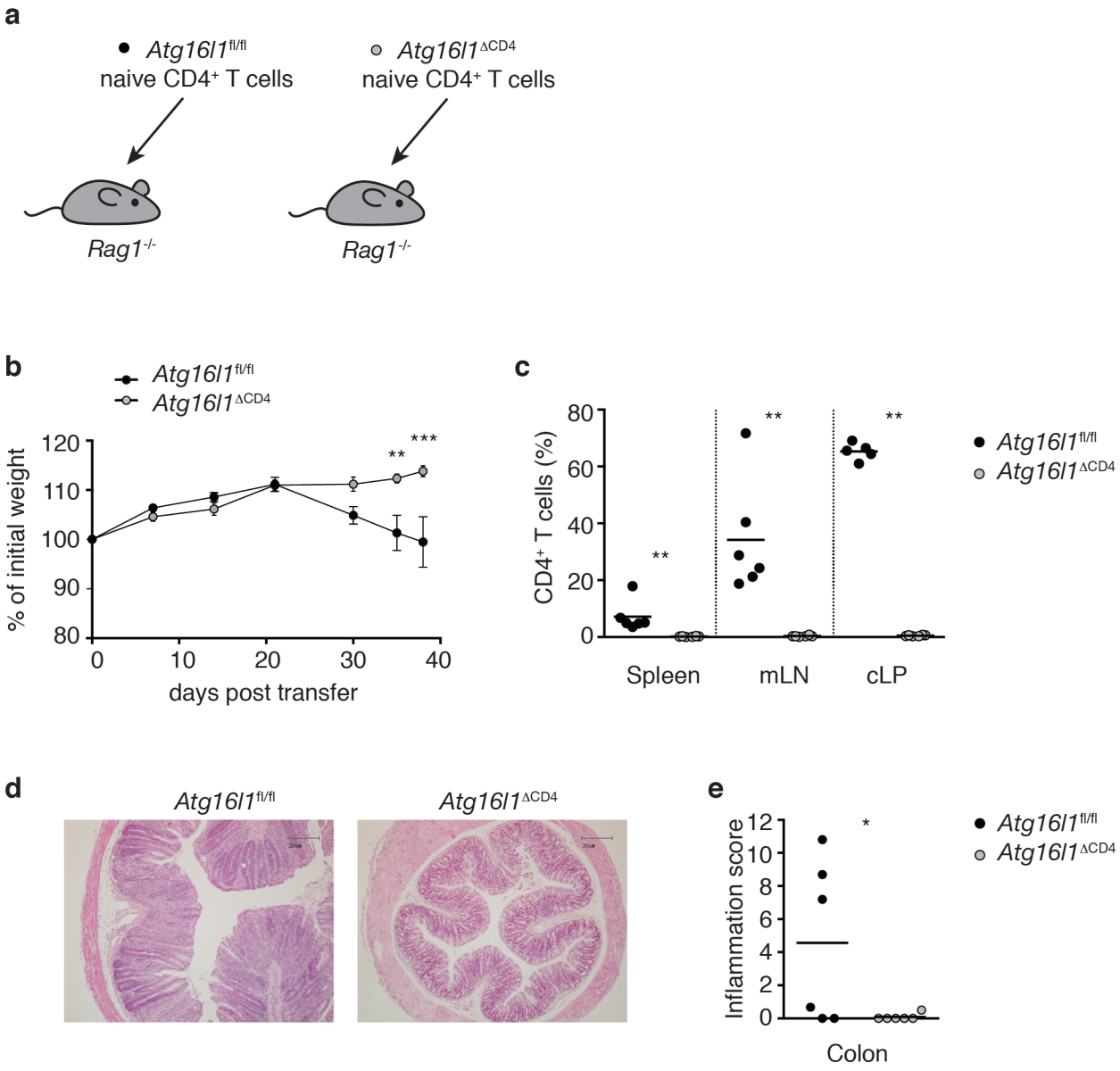
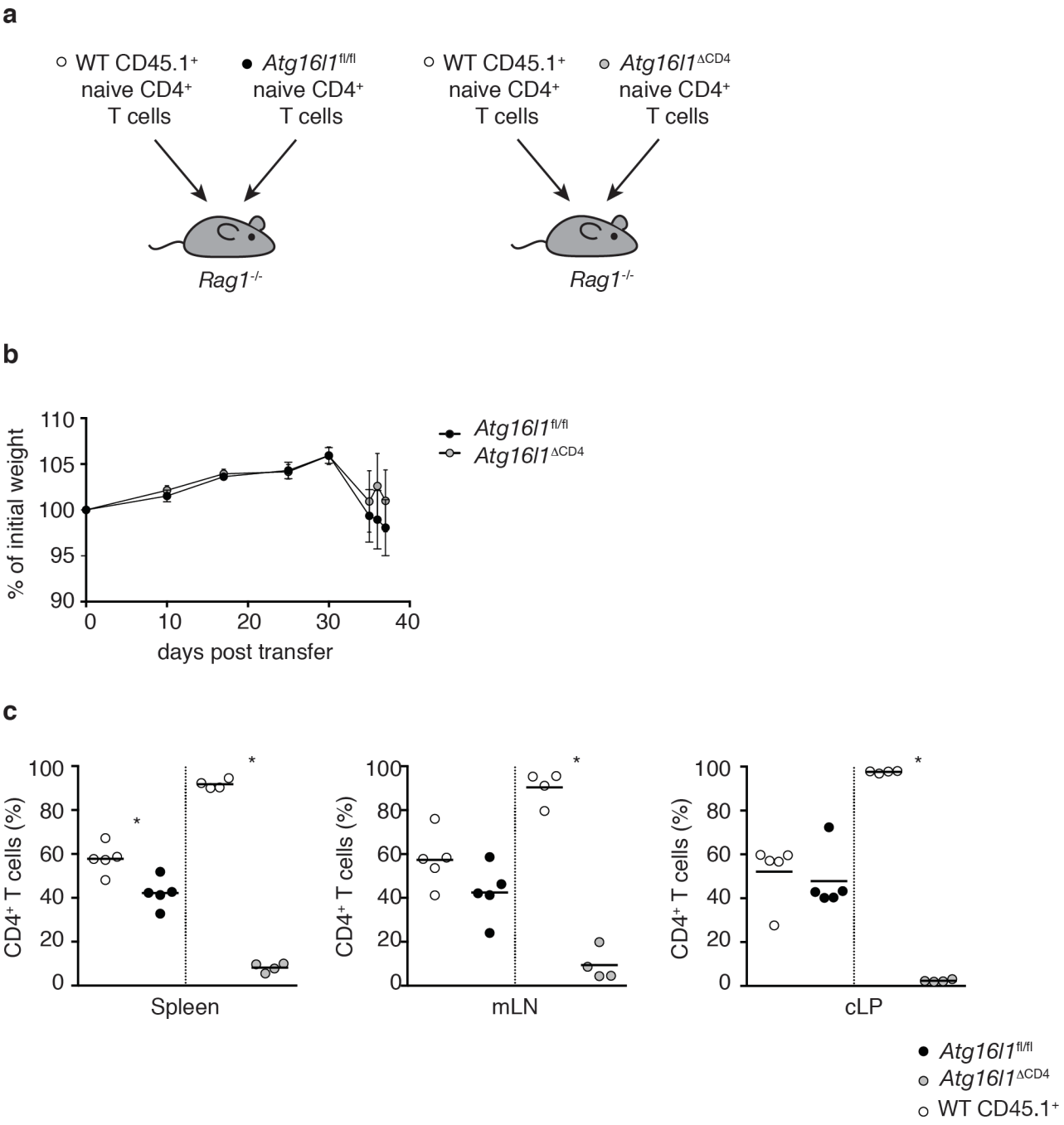

a
Atg16l1fl/fl
naive CD4+ T cells
Atg16l1ΔCD4
naive CD4+ T cells
Rag1-/-
Rag1-/-
b
Atg16l1fl/fl
Atg16l1ΔCD4
% of initial weight
120
110
100
90
80
0
10
20
30
40
days post transfer
**
***
c
CD4+ T cells (%)
80
60
40
20
0
**
**
**
Spleen
mLN
cLP
Atg16l1fl/fl
Atg16l1ΔCD4
d
Atg16l1fl/fl
Atg16l1ΔCD4
e
Inflammation score
12
10
8
6
4
2
0
*
Colon
Atg16l1fl/fl
Atg16l1ΔCD4

Figure 8 The reconstitution defect of *Atg16l1*-deficient CD4$^+$ T cells is cell-intrinsic

Rag1$^{-/-}$ recipient mice were injected i.p. with a 1:1 ratio of FACS-purified naïve splenic CD4$^+$ CD25$^-$ CD45RB^{hi} T cells from WT CD45.1$^+$ mice and either *Atg16l1*$^{\Delta CD4}$ mice or *Atg16l1*$^{fl/fl}$ mice (total of 4x10^5 cells/mouse). Recipients were sacrificed upon development of clinical signs of disease.

(a) Schematic representation of the experimental design. **(b)** Body weight curves of *Rag1*$^{-/-}$ recipients of WT CD45.1$^+$ plus *Atg16l1*$^{\Delta CD4}$ naïve CD4$^+$ T cells, or WT CD45.1$^+$ plus *Atg16l1*$^{fl/fl}$ naïve CD4$^+$ T cells. **(c)** Frequencies of WT CD45.1$^+$, *Atg16l1*$^{\Delta CD4}$ or *Atg16l1*$^{fl/fl}$ CD4$^+$ T cells as a proportion of total CD4$^+$ T cells in the spleen, mLN and cLP of *Rag1*$^{-/-}$ recipients.

Data are from one experiment with 4-5 mice per group. Data shown are means ± s.e.m (b). Each dot represents an individual mouse and horizontal bars denote means (c). Statistical significance was determined using two-way analysis of variance (ANOVA) with Bonferroni's correction for multiple comparisons (b) or using the Mann Whitney test (c), * $p<0.05$.


a
WT CD45.1+ naive CD4+ T cells
Atg16l1fl/fl naive CD4+ T cells
WT CD45.1+ naive CD4+ T cells
Atg16l1ΔCD4 naive CD4+ T cells
Rag1-/-
Rag1-/-
b
% of initial weight
110
105
100
95
90
0
10
20
30
40
days post transfer
Atg16l1fl/fl
Atg16l1ΔCD4
c
CD4+ T cells (%)
100
80
60
40
20
0
Spleen
mLN
cLP
*
Atg16l1fl/fl
Atg16l1ΔCD4
WT CD45.1+

3.5 *Atg16l1*$^{\Delta CD4}$ mice exhibit increased susceptibility to T cell-mediated experimental colitis

Results from the T cell transfer experiments suggested that *Atg16l1*-deficient CD4$^+$ T cells were unable to drive intestinal inflammation. However, a cardinal feature of the T cell transfer model is a vigorous lymphopaenia-driven proliferation of naïve CD4$^+$ T cells, which the *Atg16l1*-deficient T cells were not able to perform. In contrast, at steady state CD4$^+$ T cells are present, although reduced in number. Therefore we wondered whether *Atg16l1*-deficient CD4$^+$ T cells are able to induce pathology in a more homeostatic setting. To assess that we used a CD4$^+$ T cell-mediated IBD model based on infection with *Helicobacter hepaticus* (*H. hepaticus*). *H. hepaticus* is a gram-negative, micro-aerophilic, helical bacterium that can persistently colonize the murine caecum, colon and liver (Fox *et al.*, 1994). Oral infection in genetically susceptible mouse strains, such as *Il10*$^{-/-}$ mice, results in chronic inflammation of caecum and colon (typhlocolitis) (Burich *et al.*, 2001; Maloy *et al.*, 2003; Maggio-Price *et al.*, 2006). However, WT mice are resistant to *H. hepaticus* elicited intestinal inflammation despite persistent colonization, largely due to generation of *H. hepaticus*-specific Treg cells that are able to control effector responses towards the bacteria (Kullberg *et al.*, 2002). Thus, perturbation of IL-10 dependent regulatory pathways reveals pathogenic effector CD4$^+$ T cell responses towards *H. hepaticus* in WT mice, leading to severe intestinal inflammation (Burich *et al.*, 2001; Kullberg *et al.*, 2006). Pathology in this model is driven by IL-23 dependent CD4$^+$ T cells responses characterized by accumulation of Th1 and Th17 cells in the intestinal lamina propria (Kullberg *et al.*, 2006).

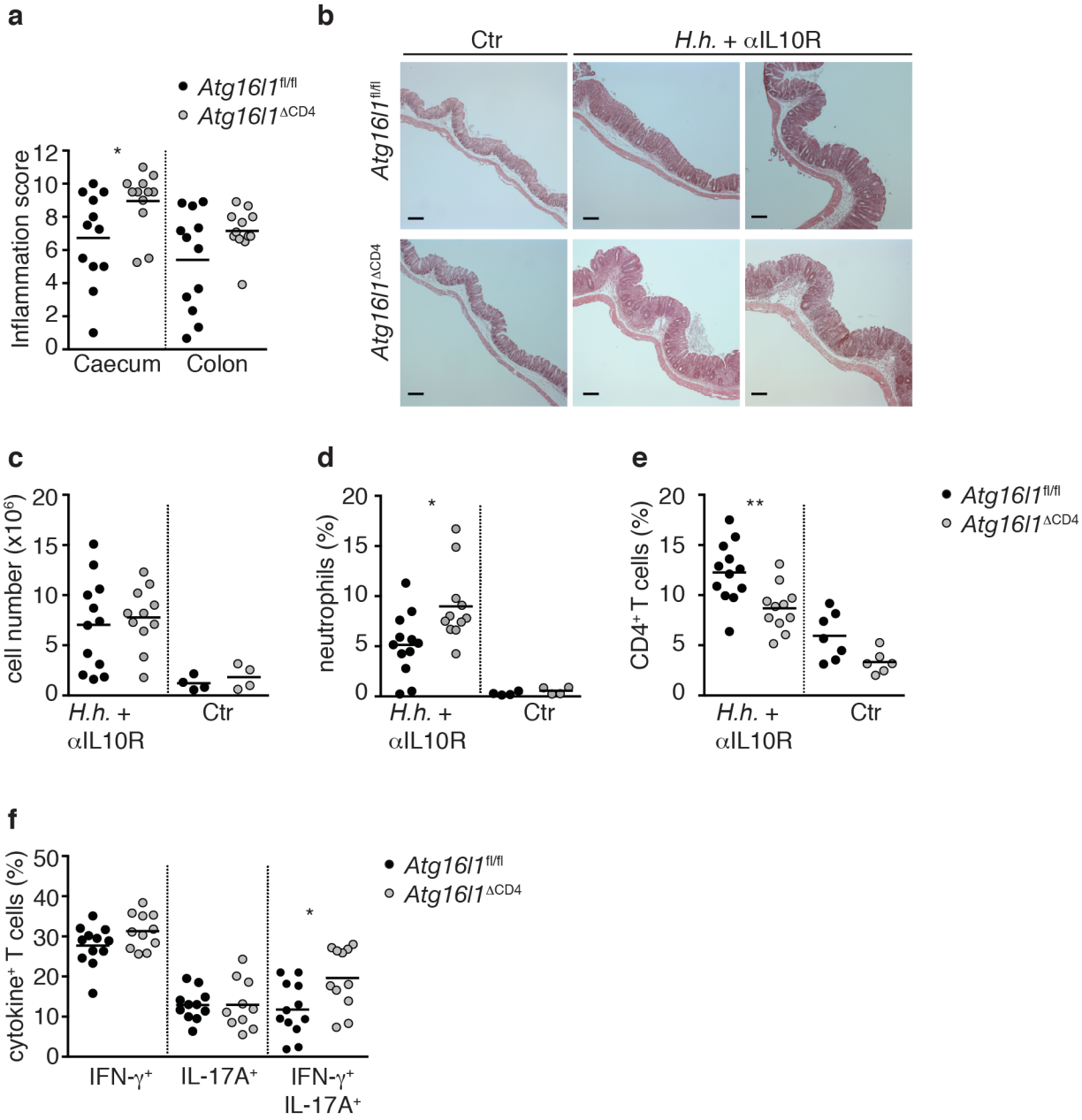
We therefore attempted to induce $CD4^+$ T cell-mediated typhlocolitis in *Atg16l1*$^{fl/fl}$ and *Atg16l1*$^{\Delta CD4}$ mice using oral infection with *H. hepaticus* and concomitant administration of blocking anti-IL-10R antibody (Burich *et al.*, 2001; Kullberg *et al.*, 2006). Somewhat to our surprise, in this model, *Atg16l1*$^{\Delta CD4}$ mice developed severe intestinal inflammation and even exhibited mildly increased typhlitis when compared to control *Atg16l1*$^{fl/fl}$ mice (**Figure 9a,b**). In addition, *Atg16l1*$^{\Delta CD4}$ mice showed marked leukocyte and $CD4^+$ T cell infiltration in the cLP and increased neutrophil accumulation relative to *Atg16l1*$^{fl/fl}$ control littermates (**Figure 9c-e**). We also saw significant increase in the frequencies of double positive IFN-γ^+ IL-$17A^+$ $CD4^+$ T cells in the colonic lamina propria of *Atg16l1*$^{\Delta CD4}$ mice (**Figure 9f**). Thus, *Atg16l1* deletion does not completely prevent $CD4^+$ T cells from expanding and driving chronic intestinal inflammation in the context of severe inflammatory stimuli.

Figure 9 *Atg16l1*$^{\Delta CD4}$ mice show increased susceptibility to T cell-mediated experimental colitis

Cohorts of *Atg16l1*$^{\Delta CD4}$ and *Atg16l1*$^{fl/fl}$ littermates were infected with *Helicobacter hepaticus* by oral gavage (three feeds of 1x10^8 CFU) and treated with anti-IL-10R mAb (1mg/mouse *i.p.* given weekly; *H.h* + αIL10R) or left untreated (Ctr). Two weeks post-infection mice were sacrificed for analyses.

(a) Caecum and colon histopathology scores in *H.h* + αIL10R treated *Atg16l1*$^{\Delta CD4}$ and *Atg16l1*$^{fl/fl}$ littermates. **(b)** Representative photomicrographs of H&E stained caecum of untreated Ctr (left panels) or *H.h* + αIL10R treated (middle and right panels) *Atg16l1*$^{\Delta CD4}$ and *Atg16l1*$^{fl/fl}$ littermates, scale bar 150μm. **(c)** Total lamina propria leukocyte numbers and frequencies of **(d)** neutrophils (Gr1^{hi} CD11b$^+$) and **(e)** CD4$^+$ T cells in cLP isolated from untreated Ctr or *H.h* + αIL10R treated *Atg16l1*$^{\Delta CD4}$ and *Atg16l1*$^{fl/fl}$ littermates. (**f**) Frequencies of IFN-γ$^+$, IL-17A$^+$ or double positive IFN-γ$^+$ IL-17A$^+$ CD4$^+$ T cells isolated from *H.h* + αIL10R treated *Atg16l1*$^{\Delta CD4}$ and *Atg16l1*$^{fl/fl}$ littermates (gated on CD4$^+$ TCRβ$^+$ Foxp3$^-$ cells).

Data are combined from two or three independent experiments with at least 2 mice per group. Each dot represents an individual mouse and horizontal bars denote means. Statistical significance was determined using the Mann Whitney test, * $p<0.05$; ** $p<0.01$.


a
Atg16l1fl/fl
Atg16l1ΔCD4
Inflammation score
Caecum
Colon
b
Ctr
H.h. + αIL10R
Atg16l1fl/fl
Atg16l1ΔCD4
c
cell number (x10⁶)
H.h. + αIL10R
Ctr
d
neutrophils (%)
e
CD4⁺ T cells (%)
f
cytokine⁺ T cells (%)
IFN-γ⁺
IL-17A⁺
IFN-γ⁺ IL-17A⁺

3.6 Discussion

As *Atg16l1*$^{\Delta CD4}$ mice had not been previously described, the initial characterisation of *Atg16l1*$^{\Delta CD4}$ mice, as well as the responses of *Atg16l1*-deficient CD4^{+} T cells *in vitro* and *in vivo,* were described in this chapter.

Autophagy is an essential homeostatic process for all eukaryotic cells, including T cells. Therefore, genetic deletion of essential autophagy genes early during T cell development, using CD4 or Lck promoter driven Cre-lox technology, can mediate major changes in autophagy-deficient T cells, including alterations in gene expression (Stephenson *et al.*, 2009). Arguably, this makes it difficult to study the function of essential autophagy genes in isolation on a particular process or step of T cell development. In order to avoid this complication, several groups attempted to use an inducible system to selectively knock out autophagy genes at a chosen time point (Jia and He, 2011; Schlie *et al.*, 2015), or used the granzyme B promoter to selectively drive Cre expression in mature CD8^{+} T cells (Xu *et al.*, 2014). However, it is important to remember that in terms of revealing how polymorphisms in autophagy genes are linked to disease susceptibility, the system where autophagy is perturbed from the beginning of T cell development may be more physiologically relevant. In this study, we were interested in investigating the overall impact of *Atg16l1* and autophagy on the homeostasis of intestinal CD4^{+} T cells *in vivo*; therefore we argue that the CD4-Cre system was an appropriate one to use for this purpose.

Changes in the central and peripheral T cell compartment in the absence of several essential autophagy genes have been previously reported (Bronietzki *et al.*, 2015). The requirement for autophagy in the thymic development seems to be dependent on the system that was used to generate T cell specific deletion of autophagy genes. While mice where Cre recombinase expression was driven by the Lck promoter (Cre

expression occurs at the double negative stage) showed a mild, but significant, reduction in thymocyte numbers (Pua *et al.*, 2009; Stephenson *et al.*, 2009), mice where the CD4 promoter was used to drive Cre expression (resulting in excision during the later double positive stage) did not show any significant changes in thymocyte development (Kovacs *et al.*, 2012; Willinger and Flavell, 2012; Puleston *et al.*, 2014). This is in line with our data, as we did not observed any perturbation in thymocyte frequencies or numbers. Thus, autophagy seems largely dispensable for late thymic $CD4^+$ and $CD8^+$ T cell development.

In contrast, almost all previous studies where the CD4 or LCK promoters were used to drive selective deletion of key autophagy genes in T cells, such as *Atg3, Atg5, Atg7, Beclin1* or *Vps34*, showed marked reductions in frequencies and numbers of peripheral $CD4^+$ and $CD8^+$ T cells (Pua *et al.*, 2009; Stephenson *et al.*, 2009; Jia and He, 2011; Kovacs *et al.*, 2012; Willinger and Flavell, 2012; Parekh *et al.*, 2013; Puleston *et al.*, 2014). Here, for the first time, the role of the essential autophagy gene *Atg16l1* in $CD4^+$ T cell homeostasis and function was examined. Consistent with these previous studies, T cell-specific deletion of *Atg16l1* led to significantly decreased peripheral $CD4^+$ and $CD8^+$ T cell numbers in the spleen and mLN. Moreover, analysis of the intestinal lamina propria T cell populations revealed marked reductions of $CD4^+$ and $CD8^+$ T cell numbers by *Atg16l1* deletion, indicating that autophagy pathway is essential for the maintenance of T cells in the small intestinal and colonic mucosa.

To date, only a few studies have addressed the role of autophagy in T cell-mediated effector responses *in vivo*. However, mice with T cell-specific *Beclin-1* deficiency were found to be resistant to the development of experimental autoimmune encephalomyelitis (EAE), a Th1 and Th17 driven disease (Kovacs *et al.*, 2012). In contrast, $CD8^+$ T cells lacking *Atg5* or *Atg7* were capable of mounting potent effector CTL responses during viral infection (Puleston *et al.*, 2014; Xu *et al.*, 2014), however

generation of the $CD8^+$ T cell memory compartment was greatly impaired by autophagy deficiency (Puleston *et al.*, 2014; Xu *et al.*, 2014; Schlie *et al.*, 2015). In our hands, *Atg16l1*-deficient $CD4^+$ T cells were capable of expanding and driving chronic intestinal inflammation initiated by administration of anti-IL-10R mAb and *H. hepaticus* infection. Indeed, in this experimental IBD model, caecal pathology was significantly increased in $Atg16l1^{\Delta CD4}$ mice compared to $Atg16l1^{fl/fl}$ control littermates. This suggests that *Atg16l1*-deficient $CD4^+$ T cells have pathogenic potential *in vivo* and might indicate that, similarly to $CD8^+$ CTL responses, autophagy could be dispensable for the early activation and expansion of $CD4^+$ effector T cells in the intestinal lamina propria. This result was initially surprising, since we were also able to confirm previous reports (Pua *et al.*, 2007; Stephenson *et al.*, 2009; Jia and He, 2011) of a strong proliferation defect in autophagy-deficient $CD4^+$ T cells when activated *in vitro*. We also found that *Atg16l1*-deficient naive $CD4^+$ T cells could not reconstitute lymphopaenic hosts or mediate colitis after adoptive transfer and showed that this defect was cell-intrinsic. The discrepancy between the effects that *Atg16l1*-deficiency has on $CD4^+$ T cell proliferation in these experiments could be due to the different conditions of TCR activation; the strength of TCR signalling and presence of co-stimulatory signals. While *in vitro* priming with high dose of anti-CD3 and anti-CD28 or *in vivo* expansion in lymphopaenic *Rag*1-/- hosts would result in a very strong T cell activation, the conditions would be different in the setting of chronic intestinal inflammation during the *H. hepaticus* infection in $Atg16l1^{\Delta CD4}$ mice, where inflammation is additionally accompanied by innate cell activation and increased B cell responses.

The question of when autophagy is activated in T cells remains controversial. While autophagy is known to be negatively regulated by mTORC1 signalling in many cell types, and therefore inversely correlated with cell proliferation (Levine *et al.*, 2011), early *in vitro* studies indicated that TCR triggering induces autophagy in

T lymphocytes and reported that chemical or genetic blockage of the autophagy pathway impaired T cell activation and proliferation (Li *et al.*, 2006; Pua *et al.*, 2007; Pua *et al.*, 2009; Hubbard *et al.*, 2010). In several studies, these defects in autophagy-deficient T cells were linked to impaired organelle homeostasis, particularly mitochondria homeostasis, and were associated with an increase in ROS production (Pua *et al.*, 2009; Jia and He, 2011; Willinger and Flavell, 2012). However, whether this indeed is the mechanistic explanation for the decreased fitness of autophagy deficient $CD4^+$ T cells *in vivo* remains unclear, as other studies did not observe any increase in mitochondrial mass or ROS production (McLeod *et al.*, 2011; Kovacs *et al.*, 2012), and mitochondria were shown to be excluded from the autophagosome degradation upon activation of WT $CD4^+$ T cells (Hubbard *et al.*, 2010). A further complication arises from the findings that although increased ROS can be detrimental for the T cells (Hildeman *et al.*, 1999), ROS production is normally increased after TCR triggering and is in fact required for T cell proliferation, particularly in $CD8^+$ T cells (Chaudhri *et al.*, 1988; Devadas *et al.*, 2002; Sena *et al.*, 2013; Okoye *et al.*, 2015). In our experiments we did not observe substantial increase in mitochondrial mass in unstimulated or *in vitro* activated *Atg16l1*-deficient $CD4^+$ T cells. Indeed, adequate mitochondria functionality in unstimulated *Atg16l1*-deficient $CD4^+$ T cells could also be indirectly inferred from our metabolic analysis, as naïve $CD4^+$ T cells isolated from *Atg16l1*$^{\Delta CD4}$ mice showed comparable or increased levels of OCR as WT control naïve $CD4^+$ T cells, indicating that mitochondrial respiration is present in freshly isolated *Atg16l1*-deficient $CD4^+$ T cells. However, during prolonged activation *Atg16l1*-deficient $CD4^+$ T cells could not sustain elevated OXPHOS levels, which could be a result of defective mitochondria function. Therefore a more comprehensive analysis of the mitochondrial functionality and ROS production in activated *Atg16l1*-deficient $CD4^+$ T will need to be perform in order to draw definitive conclusions. Interestingly, several studies reported that the

difference in mitochondria mass between autophagy-deficient T cells and control T cells was more pronounced in $CD8^+$ T cells than in $CD4^+$ T cells (Pua *et al.*, 2009; Stephenson *et al.*, 2009; Willinger and Flavell, 2012). Therefore, it could be speculated that defective mitophagy could be more detrimental to $CD8^+$ T cell than to $CD4^+$ T cell homeostasis.

More recently, detailed analysis of autophagy induction in $CD8^+$ T cells *in vivo* was performed by Xu *et al.* (Xu *et al.*, 2014). By analysing virus-specific $CD8^+$ T cells during infection with lymphocytic choriomeningitis virus (LCMV) this group demonstrated that autophagy was initially downregulated when $CD8^+$ T cells were actively proliferating during the initial expansion phase. However, autophagy was upregulated upon entry into the contraction phase, when proliferation of $CD8^+$ T cells was decreasing, and autophagy-deficient $CD8^+$ T cells did not efficiently differentiate into memory $CD8^+$ T cells (Xu *et al.*, 2014). Whether the same dynamic regulation of autophagy induction is true during $CD4^+$ T cell effector responses remains to be determined, however our data from the *H. hepaticus* IBD model are in line with the hypothesis that early differentiation into effector T cells *in vivo* might not require autophagy. Additionally, our results from *in vitro* analysis indicated that *Atg16l1*-deficient $CD4^+$ T cells were capable of mounting the initial response to TCR triggering *in vitro*, as we found that upregulation of activation markers, secretion of IL-2, increased glucose uptake, OXPHOS and glycolysis levels were all comparable (or increased) in *Atg16l1*-deficient $CD4^+$ T cells in comparison to WT control $CD4^+$ T cells early upon activation. These results argue that autophagy might be dispensable in initiating these early responses to TCR triggering in $CD4^+$ T cells. However, while metabolic changes associated with T cell activation were initially present in *Atg16l1*-deficient $CD4^+$ T cells, prolonged *in vitro* activation led to dramatic change in the metabolic capabilities of *Atg16l1*-deficient $CD4^+$ T cells. Indeed, in contrast to control $CD4^+$ T cells, *Atg16l1*-deficient $CD4^+$ T cells were unable to sustain elevated

OXPHOS, whereas their levels of glycolysis remained largely comparable with control cells. It could be speculated that an impaired ability to use OXPHOS for energy production would impact on ATP levels and could lead to decreased survival or expansion of *Atg16l1*-deficient $CD4^+$ T cells. In line with this hypothesis, we observed a decrease in $CD44^+$ $CD62L^-$ activated/memory $CD4^+$ T cells in the cLP in $Atg16l1^{\Delta CD4}$ mice, suggesting that autophagy might be largely dispensable for naïve T cell survival, but becomes important after activation *in vivo*, particularly in sites of continuous antigen exposure like the intestinal lamina propria.

Altogether, data presented in this chapter summarise the initial characterisation of $Atg16l1^{\Delta CD4}$ mice and *Atg16l1*-deficient $CD4^+$ T cells. We established a crucial role of *Atg16l1* in peripheral $CD4^+$ T cell expansion and maintenance, especially in intestinal lamina propria $CD4^+$ T cells. However, the data from the T cell driven chronic colitis and typhlitis model suggest that *Atg16l1*-deficient $CD4^+$ T cells could harbour pathogenic potential, as $Atg16l1^{\Delta CD4}$ mice developed a severe intestinal inflammation

Chapter 4. Selective deletion of *Atg16l1* in T cells results in spontaneous intestinal inflammation, characterised by dysregulated Th2 and Treg responses

4.1 Introduction

Having performed the initial characterisation of the *Atg16l1*$^{\Delta CD4}$ mice and *Atg16l1*-deficient CD4^{+} T cells we sought to investigate in more detail how autophagy governs intestinal CD4^{+} T cell populations. This examination revealed selective enhancement of Th2 responses and production of antibodies towards dietary and commensal antigens in *Atg16l1*$^{\Delta CD4}$ mice, which was accompanied by a progressive loss of peripheral Treg cells, especially in the gut.

The general importance of Treg cell responses in maintaining intestinal homeostasis has been discussed in Chapter 1. The induction of Th2 responses at mucosal sites is less well understood, but recent findings, mainly from airway inflammatory models, have begun to elucidate the mechanisms responsible. Type 2 responses can be initiated *in vivo* by epithelial cell–derived alarmins, including TSLP, IL-25, GM-CSF and IL-33, which are released following tissue injury, allergen exposure, or after pathogen recognition through various PRR. These epithelial derived factors can directly activate ILC2, which then proliferate and secrete IL-5 and IL-13, contributing to anti-helminth defence or allergic inflammation (Mjosberg *et al.*, 2011; Kim *et al.*, 2013a). At the same time, epithelial derived factors stimulate DC to upregulate OX40L, downregulate IL-12 production in an IRF4-dependent manner, and upregulate the Th2 chemo-attractants CCL17 and CCL22, all of which will promote adaptive Th2 responses (Gao *et al.*, 2013; Akbari *et al.*, 2014; Hammad and Lambrecht, 2015). In

addition, epithelial alarmins can also activate basophils, which in turn secrete IL-4 and, together with DC, can act as antigen-presenting cells to drive Th2 cell differentiation (Paul and Zhu, 2010). Within the tissue, effector Th2 cells typically express both IL-13 and IL-4 (Liang *et al.*, 2012) and protection against helminths and ectoparasites has long been considered as the oldest evolutionary task of type 2 immunity. However, thanks to recent advances in this field, more attention has focussed on the homeostatic functions of type 2 responses, including metabolic regulation and tissue remodelling (Cheng and Locksley, 2015). An interesting example was provided recently where it was demonstrated that enhanced ILC2 responses boosted epithelial barrier immunity during nutrient depravation (Spencer *et al.*, 2014). Allergic reactions have traditionally been viewed as a misdirected, detrimental type 2 responses that would normally target macroparasites, but in recent years evidence has been provided that allergic responses are a programmed reaction of the immune system in defence against environmental toxins, challenging the previous, long-standing dogma (Palm *et al.*, 2012). Nevertheless, improperly targeted allergic reactions can have a profoundly detrimental effect on the host. With regards to the intestinal mucosa, dysregulated type 2 responses can drive food hypersensitivities and allergies. A wide spectrum of disorders characterised by sensitization to food antigens and Th2 skewing of the immune system falls under the umbrella of food allergies. Adverse reactions can be IgE-mediated (immediate symptoms), cell-mediated (delayed symptoms) or a combination of both (Wang and Sampson, 2011). Under homeostatic circumstances, humoral and cellular responses towards food antigens are suppressed by the induction of oral tolerance; a process predominantly mediated by intestinal mononuclear phagocytes and involving induction of Treg cells (Chehade and Mayer, 2005). As such, food allergies are thought to result from a loss or failure to induce oral tolerance (Wang and Sampson, 2011), although the exact mechanism of how this occurs remains elusive. There is

genetic and immunological evidence for an important role for Treg cells and TGF-β_1 in preventing food hypersensitivities (Zhang *et al.*, 2001a; Akdis *et al.*, 2004; Karlsson *et al.*, 2004; Torgerson *et al.*, 2007; Frischmeyer-Guerrerio *et al.*, 2013) and it has been postulated that defective control of Th2 responses by Treg cells is one of the underling causes of allergic diseases. Furthermore, it was recently shown that allergen-specific Treg cells may undergo pathogenic reprogramming towards a Th2-like phenotype, characterised by increased expression of Gata3 and IL-4 (Noval Rivas *et al.*, 2015). In addition, recent studies have begin to identify characteristics of Treg cells that preferentially mediate inhibition of Th2 responses and this appears to involve expression of protein kinase CK2 (Ulges *et al.*, 2015) and lack of expression of the co-inhibitory molecule TIGIT (Joller *et al.*, 2014). However, if these markers truly identify a separate population of local Treg cells that selectively control Th2 cells in the intestine remains to be established, as the studies discussed above focused mainly on Th2-driven airway inflammation.

Building on our initial characterisation of the disease phenotype of *Atg16l1*$^{\Delta CD4}$ mice, the following two result chapters focus on characterising changes in the intestinal CD4$^+$ T cell populations caused by *Atg16l1*-deficiency and on investigation of the mechanism behind the differential effect of autophagy deficiency on mucosal Th2 and Treg cells.

4.2 *Atg16l1*$^{\Delta CD4}$ mice develop spontaneous chronic intestinal inflammation

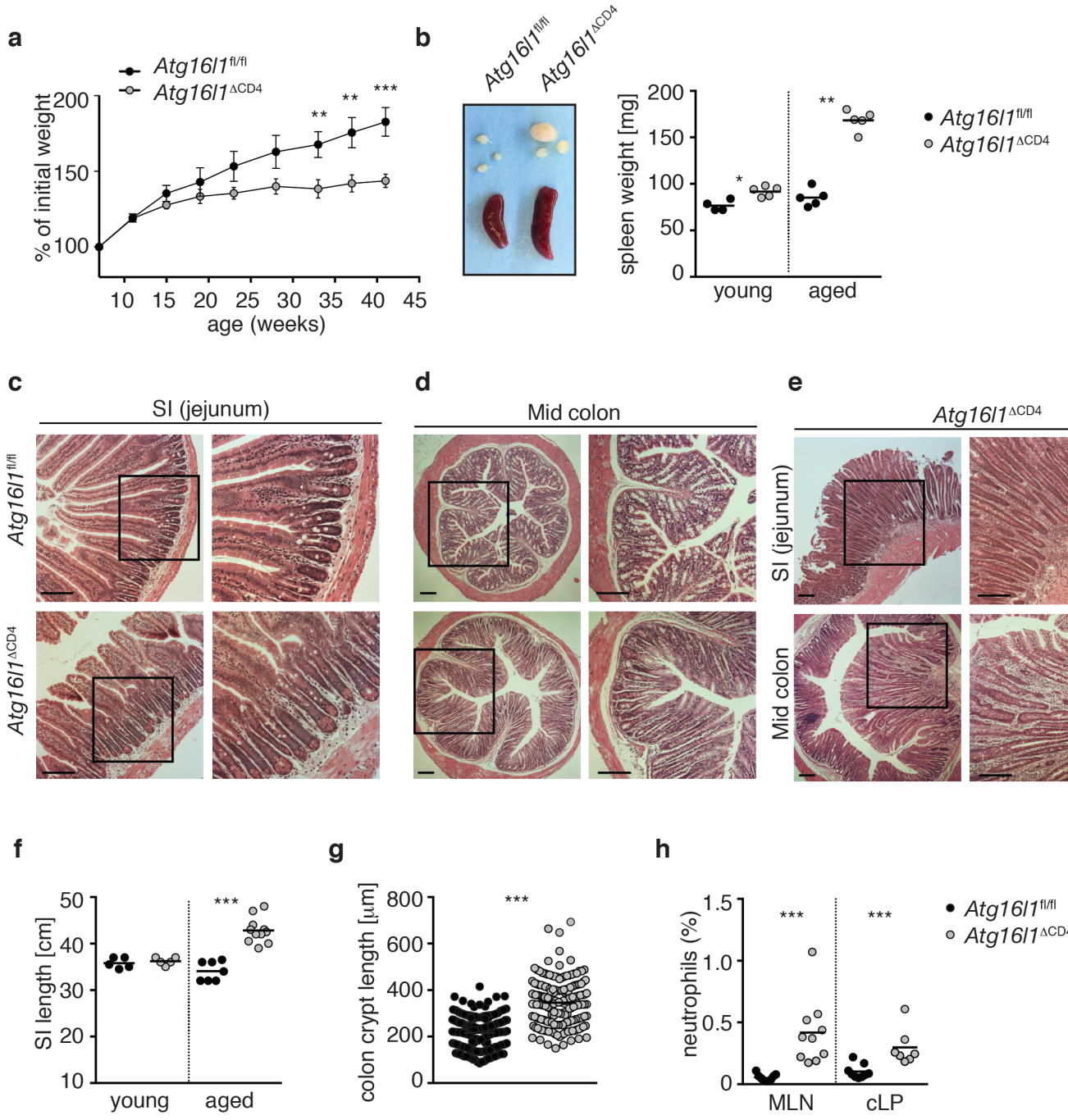
We investigated how deletion of *Atg16l1* in T cells impacted on intestinal physiology. *Atg16l1*$^{\Delta CD4}$ mice appeared normal at birth and initially gained weight in a manner comparable to littermate control *Atg16l1*$^{fl/fl}$ mice (**Figure 10a**). However, from around 5 months of age onwards, *Atg16l1*$^{\Delta CD4}$ mice stopped gaining weight (**Figure**

10a) and developed marked splenomegaly and lymphadenopathy (**Figure 10b**). Moreover, *Atg16l1*$^{\Delta CD4}$ mice developed chronic intestinal pathology that progressed with age (**Figure 10c-g)**. *Atg16l1*$^{\Delta CD4}$ mice exhibited significant inflammation of both the small intestine (SI) and colon, characterized by; increased SI length; marked lengthening of crypts, shortening of villi and leukocyte infiltration in the SI; and epithelial hyperplasia in the colon (**Figure 10c,d,f,g).** In addition, about 20% of aged *Atg16l1*$^{\Delta CD4}$ mice showed signs of abnormal growth of the intestinal epithelium (**Figure 10e**). In line with these histopathological changes *Atg16l1*$^{\Delta CD4}$ mice had increased frequencies of neutrophils in the colonic lamina propria (cLP) and mesenteric lymph nodes (mLN), indicative of ongoing inflammation (**Figure 10h**). In contrast, age-matched *Atg16l1*$^{fl/fl}$ littermate controls continued to gain weight as they aged and did not exhibit any intestinal abnormalities or signs of immune activation (**Figure 10a-h**). Together, these results indicate that T cell-specific *Atg16l1* deletion resulted in late-onset, spontaneous chronic inflammation of the gastrointestinal tract that was accompanied by systemic immune activation.

Figure 10 Aged *Atg16l1*$^{\Delta CD4}$ mice develop spontaneous intestinal inflammation

(a) Body weight curves of *Atg16l1*$^{\Delta CD4}$ and *Atg16l1*$^{fl/fl}$ littermates. **(b)** Spleen weights and representative images of spleens and mesenteric lymph nodes (mLN) from young (8-12 weeks) and aged (>5 months) *Atg16l1*$^{\Delta CD4}$ and *Atg16l1*$^{fl/fl}$ littermates. **(c,d)** Representative photomicrographs of haematoxylin and eosin (H&E) stained sections of **(c)** jejunum and **(d)** mid-colon from aged *Atg16l1*$^{\Delta CD4}$ and *Atg16l1*$^{fl/fl}$ littermates, scale bar 150μm. **(e)** Representative H&E stained sections of jejunum (top) and mid-colon (bottom) from aged *Atg16l1*$^{\Delta CD4}$ mice, showing abnormal crypt morphology, scale bar 150μm. **(f,g)** Quantification of **(f)** SI lengths in young and aged *Atg16l1*$^{\Delta CD4}$ and *Atg16l1*$^{fl/fl}$ littermates and **(g)** mid-colon crypt lengths in aged *Atg16l1*$^{\Delta CD4}$ and *Atg16l1*$^{fl/fl}$ littermates. **(h)** Frequencies of neutrophils (Gr1^{hi} CD11b^{+} live cells) in mLN and cLP from aged *Atg16l1*$^{\Delta CD4}$ and *Atg16l1*$^{fl/fl}$ littermates.

Data are representative of at least three independent experiments (a-e), or combined from two (f,h) or three (g) independent experiments, with at least 3 mice per group. Data shown are means ± s.e.m (a). Each dot represents an individual mouse and horizontal bars denote means (b,f,h). In (g) each dot represents an individual crypt measurement and horizontal bars denote means. Statistical significance was determined using two-way analysis of variance (ANOVA) with Bonferroni's correction for multiple comparisons (a) or the Mann Whitney test (b,f-h), ** $p<0.01$; *** $p<0.001$.


a
Atg16l1fl/fl
Atg16l1ΔCD4
% of initial weight
200
150
100
10 15 20 25 30 35 40 45
age (weeks)
**
**
***
b
Atg16l1fl/fl
Atg16l1ΔCD4
spleen weight [mg]
200
150
100
50
0
young
aged
*
**
Atg16l1fl/fl
Atg16l1ΔCD4
c
SI (jejunum)
Atg16l1fl/fl
Atg16l1ΔCD4
d
Mid colon
e
Atg16l1ΔCD4
SI (jejunum)
Mid colon
f
SI length [cm]
50
40
30
20
10
young
aged
***
g
colon crypt length [µm]
800
600
400
200
0
***
h
neutrophils (%)
1.5
1.0
0.5
0
MLN
cLP
***
***
Atg16l1fl/fl
Atg16l1ΔCD4

4.3 *Atg16l1*$^{\Delta CD4}$ mice display elevated Th2 inflammatory responses at the intestinal mucosa

To characterize the effects of *Atg16l1* on intestinal and systemic T cell homeostasis independently from any confounding effects of ongoing tissue inflammation, we analysed young (8-12 weeks old) *Atg16l1*$^{\Delta CD4}$ mice before the onset of inflammatory pathology or systemic symptoms. We observed comparable frequencies of IFN-γ^+ Th1 and IL-17A$^+$ Th17 populations in the cLP of *Atg16l1*$^{\Delta CD4}$ mice and *Atg16l1*$^{fl/fl}$ littermates (**Figure 11a**). However, due to the overall decrease in colonic CD4$^+$ T cell number in *Atg16l1*$^{\Delta CD4}$ mice (**Figure 4a**), total intestinal Th1 and Th17 numbers were significantly decreased in comparison to *Atg16l1*$^{fl/fl}$ littermates (**Figure 11b**). In contrast, both frequencies and total numbers of IL-13$^+$ Th2 cells were significantly increased in cLP of *Atg16l1*$^{\Delta CD4}$ mice (**Figure 11a-c**). These IL-13$^+$ cells were *bona fide* Th2 cells, as they co-expressed the lineage-specifying transcription factor Gata3 (Zheng and Flavell, 1997) (**Figure 11c**). The increased Th2 cell accumulation observed in *Atg16l1*$^{\Delta CD4}$ mice occurred mainly within the intestinal LP, as Gata3$^+$ Th2 cell frequencies were only slightly increased in the mLN and were unchanged in the spleen (**Figure 11d**). However, the functional effects of increased intestinal Th2 responses in young *Atg16l1*$^{\Delta CD4}$ mice extended beyond the intestinal LP, as evidenced by increased eosinophil frequencies in the spleen and elevated serum levels of mast cell protease 1 (MCPT-1), a marker of intestinal mast cell activation (Wastling *et al.*, 1998) (**Figure 12a,b,c**). Overall, these results demonstrate that deficiency of *Atg16l1* in T cells led to an accumulation of Th2 cells in the intestinal LP prior to the onset of overt pathology.

Figure 11 Young *Atg16l1*$^{\Delta CD4}$ mice exhibit selective expansion of Th2 cells in the intestine

Cohorts of young (8-12 weeks old) *Atg16l1*$^{\Delta CD4}$ and *Atg16l1*$^{fl/fl}$ littermates were sacrificed and intestinal and systemic CD4$^+$ T cell populations analysed by FACS. **(a)** Frequencies and **(b)** total numbers of Th1 (IFN-γ^+), Th17 (IL-17A$^+$) and Th2 (IL-13$^+$) cells in cLP (gated on CD4$^+$ T cells). **(c)** Representative FACS plots of Gata3 and IL-13 (top) or IFN-γ and IL-17A (bottom) expression by cLP CD4$^+$ T cells (gated on CD4$^+$ TCRβ^+ Foxp3$^-$ live cells). **(d)** Frequencies of Gata3$^+$ CD4$^+$ Foxp3$^-$ T cells (gated on CD4$^+$ TCRβ^+ Foxp3$^-$ cells).

Data are combined from three or more independent experiments with at least 2 mice per group (a,b,d), or are representative of four independent experiments with at least 4 mice per group (c). Each dot represents an individual mouse and horizontal bars denote means. Numbers indicate percentage of cells in gates or quadrants. Statistical significance was determined using the Mann Whitney test, * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

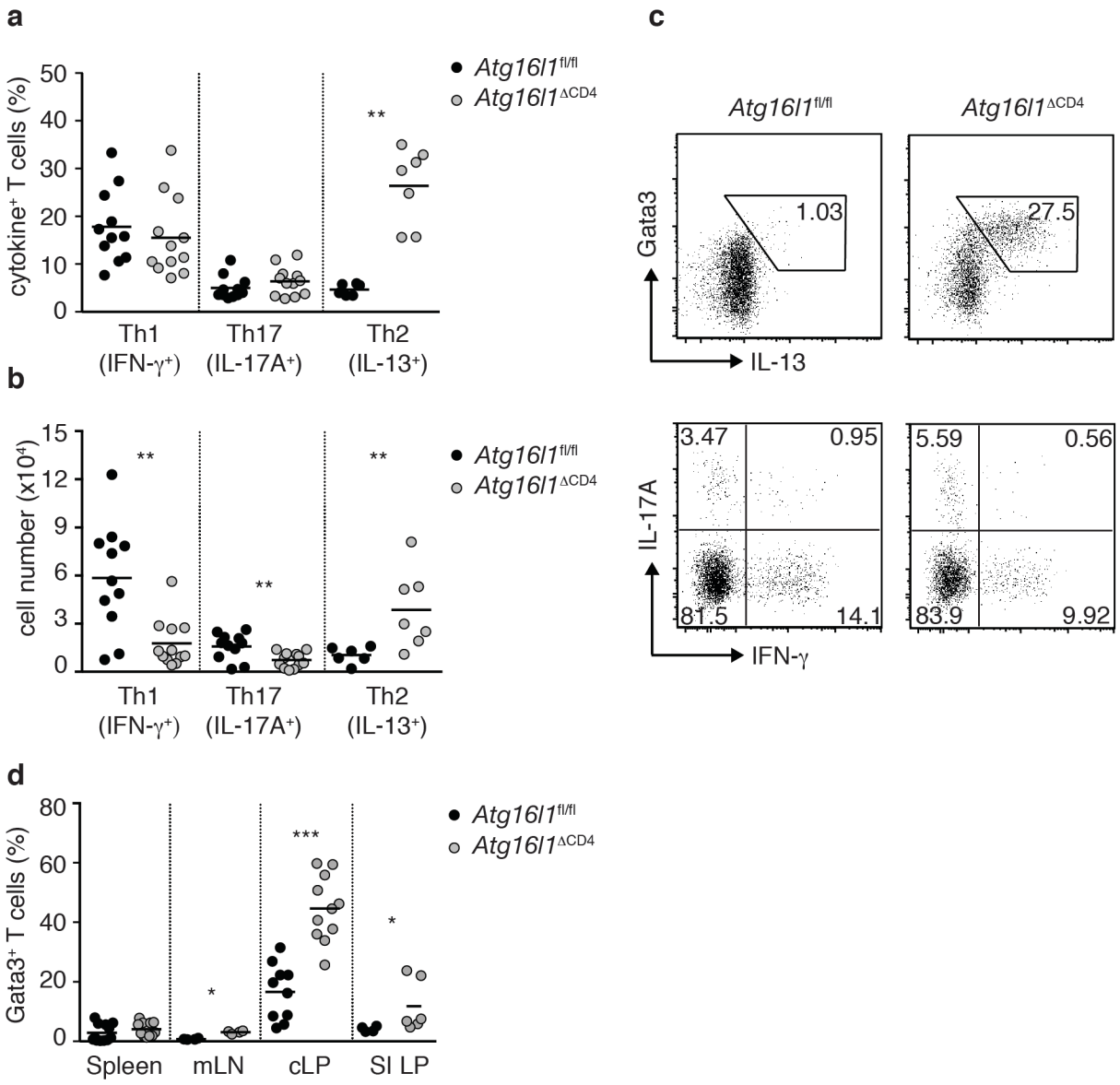
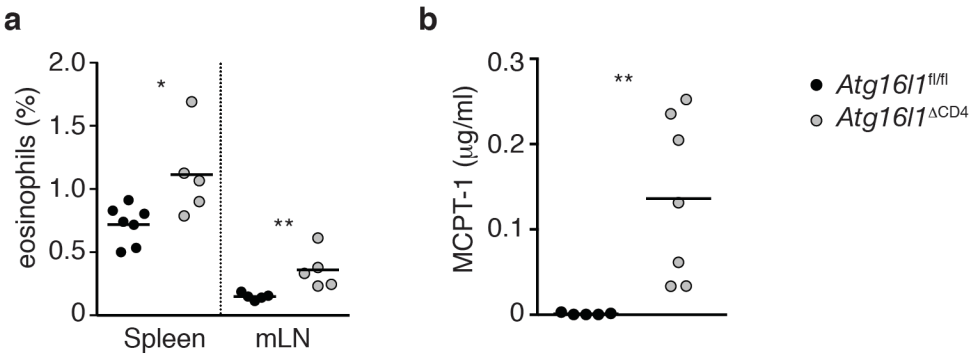

a
cytokine+ T cells (%)
50
40
30
20
10
0
**
Th1
(IFN-γ+)
Th17
(IL-17A+)
Th2
(IL-13+)
Atg16l1fl/fl
Atg16l1ΔCD4
b
cell number (x10^4)
15
12
9
6
3
0
**
**
**
Th1
(IFN-γ+)
Th17
(IL-17A+)
Th2
(IL-13+)
Atg16l1fl/fl
Atg16l1ΔCD4
c
Atg16l1fl/fl
Atg16l1ΔCD4
Gata3
1.03
27.5
IL-13
IL-17A
3.47
0.95
81.5
14.1
5.59
0.56
83.9
9.92
IFN-γ
d
Gata3+ T cells (%)
80
60
40
20
0
*
***
*
Spleen
mLN
cLP
SI LP
Atg16l1fl/fl
Atg16l1ΔCD4

Figure 12 Systemic effects of elevated Th2 responses in young *Atg16l1*$^{\Delta CD4}$ mice

Cohorts of young (8-12 weeks old) *Atg16l1*$^{\Delta CD4}$ and *Atg16l1*$^{fl/fl}$ littermates were sacrificed for analysis. **(a)** Frequencies of eosinophils (Ly6C^{low} Ly6G^{low} CD11b^{+} F4/80^{-}) in spleen and mLN were determined by FACS. **(b)** Serum MCPT-1 levels were measured by ELISA.

Data are representative of (a), or combined from (b), two independent experiments with at least 2 mice per group. Each dot represents an individual mouse and horizontal bars denote means. Statistical significance was determined using the Mann Whitney test, * $p<0.05$; ** $p<0.01$.


a
b
eosinophils (%)
2.0
1.5
1.0
0.5
0
*
**
Spleen
mLN
MCPT-1 (µg/ml)
0.3
0.2
0.1
0
**
Atg16l1fl/fl
Atg16l1ΔCD4

4.4 Elevated humoral responses to environmental antigens in *Atg16l1*$^{\Delta CD4}$ mice

To further investigate the consequences of exacerbated intestinal Th2 responses in *Atg16l1*$^{\Delta CD4}$ mice, we analysed Th2 associated antibodies. Whilst at the limit of detection in *Atg16l1*$^{fl/fl}$ littermates, serum IgE concentrations were significantly elevated in young *Atg16l1*$^{\Delta CD4}$ mice and increased further as the mice aged (**Figure 13a**). Furthermore, levels of serum IgG_1 and IgA in aged *Atg16l1*$^{\Delta CD4}$ mice were also significantly elevated relative to *Atg16l1*$^{fl/fl}$ littermates, whereas levels of antibody isotypes that are not directly associated with Th2 help, including IgG_{2b}, IgG_{2c} and IgM, were comparable (**Figure 13b).** As observed with IgE, elevated levels of serum IgG_1 and IgA were already detectable in young *Atg16l1*$^{\Delta CD4}$ mice (**Figure 13c**). Thus, *Atg16l1*$^{\Delta CD4}$ mice displayed selective increases in Th2-associated antibody isotypes that preceded the onset of intestinal pathology and progressed with age.

Consistent with their dysregulated humoral responses aged *Atg16l1*$^{\Delta CD4}$ mice had greatly enlarged Peyer's patches in the small intestine (**Figure 14a**). Young *Atg16l1*$^{\Delta CD4}$ mice also showed changes in systemic B cell compartments in the spleen and mLN, as they had higher frequencies of GC B cells, memory B cells and plasma cells when compared to *Atg16l1*$^{fl/fl}$ littermates (**Figure 14b**).

Figure 13 *Atg16l1*$^{\Delta CD4}$ mice have elevated serum levels of IgE, IgG1 and IgA

Cohorts of age-matched *Atg16l1*$^{\Delta CD4}$ and *Atg16l1*$^{fl/fl}$ littermates were bled and serum antibody isotypes measured by ELISA. **(a)** Serum IgE concentrations in young and aged mice. **(b)** Serum antibody IgA, IgM, IgG_1, IgG_{2b} and IgG_{2c} isotype levels in aged littermates (>5 months). **(c)** Serum antibody IgA and IgG_1 isotype levels in young littermates (7-12weeks).

Data are representative of at least two independent experiments with at least 3 mice per group (a,b), or are from one experiment with at least 4 mice per group (c). Each dot represents an individual mouse and horizontal bars denote means (a). Serum isotype levels are shown as means ± s.e.m (b,c). Statistical significance was determined using the Mann Whitney test (a) or two-way analysis of variance (ANOVA) with Bonferroni's correction for multiple comparisons (b,c), * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

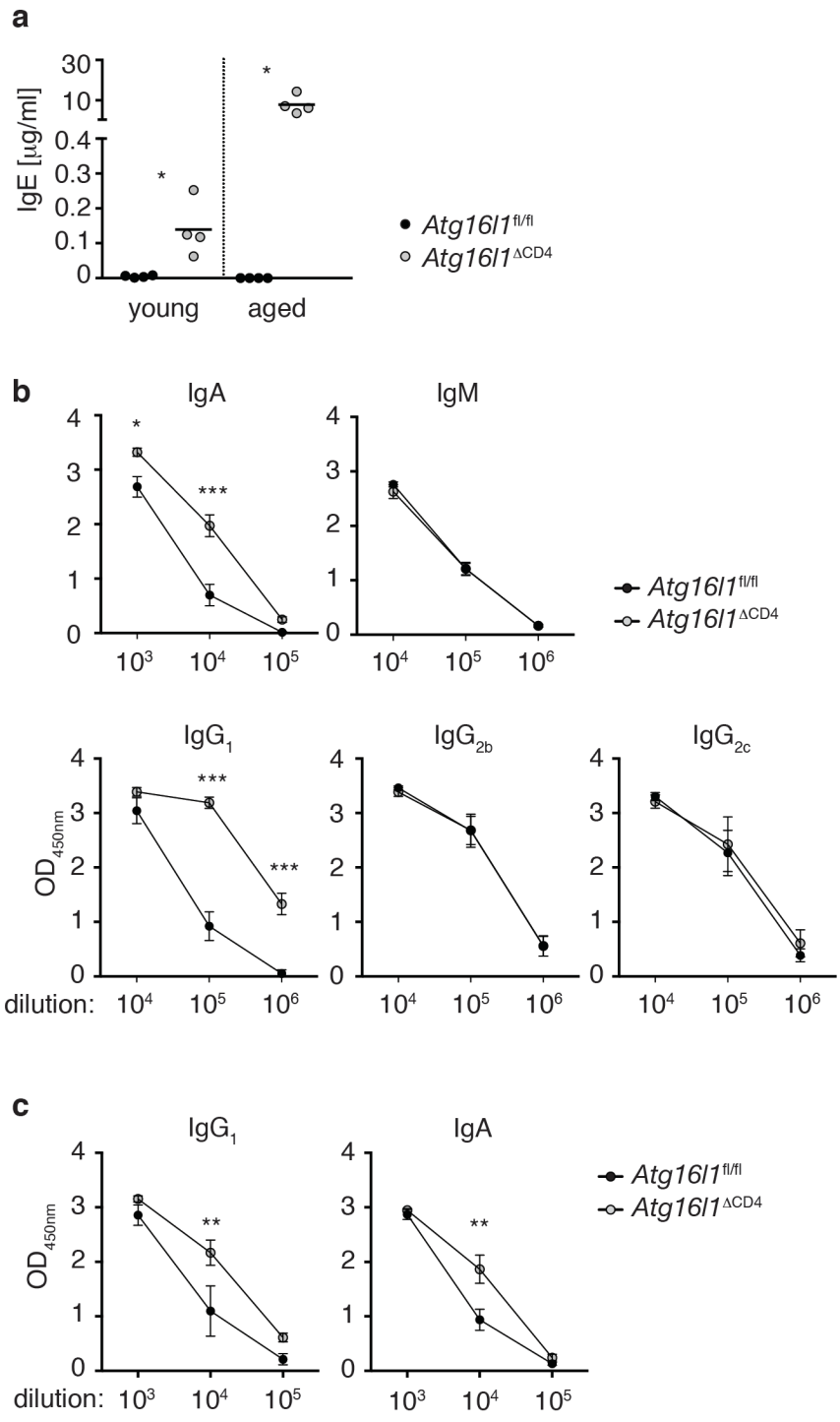
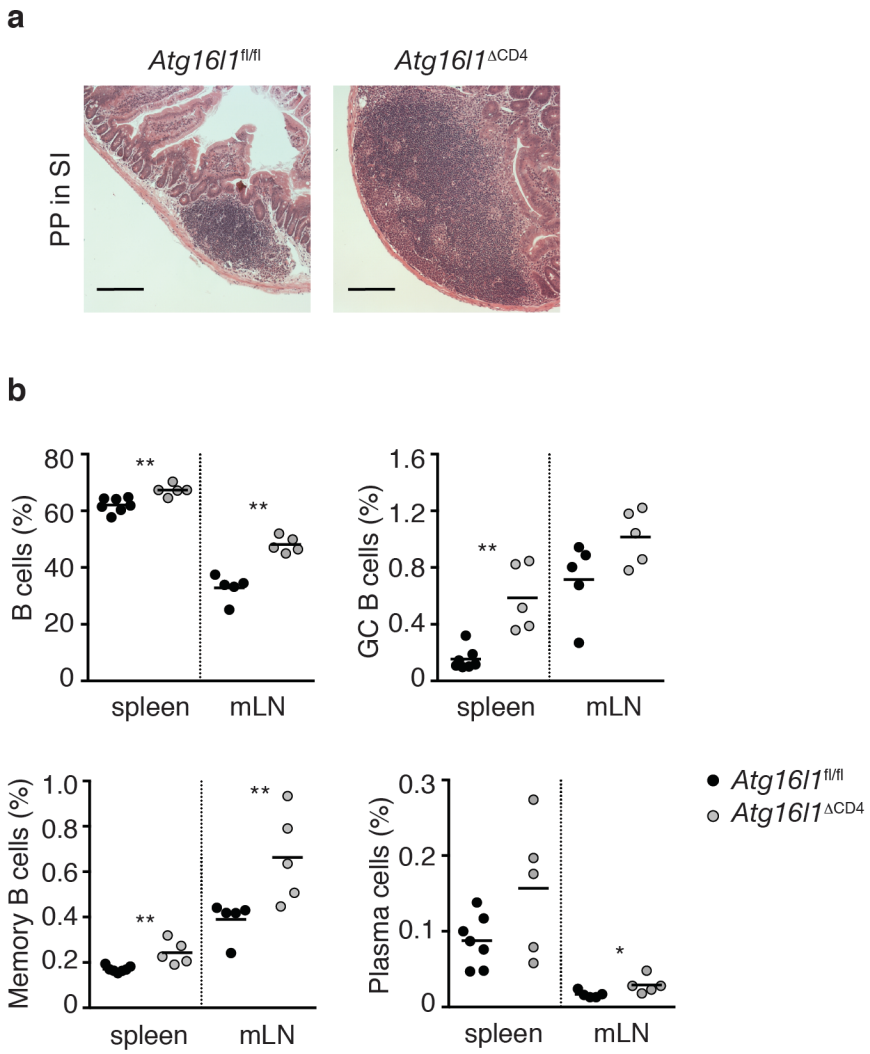

a
IgE [µg/ml]
30
10
0.4
0.3
0.2
0.1
0
young
aged
Atg16l1fl/fl
Atg16l1ΔCD4
b
IgA
IgM
IgG1
IgG2b
IgG2c
OD450nm
dilution:
10³
10⁴
10⁵
10⁶
c
IgG1
IgA

Figure 14 *Atg16l1*$^{\Delta CD4}$ mice have an altered B cell compartment

Cohorts of age-matched *Atg16l1*$^{\Delta CD4}$ and *Atg16l1*$^{fl/fl}$ littermates were sacrificed and lymphoid tissues analysed using histological staining and FACS. **(a)** Representative photomicrographs of H&E stained sections of Peyer's patch (PP) in the SI (jejunum) of aged littermates (>5 months), scale bar 150μm. **(b)** Frequencies of B cells (B220$^+$), germinal center B cells (GC: B220$^+$ GL7$^+$ CD95$^+$), memory B cells (B220$^+$ GL7$^-$ IgM$^-$ IgG$^+$) and plasma cells (CD138$^+$) in the spleen and mLN of young (8-12 weeks) littermates (gated on live CD45+ cells).

Data are representative of two independent experiments with 5 mice per group (a), or from one experiment with at least 5 mice per group (b). Each dot represents an individual mouse and horizontal bars denote means (b). Statistical significance was determined using the Mann Whitney test (a) * $p<0.05$; ** $p<0.01$.

Analysis in (b) was done in collaboration with Dr Lucie Abeler-Dörner and Dr Adam Laing (King's College London).


a
Atg16l1fl/fl
Atg16l1ΔCD4
PP in SI
b
B cells (%)
GC B cells (%)
Memory B cells (%)
Plasma cells (%)
spleen
mLN
Atg16l1fl/fl
Atg16l1ΔCD4

Aberrant intestinal Th2 responses are often associated with food hypersensitivities (Berin and Sampson, 2013), we therefore examined whether elevated humoral responses in *Atg16l1*$^{\Delta CD4}$ mice were directed against dietary antigens. Soy is the main protein source in the chow of laboratory mice and we detected high levels of anti-soy IgG_1 and IgA in sera from aged *Atg16l1*$^{\Delta CD4}$ mice, whereas these responses were undetectable in control *Atg16l1*$^{fl/fl}$ littermates (**Figure 15a**). By contrast, we only detected marginal levels of soy-specific IgG_{2b} or IgG_{2c} in the sera from aged *Atg16l1*$^{\Delta CD4}$ mice (**Figure 15a**). Importantly, significantly elevated levels of anti-soy antibodies were already detectable in the sera of young *Atg16l1*$^{\Delta CD4}$ mice, before the onset of intestinal inflammation (**Figure 15b**). However, despite the very high levels of total serum IgE in *Atg16l1*$^{\Delta CD4}$ mice, we did not detect elevated levels of anti-soy IgE (data not shown). The absence of soy-specific IgE could be due to the inhibiting effects of persistent exposure to high dose antigens on IgE responses (Sudowe *et al.*, 1997; Riedl *et al.*, 2005). Therefore, to test whether an IgE response could be mounted during a transient exposure to dietary antigen, we fed young *Atg16l1*$^{\Delta CD4}$ and *Atg16l1*$^{fl/fl}$ littermates with ovalbumin (OVA), either alone or in combination with the mucosal adjuvant cholera toxin (CT) (**Figure 16a**). As expected, anti-OVA IgE responses were undetectable in control *Atg16l1*$^{fl/fl}$ littermates fed OVA alone and were only slightly increased by co-administration of CT (**Figure 16b**). In contrast, *Atg16l1*$^{\Delta CD4}$ mice exhibited significantly elevated levels of anti-OVA IgE after being fed OVA alone and developed >10-fold higher levels of OVA-specific IgE after feeding of OVA with CT (**Figure 16b**). Together, these results indicate that *Atg16l1*$^{\Delta CD4}$ mice mounted aberrant Th2-associated antibody responses towards otherwise innocuous dietary protein antigens.

We next investigated whether the spontaneous Th2-associated antibody responses in *Atg16l1*$^{\Delta CD4}$ mice also extended to other antigens present in the gut lumen, such as commensal antigens. Thus, we measured antibodies directed against the flagellin

antigen CBir1, produced by commensal bacteria belonging to *Clostridia* cluster XIVa (Lodes *et al.*, 2004), which is of particular interest as antibodies against flagellin are readily detected in sera of IBD patients (Lodes *et al.*, 2004). As an additional control in these experiments, we also obtained sera from GF mice, which lack all commensal microbiota. As expected, we did not detect any anti-CBir1 antibody responses in the control sera of GF mice (**Figure 17a**). However, we detected significantly higher levels of CBir1-specific IgG_1 and IgA in sera of aged *Atg16l1*$^{\Delta CD4}$ mice compared to control *Atg16l1*$^{fl/fl}$ littermates, whereas anti-CBir1 IgG_{2b} and IgG_{2c} levels were comparably low in both groups (**Figure 17a**). Again, we found that CBir1-specific IgG_1 and IgA were already detectable in young *Atg16l1*$^{\Delta CD4}$ mice (**Figure 17b**). This indicates that *Atg16l1*$^{\Delta CD4}$ mice also mounted exacerbated Th2 associated humoral responses to commensal bacterial antigens. To examine whether exacerbated antibody responses were observed towards all types of intestinal antigens, we infected *Atg16l1*$^{\Delta CD4}$ and control mice with intestinal pathogens that are known to elicit strong antibody responses: gram-negative bacterium *H. hepaticus* and nematode parasite *Trichuris muris*. Increased Th2 cell-associated antibody responses were not observed in *Atg16l1*$^{\Delta CD4}$ mice following oral infection either with the *H. hepaticus* or with the *T. muris* (**Figure 17c,d**). Altogether, these results suggest that the abnormal IgG_1 and IgA responses in *Atg16l1*$^{\Delta CD4}$ mice are selectively induced towards commensal and dietary antigens.

Figure 15 Serum IgG_1 and IgA antibodies in *Atg16l1*$^{\Delta CD4}$ mice recognize dietary antigen

Cohorts of age-matched *Atg16l1*$^{\Delta CD4}$ and *Atg16l1*$^{fl/fl}$ littermates were bled and serum soy-specific antibody isotypes measured by ELISA. **(a,b)** Levels of soy-specific IgA, IgG_1, IgG_{2b}, IgG_{2c} antibodies in **(a)** aged (>5 months) and **(b)** young (8-12weeks) littermates.

Data are representative of three independent experiments with at least 3 mice per group (a), or from one experiment (b). Each dot represents an individual mouse and horizontal bars denote means. Statistical significance was determined using the Mann Whitney test; * $p<0.05$; *** $p<0.001$.

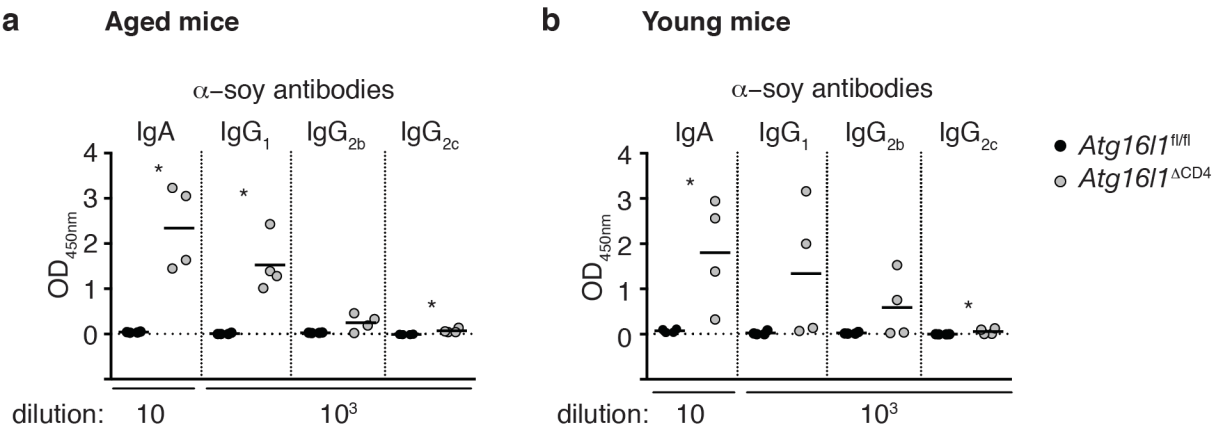
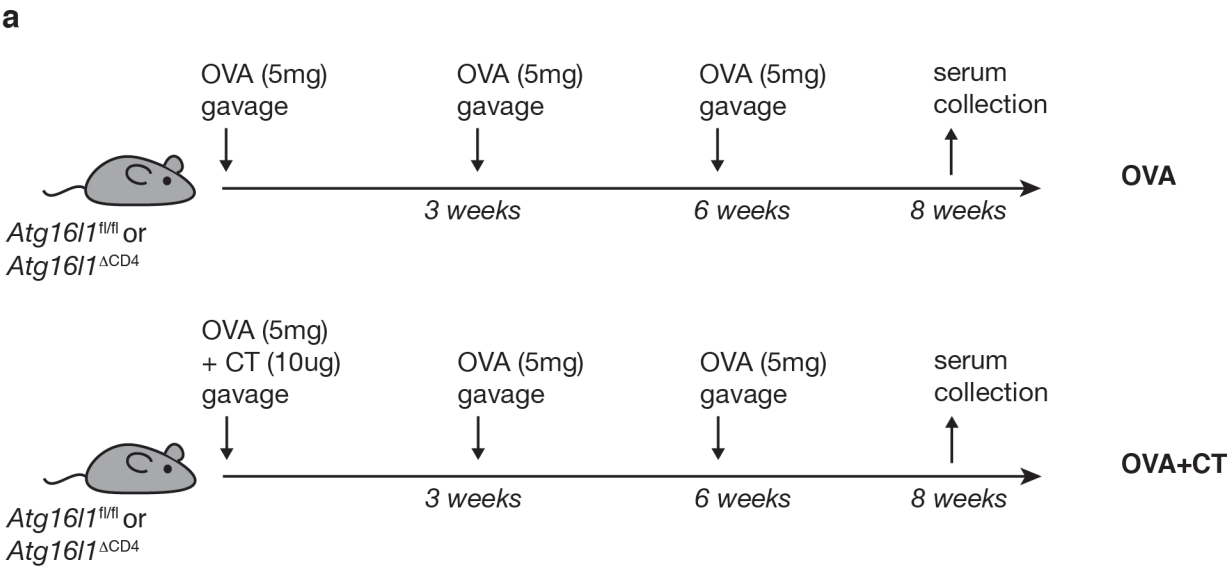

a
Aged mice
α–soy antibodies
IgA
IgG1
IgG2b
IgG2c
OD450nm
4
3
2
1
0
dilution:
10
10³
b
Young mice
α–soy antibodies
IgA
IgG1
IgG2b
IgG2c
OD450nm
4
3
2
1
0
dilution:
10
10³
Atg16l1fl/fl
Atg16l1ΔCD4

Figure 16 *Atg16l1*$^{\Delta CD4}$ mice mount IgE responses against an orally administered protein antigen

Cohorts of young (8-12 weeks old) *Atg16l1*$^{\Delta CD4}$ and *Atg16l1*$^{fl/fl}$ littermates were fed three times with 5mg ovalbumin (OVA) alone, or with 10ug cholera toxin (CT) included with the first feed, and OVA-specific serum IgE responses measured by ELISA. **(a)** Schematic representation of the experimental design. **(b)** Levels of OVA-specific serum IgE 8 weeks after the first OVA feed.

Data are combined from two independent experiments with at least 3 mice per group. Each dot represents an individual mouse and horizontal bars denote means. Statistical significance was determined using the Mann Whitney test, ** $p<0.01$; *** $p<0.001$.


a
OVA (5mg)
gavage
OVA (5mg)
gavage
OVA (5mg)
gavage
serum
collection
3 weeks
6 weeks
8 weeks
OVA
Atg16l1fl/fl or
Atg16l1ΔCD4
OVA (5mg)
+ CT (10ug)
gavage
OVA (5mg)
gavage
OVA (5mg)
gavage
serum
collection
3 weeks
6 weeks
8 weeks
OVA+CT
Atg16l1fl/fl or
Atg16l1ΔCD4

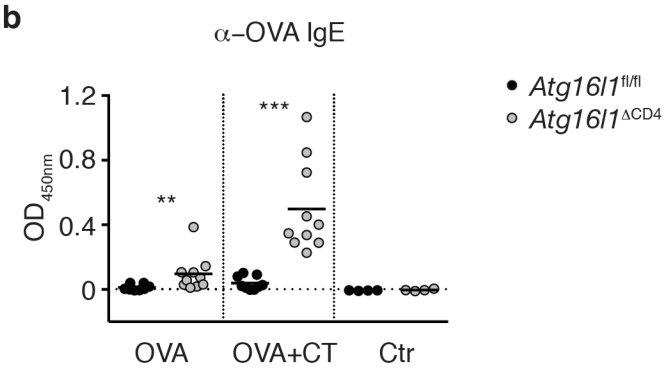

b
α–OVA IgE
OD450nm
1.2
0.8
0.4
0
**
***
OVA
OVA+CT
Ctr
Atg16l1fl/fl
Atg16l1ΔCD4

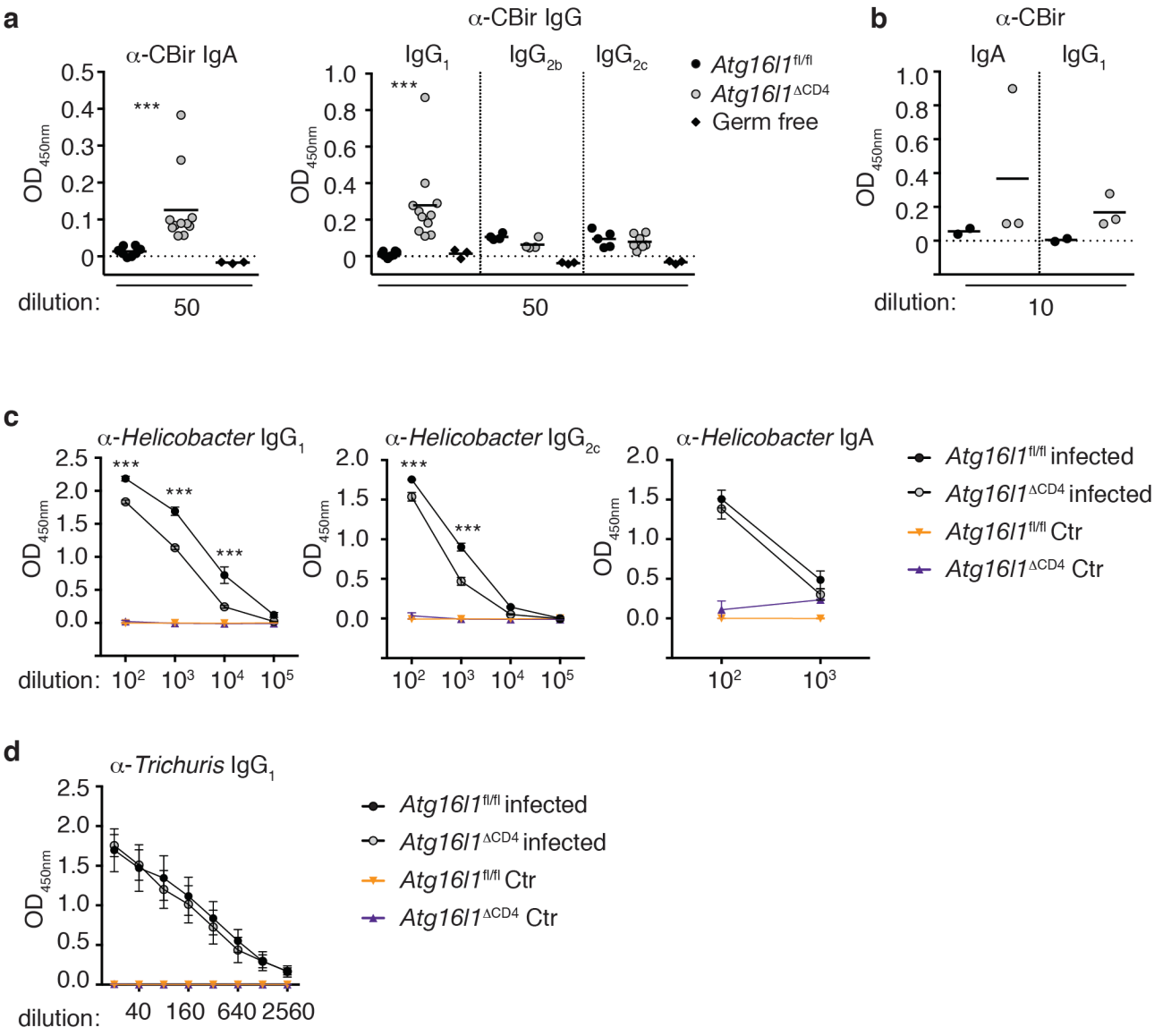
Figure 17 Serum IgG_1 and IgA antibodies in *Atg16l1*$^{\Delta CD4}$ mice recognize commensal antigen

Cohorts of age-matched *Atg16l1*$^{\Delta CD4}$ and *Atg16l1*$^{fl/fl}$ littermates were bled and serum soy-specific antibody isotypes measured by ELISA. **(a)** Levels of CBir1-specific IgA, IgG_1, $IgG_{2b,}$ IgG_{2c} antibodies in serum of aged (>5 months) littermates or germ-free mice measured by ELISA. (**b**) Levels of CBir1-specific IgA and IgG_1 antibodies in serum of young (8-12weeks) littermates.

Cohorts of young (8-12 weeks) *Atg16l1*$^{\Delta CD4}$ and *Atg16l1*$^{fl/fl}$ littermates were infected by oral gavage with *Helicobacter hepaticus* (three feeds of $1x10^8$ CFU) or *Trichuris muris* (200 eggs) and serum antibody responses determined by ELISA. **(c)** Levels of *H. hepaticus*-specific IgG_1, IgG_{2c} and IgA antibodies at d21 post-infection. **(d)** Levels of *T. muris*-specific IgG_1 at day 34 post-infection.

Data are combined from three independent experiments with at least 3 mice per group (a), are from one experiment with at least 2 mice per group (b,c), or are representative of two independent experiments with 5 mice per group (d). Each dot represents an individual mouse and horizontal bars denote means (a,b). Data represent mean ± s.e.m (c,d). Statistical significance was determined using the Mann Whitney test (a,b) or using two-way analysis of variance (ANOVA) with Bonferroni's correction for multiple comparisons, statistical significance was determined between infected groups (b,c), * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

Analysis in (d) was done in collaboration with Prof Richard Grencis and Dr Simon Forman (University of Manchester).


a
α-CBir IgA
α-CBir IgG
IgG1
IgG2b
IgG2c
OD450nm
***
dilution:
50
Atg16l1fl/fl
Atg16l1ΔCD4
Germ free
b
α-CBir
IgA
IgG1
dilution:
10
c
α-Helicobacter IgG1
α-Helicobacter IgG2c
α-Helicobacter IgA
dilution: 10² 10³ 10⁴ 10⁵
Atg16l1fl/fl infected
Atg16l1ΔCD4 infected
Atg16l1fl/fl Ctr
Atg16l1ΔCD4 Ctr
d
α-Trichuris IgG1
dilution: 40 160 640 2560

4.5 Impaired Foxp3$^+$ Treg cell homeostasis in the intestinal mucosa of *Atg16l1*$^{\Delta CD4}$ mice

Elevated mucosal Th2 responses have been described in mice with Treg cell defects, including those selectively affecting peripherally induced Treg cells (pTreg) (Mucida *et al.*, 2005; Curotto de Lafaille *et al.*, 2008; Josefowicz *et al.*, 2012b). We therefore assessed Foxp3$^+$ Treg cell populations in young *Atg16l1*$^{\Delta CD4}$ mice. We found that thymic development of Foxp3$^+$ Treg cells was not diminished in *Atg16l1*$^{\Delta CD4}$ mice compared with *Atg16l1*$^{fl/fl}$ littermates (**Figure 18a,b**). In contrast, we observed a small, but significant, decrease in frequencies and total numbers of Foxp3$^+$ Treg cells in the spleen and mLN of *Atg16l1*$^{\Delta CD4}$ mice compared with *Atg16l1*$^{fl/fl}$ littermates (**Figure 18c,d**). However, the intestinal Treg compartment was most severely affected by *Atg16l1* deficiency, as the frequencies of both colonic and SI LP Foxp3$^+$ Treg cells were reduced by around two-fold relative to *Atg16l1*$^{fl/fl}$ littermates (**Figure 18b,c**). Taking into account the decreased frequencies of CD4$^+$ T cells in *Atg16l1*$^{\Delta CD4}$ mice (**Figure 4a**), this equated to a reduction in Treg cell numbers by around 10-fold in the colonic LP and 4-fold in SI LP of *Atg16l1*$^{\Delta CD4}$ mice (**Figure 18d**).

Given that Th2 cell cytokines have been demonstrated to inhibit induction of Foxp3$^+$ Treg cells (Dardalhon *et al.*, 2008), it was possible that dysregulated Treg cell homeostasis in the *Atg16l1*$^{\Delta CD4}$ mice was a consequence of increased levels of Th2 cytokines at the intestinal mucosa. Therefore, to establish if reduced Foxp3$^+$ Treg cell frequencies in *Atg16l1*$^{\Delta CD4}$ mice were due to a cell-intrinsic defect or were a consequence of dysregulated effector T cell homeostasis, we generated mixed bone marrow (BM) chimeras by reconstituting irradiated *Rag1*$^{-/-}$ mice with a 1:1 mixture of BM cells from *Atg16l1*$^{\Delta CD4}$ or *Atg16l1*$^{fl/fl}$ mice (CD45.2$^+$) together with congenic WT C57BL/6 BM cells (CD45.1$^+$) (**Figure 19a**). Reconstitution of total CD4$^+$ T cells derived

from *Atg16l1*$^{\Delta CD4}$ and *Atg16l1*$^{fl/fl}$ BM cells was comparable in the thymus, however in peripheral lymphoid organs, reconstitution by *Atg16l1*-deficient CD4$^+$ T cells was significantly less efficient (**Figure 19b,c**). In particular, *Atg16l1*-deficient CD4$^+$ T cells in the cLP of reconstituted hosts accounted for less than 3% of total CD4$^+$ T cells, confirming the importance of autophagy for intestinal CD4$^+$ T cell homeostasis (**Figure 19b,c**).

Furthermore, examination of CD4$^+$ Foxp3$^+$ Treg cells in mixed BM chimeras demonstrated that *Atg16l1*$^{fl/fl}$ BM cells were able to efficiently develop into peripheral Foxp3$^+$ Treg cells, whereas very few of the splenic or cLP Treg cells were derived from *Atg16l1*$^{\Delta CD4}$ BM (**Figure 20a,b**). These results indicate that *Atg16l1*-deficiency decreases the ability of Foxp3$^+$ Treg cells to compete with WT Treg cells in a cell-intrinsic manner.

Figure 18 *Atg16l1*$^{\Delta CD4}$ mice have a severe deficiency in intestinal Foxp3$^+$ Treg cells

Cohorts of young (8-12 weeks) *Atg16l1*$^{\Delta CD4}$ and *Atg16l1*$^{fl/fl}$ littermates were sacrificed and Foxp3$^+$ Treg cells assayed using FACS. **(a)** Frequencies of Foxp3$^+$ Treg cells in the thymus (gated on single positive CD4$^+$ TCRβ^+ cells). **(b)** Representative FACS plots of thymus, cLP and SI LP Foxp3$^+$ Treg cells (gated on CD4$^+$ TCRβ^+ cells). **(c)** Frequencies and **(d)** total numbers of Foxp3$^+$ Treg cells in spleen, mLN, cLP and SI LP (gated on CD4$^+$ TCRβ^+ cells).

Data are combined from two or three independent experiments with at least 3 mice per group (a,c,d), or are representative of at least two independent experiments with at least 4 mice per group (b). Each dot represents an individual mouse and horizontal bars denote means. Numbers indicate percentage of cells in gates. Statistical significance was determined using the Mann Whitney test, * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

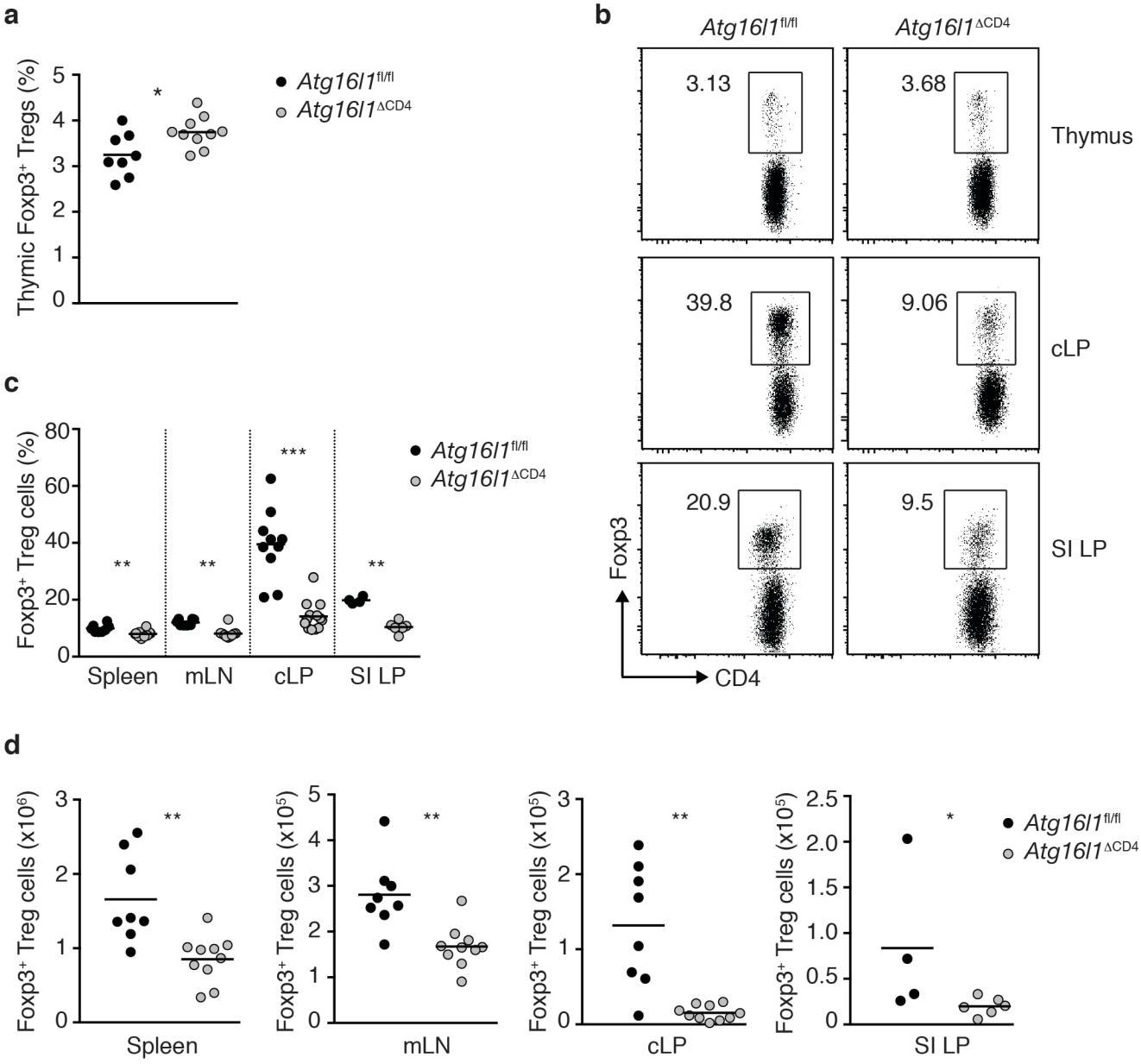
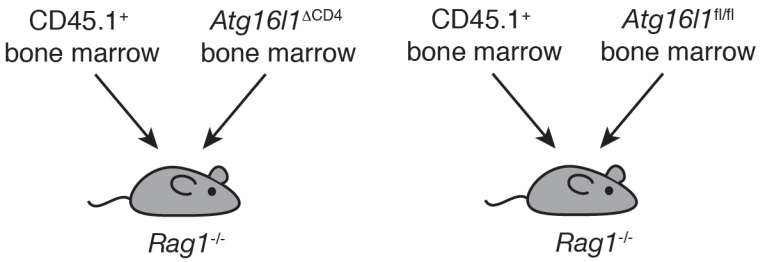

a
Thymic Foxp3+ Tregs (%)
Atg16l1fl/fl
Atg16l1ΔCD4
b
Atg16l1fl/fl
Atg16l1ΔCD4
3.13
3.68
Thymus
39.8
9.06
cLP
20.9
9.5
SI LP
Foxp3
CD4
c
Foxp3+ Treg cells (%)
Spleen
mLN
cLP
SI LP
d
Foxp3+ Treg cells (x10^6)
Foxp3+ Treg cells (x10^5)
Spleen
mLN
cLP
SI LP

Figure 19 *Atg16l1*-deficient BM cells show impaired reconstitution of peripheral lymphoid compartments in irradiation bone marrow chimeras

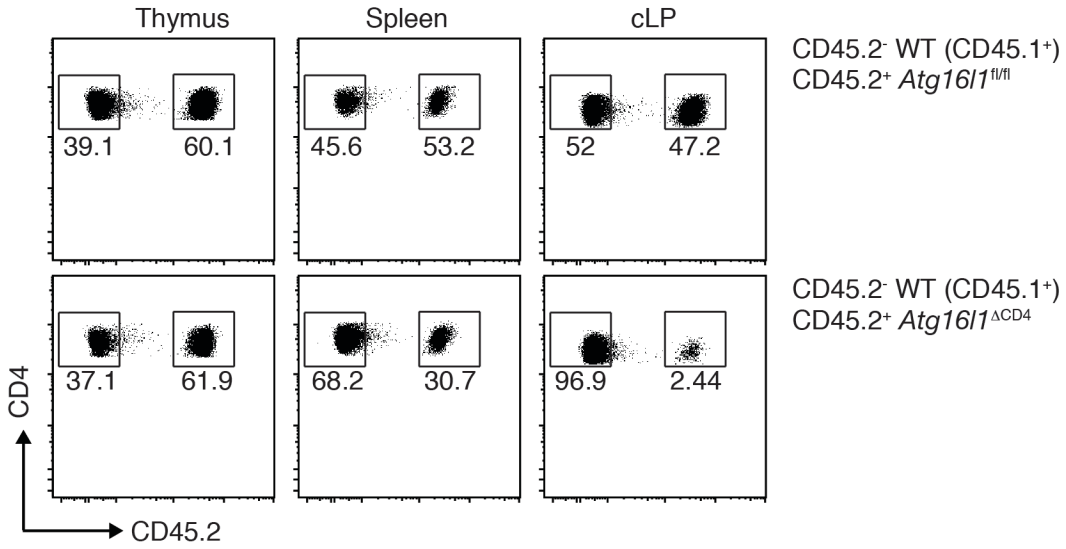
Bone marrow (BM) cells isolated from WT ($CD45.1^+$) and $Atg16l1^{fl/fl}$ or $Atg16l1^{\Delta CD4}$ ($CD45.2^+$) mice were injected i.v. at a 1:1 ratio into lethally irradiated (1100 Rad) $Rag1^{-/-}$ recipients (total of $1x10^7$ cells per mouse). BM chimeras were sacrificed 10 weeks later and reconstitution of lymphoid compartments assayed by FACS. **(a)** Experimental design for the generation of mixed BM chimeras. **(b)** Representative FACS plots showing frequencies of $Atg16l1^{\Delta CD4}$ or $Atg16l1^{fl/fl}$ ($CD45.2^+$) and WT ($CD45.2^-$) $CD4^+$ T cells in thymus, spleen and cLP of mixed BM chimeras (gated on $CD4^+$ $TCR\beta^+$ T cells). **(c)** Frequencies of $Atg16l1^{\Delta CD4}$ or $Atg16l1^{fl/fl}$ ($CD45.2^+$) $CD4^+$ T cells in mixed BM chimeras (shown as percentage of total $CD4^+$ $TCR\beta^+$ T cells).

Data are representative of two independent experiments with at least 7 mice per group. Each dot represents an individual mouse and horizontal bars denote means. Numbers indicate percentage of cells in gates. Statistical significance was determined using the Mann Whitney test, ** $p<0.01$; *** $p<0.001$.

a


CD45.1+
bone marrow
Atg16l1ΔCD4
bone marrow
CD45.1+
bone marrow
Atg16l1fl/fl
bone marrow
Rag1-/-
Rag1-/-

b


Thymus
Spleen
cLP
CD45.2- WT (CD45.1+)
CD45.2+ Atg16l1fl/fl
39.1
60.1
45.6
53.2
52
47.2
CD45.2- WT (CD45.1+)
CD45.2+ Atg16l1ΔCD4
37.1
61.9
68.2
30.7
96.9
2.44
CD4
CD45.2

c

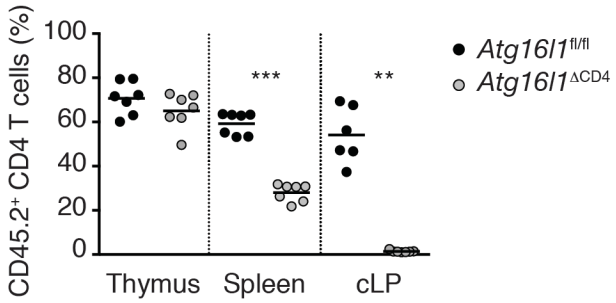
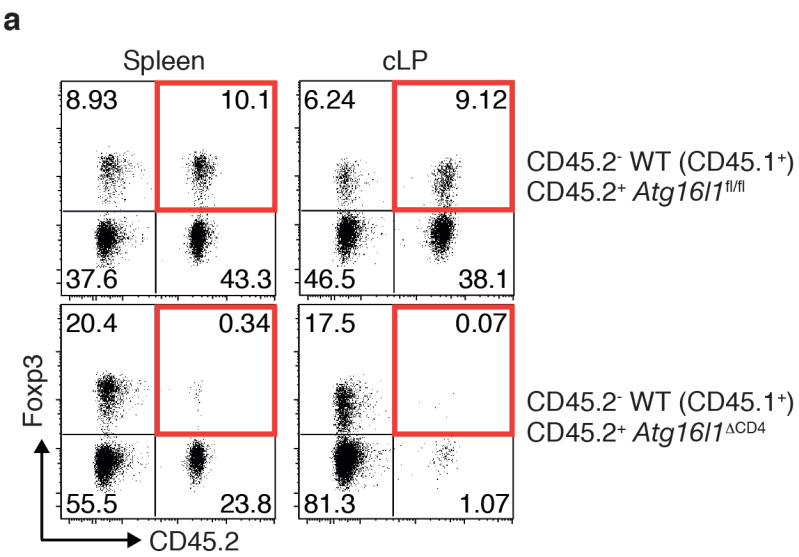
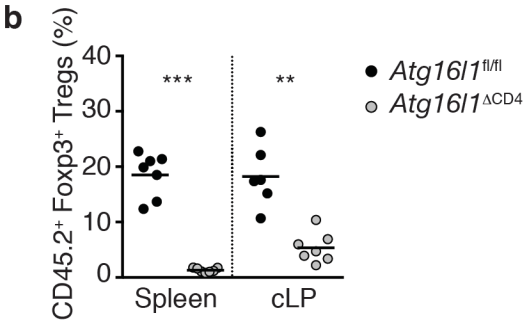

CD45.2+ CD4 T cells (%)
100
80
60
40
20
0
***
**
Thymus
Spleen
cLP
Atg16l1fl/fl
Atg16l1ΔCD4

Figure 20 Deficiency in intestinal Foxp3$^+$ Treg cells in *Atg16l1*$^{\Delta CD4}$ mice is due to cell-intrinsic mechanism

Bone marrow (BM) cells isolated from WT (CD45.1$^+$) and *Atg16l1*$^{fl/fl}$ or *Atg16l1*$^{\Delta CD4}$ (CD45.2$^+$) mice were injected i.v. at a 1:1 ratio into lethally irradiated (1100 Rad) *Rag1*$^{-/-}$ recipients (total of 1x10^7 cells per mouse). BM chimeras were sacrificed 10 weeks later and Foxp3$^+$ Treg cells assayed by FACS. **(a)** Representative FACS plots and **(b)** frequencies of Foxp3$^+$ Treg cells derived from *Atg16l1*$^{\Delta CD4}$ or *Atg16l1*$^{fl/fl}$ (CD45.2$^+$) cells in mixed BM chimeras (gated on CD4$^+$ TCRß$^+$ T cells). Highlighted top right quadrants indicate Treg cells derived from *Atg16l1*$^{fl/fl}$ or *Atg16l1*$^{\Delta CD4}$ BM.

Data are representative of two independent experiments with at least 7 mice per group. Each dot represents an individual mouse and horizontal bars denote means. Numbers indicate percentage of cells in quadrants. Statistical significance was determined using the Mann Whitney test, ** $p<0.01$; *** $p<0.001$.


a
Spleen
cLP
8.93
10.1
37.6
43.3
6.24
9.12
46.5
38.1
CD45.2- WT (CD45.1+)
CD45.2+ Atg16l1fl/fl
20.4
0.34
55.5
23.8
17.5
0.07
81.3
1.07
CD45.2- WT (CD45.1+)
CD45.2+ Atg16l1ΔCD4
Foxp3
CD45.2


b
CD45.2+ Foxp3+ Tregs (%)
40
30
20
10
0
***
**
Atg16l1fl/fl
Atg16l1ΔCD4
Spleen
cLP

Given that *Atg16l1*-deficiency significantly reduced the number of intestinal Treg cells in *Atg16l1*$^{\Delta CD4}$ mice, we hypothesized that Treg cells may be particularly reliant on autophagy compared to other Th cell subsets. Indeed, in WT mice we found that levels of autophagy were significantly higher in Foxp3^{+} Treg cells compared to their Foxp3^{-} CD4^{+} T cell counterparts in the mLN and cLP, both constitutively and after TCR activation (**Figure 21a,b**).

To get an indication whether lack of autophagy affects Treg cell functional parameters we next assessed how *Atg16l1*-deficiency affected Foxp3^{+} Treg cell phenotype. Consistent with previous reports (Campbell and Koch, 2011), intestinal CD4^{+} Foxp3^{+} Treg cells from control *Atg16l1*$^{fl/fl}$ mice did not produce effector cytokines under homeostatic conditions (**Figure 22a**). However, CD4^{+} Foxp3^{+} Treg cells from cLP and SI LP of *Atg16l1*$^{\Delta CD4}$ mice showed significantly increased expression of IFN-γ and IL-17A (**Figure 22a**). Furthermore, CD4^{+} Foxp3^{+} T cells from *Atg16l1*$^{\Delta CD4}$ mice showed higher expression of signature Treg markers CD103, CTLA-4 and ICOS (**Figure 22b**) and had significantly increased expression of Ki67 and higher levels of phosphorylated S6, suggesting that the majority were in cell cycle (**Figure 22c-e**). In contrast, we did not observe any differences in expression of CD44 or CD62L by *Atg16l1*-deficient Treg cells, suggesting that retention in the lymph nodes is not affected by the lack of autophagy (**Figure 23a,b**).

Since the gastrointestinal tract is a preferential site of pTreg conversion (Harrison and Powrie, 2013), we measured the expression of Neuropilin-1 (Nrp1) and Helios, markers that have been proposed to distinguish between thymic-derived Treg cells (Nrp1^{+}/Helios^{+}) and pTreg cells (Nrp1^{-}/Helios^{-}) (Thornton *et al.*, 2010; Weiss *et al.*, 2012; Yadav *et al.*, 2012; Singh *et al.*, 2015). We found similar frequencies of both Nrp1^{+}/Helios^{+} and Nrp1^{-}/Helios^{-} T_{reg} populations in the SI LP and cLP of *Atg16l1*$^{\Delta CD4}$ mice and *Atg16l1*$^{fl/fl}$ littermates, suggesting that *Atg16l1* deficiency has a similarly

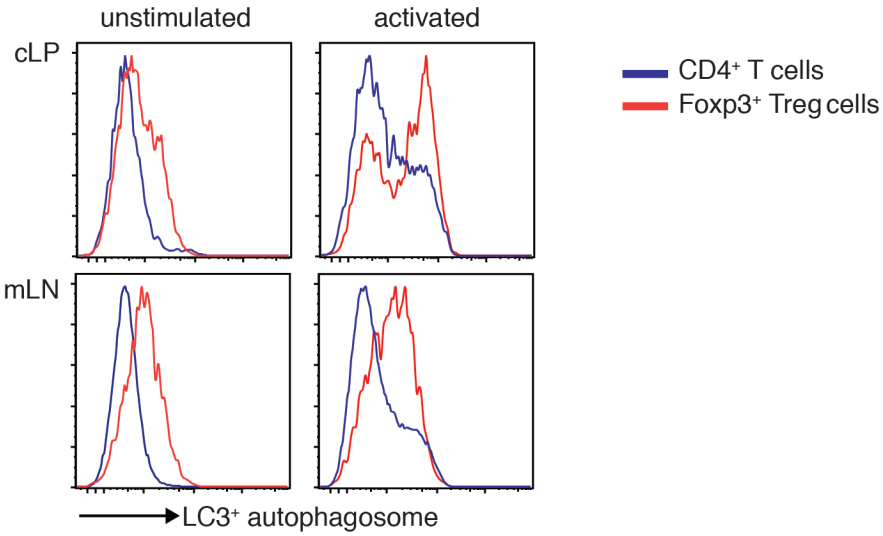
adverse effect on both thymic-derived and pT_{reg} cells in the intestine (**Figure 23c**). Taken together, these results point at the role for autophagy in the maintenance and functional regulation of intestinal Treg cells.

Figure 21 Autophagy levels are higher in Foxp3$^+$ CD4$^+$ Treg cells than in conventional CD4$^+$ T cells

CD4$^+$ T cells were purified from cLP and mLN of WT mice and analysed either directly (unstimulated) or following overnight activation with α-CD3 (5μg/ml) and α-CD28 (1μg/ml). Autophagy levels were measured by FACS. **(a)** Representative LC3 staining and **(b)** quantification of LC3$^+$ autophagosome formation by Foxp3$^-$ CD4$^+$ T cells and Foxp3$^+$ T_{reg} cells (gated on CD4$^+$ TCRβ^+ T cells).

Data are representative of two independent experiments with at least 4 mice per group. Each dot represents an individual mouse and horizontal bars denote means. Statistical significance was determined using the Mann Whitney test, ** $p<0.01$; *** $p<0.001$.

a


unstimulated
activated
cLP
mLN
CD4+ T cells
Foxp3+ Treg cells
LC3+ autophagosome

b

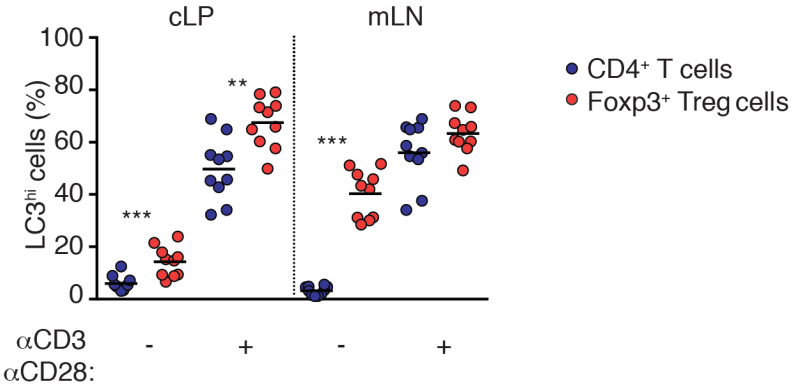

cLP
mLN
100
80
60
40
20
0
LC3hi cells (%)
***
**
***
αCD3
αCD28:
-
+
-
+
CD4+ T cells
Foxp3+ Treg cells

Figure 22 *Atg16l1*-deficient intestinal Treg cells show increased expression of effector cytokines and activation markers

Cohorts of young (8-12 weeks) *Atg16l1*$^{\Delta CD4}$ and *Atg16l1*$^{fl/fl}$ littermates were sacrificed and Foxp3$^+$ Treg cells analysed using FACS. **(a)** Frequencies of IL-17A$^+$ or IFN-γ^+ Foxp3$^+$ T_{reg} cells in the cLP and SI LP (gated on Foxp3$^+$ CD4$^+$ TCRβ^+ T cells). **(b)** Expression of CD103, CTLA4 and ICOS by cLP and splenic Foxp3$^+$ T_{reg} cells (gated on Foxp3$^+$ CD4$^+$ TCRβ^+ T cells). Numbers within graphs indicate mean fluorescence intensity (MFI). **(c)** Representative FACS plots and **(d)** frequencies of Ki67$^+$ Foxp3$^+$ T_{reg} cells in cLP (gated on Foxp3$^+$ CD4$^+$ TCRβ^+ T cells). **(e)** MFI of phosphorylated-S6 (P-S6) in Foxp3$^+$ T_{reg} cells in cLP.

Data are combined from two independent experiments with at least 3 mice per group (a), are representative of at least two independent experiments with at least 4 mice per group (c-d), or are from one experiment (e). Each dot represents an individual mouse and horizontal bars denote means. Numbers indicate MFI (b) or percentage of cells in gates (c). Statistical significance was determined using the Mann Whitney test, * $p<0.05$; ** $p<0.01$.

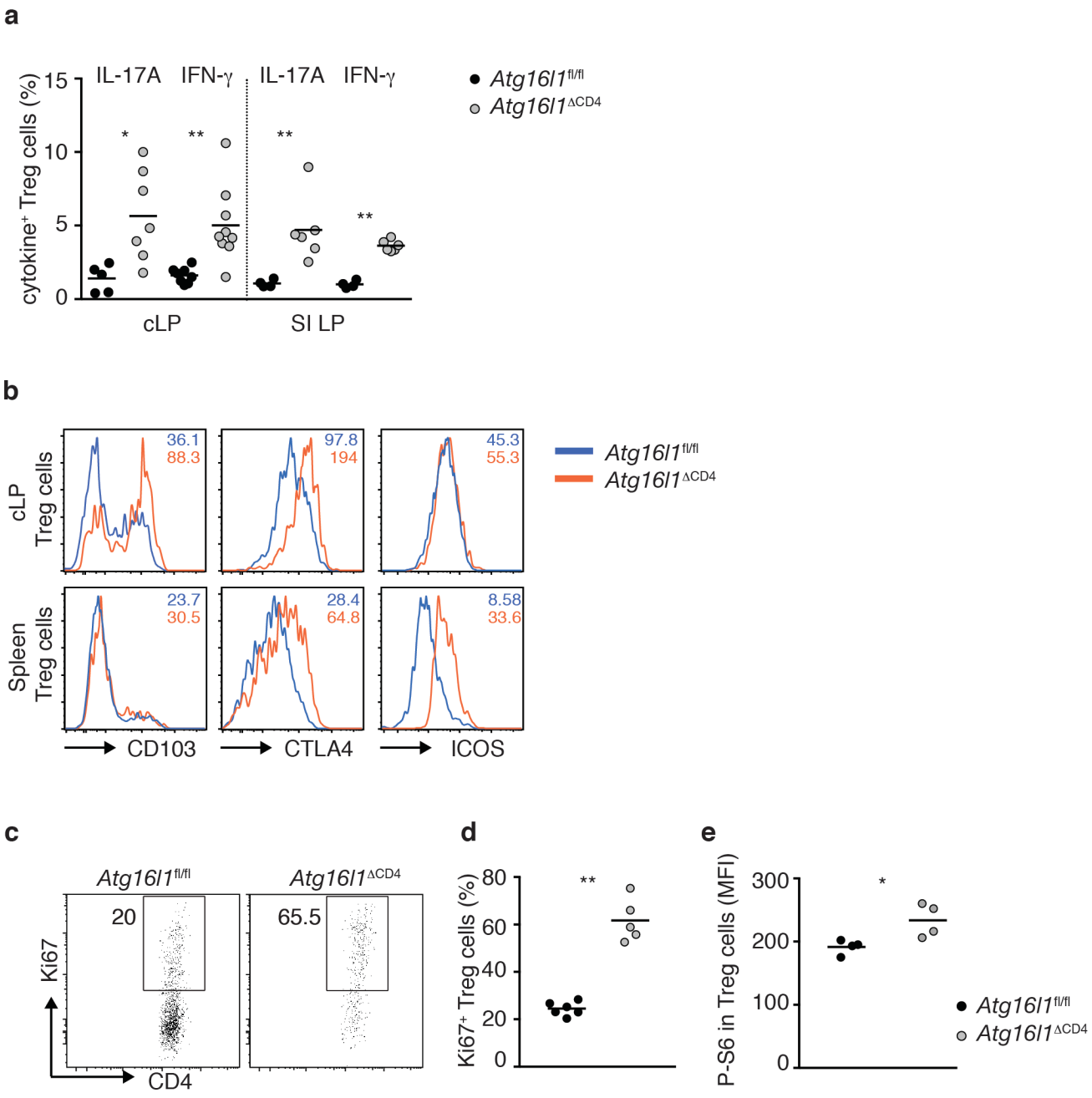
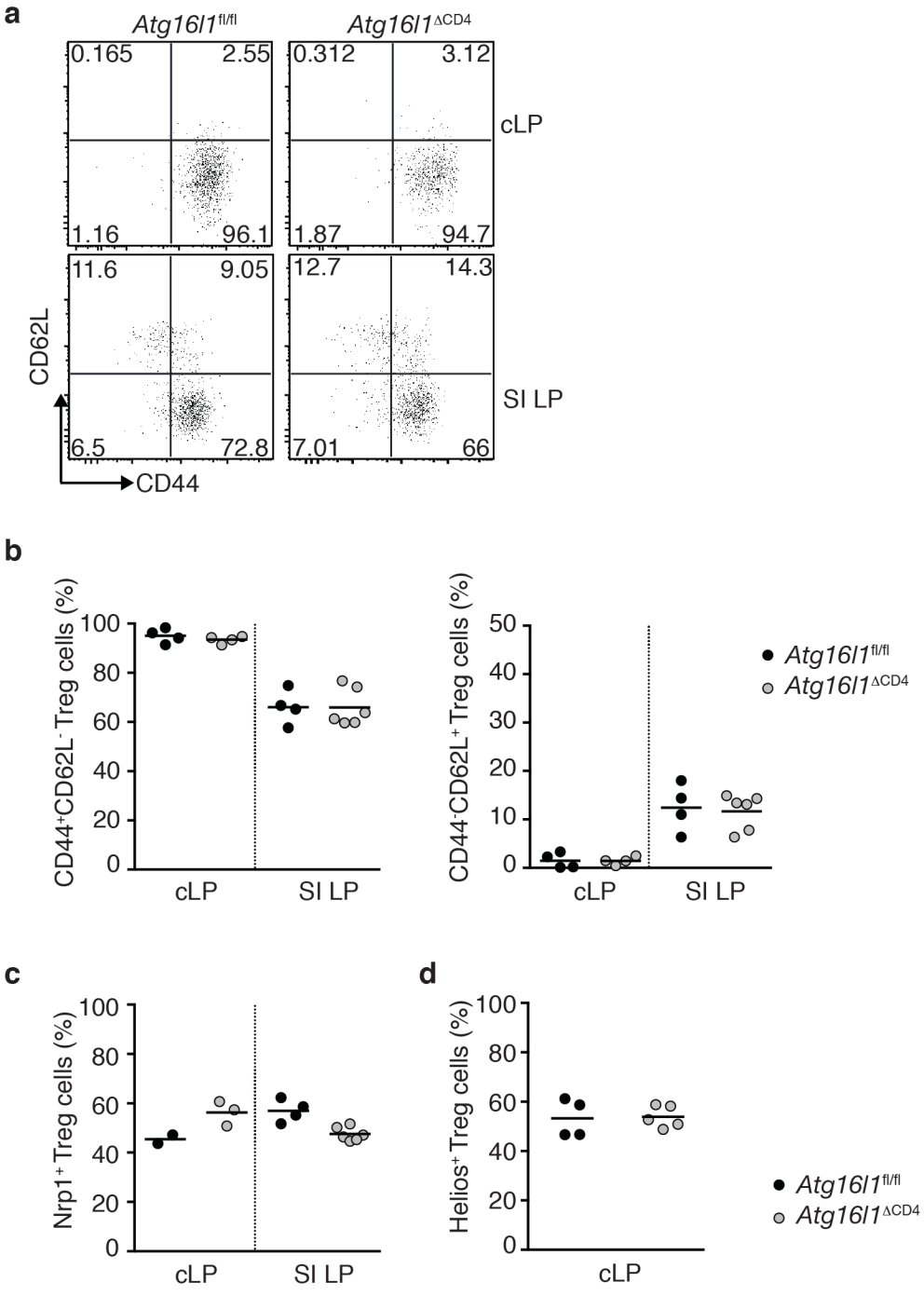

a
IL-17A
IFN-γ
IL-17A
IFN-γ
cytokine⁺ Treg cells (%)
15
10
5
0
*
**
**
**
cLP
SI LP
Atg16l1fl/fl
Atg16l1ΔCD4
b
cLP Treg cells
Spleen Treg cells
36.1
88.3
97.8
194
45.3
55.3
23.7
30.5
28.4
64.8
8.58
33.6
CD103
CTLA4
ICOS
Atg16l1fl/fl
Atg16l1ΔCD4
c
Atg16l1fl/fl
Atg16l1ΔCD4
20
65.5
Ki67
CD4
d
Ki67⁺ Treg cells (%)
80
60
40
20
0
**
e
P-S6 in Treg cells (MFI)
300
200
100
0
*
Atg16l1fl/fl
Atg16l1ΔCD4

Figure 23 *Atg16l1*-deficiency leads to reduced numbers of tTreg and pTreg cells in the intestine

Cohorts of young (8-12 weeks) *Atg16l1*$^{\Delta CD4}$ and *Atg16l1*$^{fl/fl}$ littermates were sacrificed and Foxp3$^+$ Treg cells analysed using FACS. **(a)** Representative FACS plots and **(b)** frequencies of activated (CD44$^+$ CD62L$^-$) and naïve (CD44$^-$ CD62L$^+$) Foxp3$^+$ Treg cells in the cLP and SI LP (gated on CD4$^+$ TCRβ^+ Foxp3$^+$ T cells). **(c,d)** Frequencies of **(c)** Neuropilin-1$^+$ (Nrp1$^+$) and **(d)** Helios$^+$ Foxp3$^+$ T_{reg} cells in the cLP (gated on CD4$^+$ TCRβ^+ Foxp3$^+$ T cells).

Data are combined from two independent experiments with at least 4 mice per group (b,c), are representative from two independent experiments with at least 4 mice per group (a), or are from one experiment (d). Each dot represents an individual mouse and horizontal bars denote means. Numbers indicate percentage of cells in quadrants. Statistical significance was determined using the Mann Whitney test.


a
Atg16l1fl/fl
Atg16l1ΔCD4
0.165
2.55
1.16
96.1
0.312
3.12
1.87
94.7
cLP
11.6
9.05
6.5
72.8
12.7
14.3
7.01
66
SI LP
CD62L
CD44
b
CD44+CD62L- Treg cells (%)
100
80
60
40
20
0
cLP
SI LP
CD44-CD62L+ Treg cells (%)
50
40
30
20
10
0
cLP
SI LP
Atg16l1fl/fl
Atg16l1ΔCD4
c
Nrp1+ Treg cells (%)
100
80
60
40
20
0
cLP
SI LP
d
Helios+ Treg cells (%)
100
80
60
40
20
0
cLP
Atg16l1fl/fl
Atg16l1ΔCD4

4.6 Autophagy is essential for Foxp3$^+$ Treg cells to control inflammatory responses in peripheral tissues

To selectively assess the importance of autophagy for the intestinal Treg cell compartment we crossed the *Atg16l1*$^{fl/fl}$ mice with Foxp3-Cre mice (Rubtsov *et al.*, 2010), generating *Atg16l1*$^{\Delta Foxp3}$ mice in which *Atg16l1* was selectively deleted in Foxp3$^+$ Treg cells. As expected, *Atg16l1*$^{\Delta Foxp3}$ mice showed a strong reduction of *Atg16l1* expression by splenic and cLP Foxp3$^+$ Treg cells, whereas CD4$^+$ Foxp3$^-$ T cells were unaffected (**Figure 24a**). Although *Atg16l1*$^{\Delta Foxp3}$ mice appeared normal in early life, at around 5 months of age they developed a spontaneous inflammatory disease. This was characterized by progressive weight loss, splenomegaly, lymphadenopathy and leukocyte infiltration in multiple tissues, including the liver, spleen and stomach (**Figure 24b-d**). We then assessed the Treg cell compartment in *Atg16l1*$^{\Delta Foxp3}$ mice. *Atg16l1*$^{\Delta Foxp3}$ mice had significantly decreased frequencies of Foxp3$^+$ Treg cells in the spleen and mLN compared to *Atg16l1*$^{fl/fl}$ littermates, although thymic Treg cell frequencies were similar (**Figure 25a,b**). As found in *Atg16l1*$^{\Delta CD4}$ mice, intestinal LP Foxp3$^+$ Treg cells were severely depleted in *Atg16l1*$^{\Delta Foxp3}$ mice and those remaining exhibited increased expression of effector Th cytokines, including IFN-γ, IL-17A, IL-4 and IL-13 (**Figure 25a-c**).

When we assessed the effect of *Atg16l1*-deficiency in Treg cells on the intestinal physiology, we saw that the gastrointestinal tract was strongly affected, with aged *Atg16l1*$^{\Delta Foxp3}$ mice exhibiting severe inflammation in the SI and colon that was characterized by dense leukocytic infiltration of the LP, goblet cell depletion and crypt hyperplasia (**Figure 26a,b**). Intestinal inflammation in *Atg16l1*$^{\Delta Foxp3}$ mice was characterized by accumulation of CD4$^+$ T cells in the intestinal LP (**Figure 26c-e**) and significantly increased frequencies of activated CD4$^+$ T cells in the mLN and cLP

(**Figure 26f,g**). Due to the progressive weight loss and increasing inflammation *Atg16l1*$^{\Delta Foxp3}$ mice were culled at around 5 months of age as a humane end point. Together, these results demonstrate that Foxp3^{+} Treg cell expression of *Atg16l1* is critical for Treg cell maintenance and function in peripheral tissues, including the gastrointestinal tract.

Figure 24 Aged Atg16l1$^{\Delta Foxp3}$ mice develop spontaneous multi-organ inflammatory disease

(a) CD4^{+} Foxp3^{-} T cells and Foxp3^{+} Treg cells were sorted based on surface markers and YFP expression from spleen and cLP of young (8-12 weeks) *Atg16l1*$^{\Delta Foxp3}$ and Foxp3-YFP-Cre mice and *Atg16l1* expression was assayed using Fluidigm Gene Expression system, as described in the Material and Methods section. **(b-d)** Cohorts of Atg16l1$^{\Delta Foxp3}$ and Atg16l1$^{fl/fl}$ littermates were monitored from birth and culled when Atg16l1$^{\Delta Foxp3}$ mice exhibited clinical signs of disease. **(b)** Body weights of experimental cohorts. **(c)** Spleen weights and representative images of spleen and mLN. **(d)** Representative photomicrographs of H&E stained sections of liver, spleen and stomach, scale bar 150μm.

Data are combined from three independent experiments with 2 to 5 mice per group (c), or are representative of as least two independent experiments with 3 to 5 mice per group (a,b,d). *Atg16l1* exon 3 levels are shown as mean ± s.e.m, n=3 (a). Data shown are means ± s.e.m (b). Each dot represents an individual mouse and horizontal bars denote means (c). Statistical significance was determined using two-way analysis of variance (ANOVA) with Bonferroni's correction for multiple comparisons (b) or using the Mann Whitney test (c), * $p<0.05$; *** $p<0.001$.

Analysis in (a) was done in collaboration with Dr Katja Simon and Thomas Riffelmacher (University of Oxford).

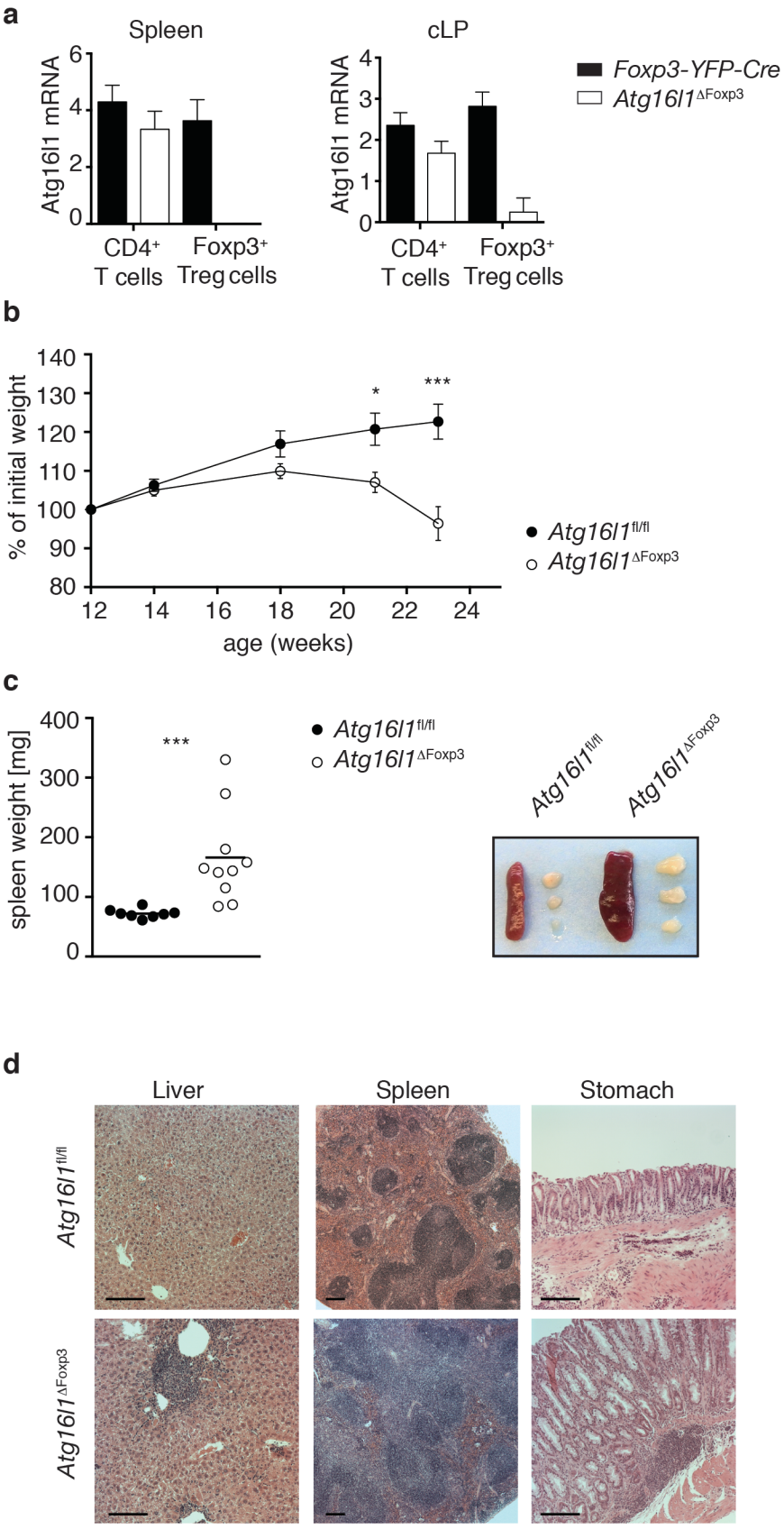

a
Spleen
cLP
Atg16l1 mRNA
CD4+ T cells
Foxp3+ Treg cells
Foxp3-YFP-Cre
Atg16l1ΔFoxp3
b
% of initial weight
age (weeks)
Atg16l1fl/fl
Atg16l1ΔFoxp3
c
spleen weight [mg]
Atg16l1fl/fl
Atg16l1ΔFoxp3
d
Liver
Spleen
Stomach
Atg16l1fl/fl
Atg16l1ΔFoxp3

Figure 25 Aged *Atg16l1*$^{\Delta Foxp3}$ mice have reduced numbers of peripheral Foxp3$^+$ Treg cells

Cohorts of aged (>5 months) *Atg16l1*$^{\Delta FoxP3}$ and *Atg16l1*$^{fl/fl}$ littermates were sacrificed and Foxp3$^+$ Treg cells analysed using FACS. **(a)** Representative FACS plots and **(b)** frequencies of Foxp3$^+$ T_{reg} cells in thymus, spleen, mLN and cLP (gated on CD4$^+$ TCRβ^+ T cells). **(c)** Frequencies of IL-17A$^+$, IFN-γ^+, IL-4$^+$ and IL-13$^+$ Foxp3$^+$ T_{reg} cells in the cLP (gated on Foxp3$^+$ CD4$^+$ TCRβ^+ T cells).

Data are representative of three independent experiments with 2 to 5 mice per group (a), or are combined from two to four independent experiments with 2 to 5 mice per group (b,c). Each dot represents an individual mouse and horizontal bars denote means. Numbers indicate percentage of cells in gates. Statistical significance was determined using the Mann Whitney test, * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

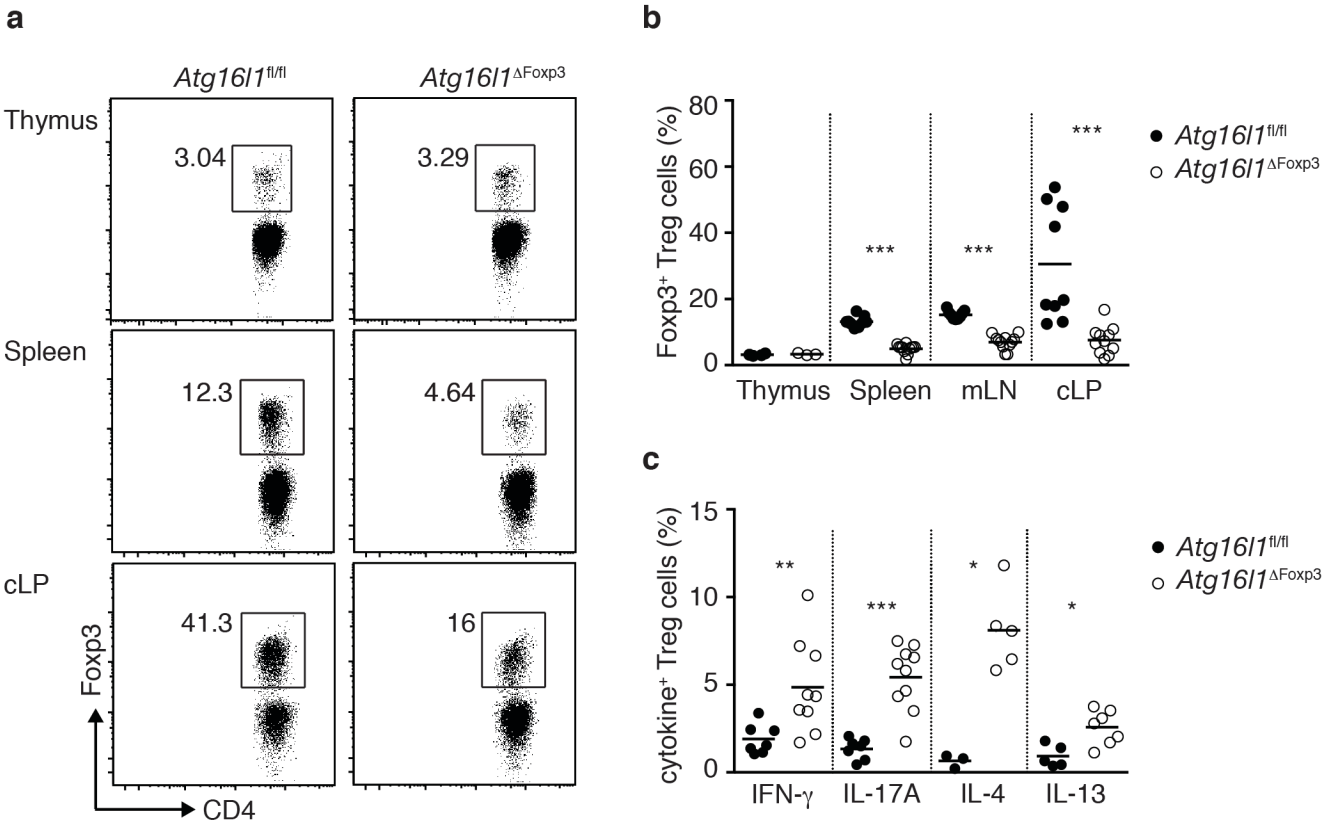
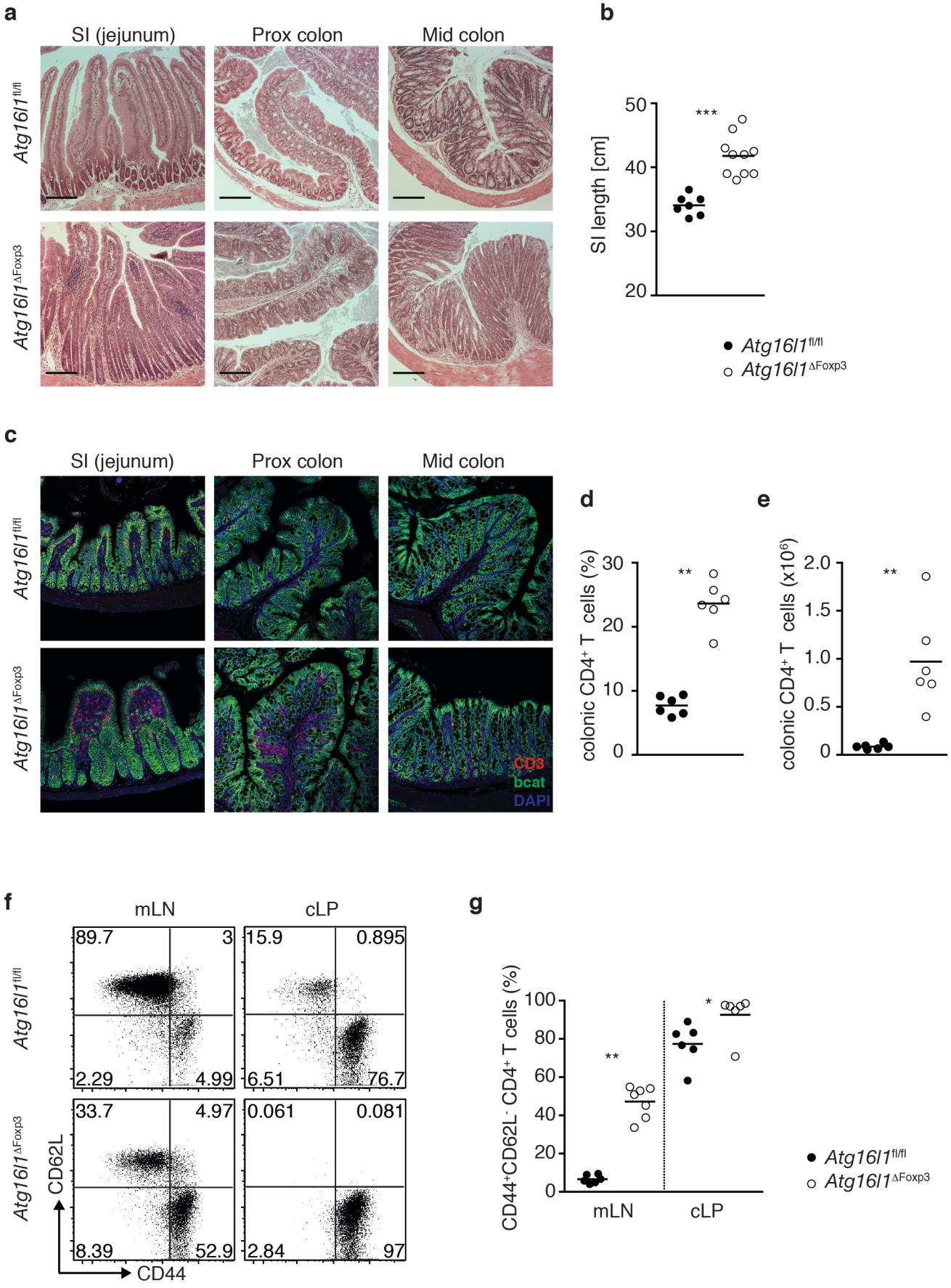

a
Atg16l1fl/fl
Atg16l1ΔFoxp3
Thymus
Spleen
cLP
3.04
3.29
12.3
4.64
41.3
16
Foxp3
CD4
b
Foxp3+ Treg cells (%)
80
60
40
20
0
***
***
***
Thymus
Spleen
mLN
cLP
Atg16l1fl/fl
Atg16l1ΔFoxp3
c
cytokine+ Treg cells (%)
15
10
5
0
**
***
*
*
IFN-γ
IL-17A
IL-4
IL-13
Atg16l1fl/fl
Atg16l1ΔFoxp3

Figure 26 Aged *Atg16l1*$^{\Delta Foxp3}$ mice develop severe intestinal inflammation.

Cohorts of aged (>5 months) Atg16l1$^{\Delta Foxp3}$ and Atg16l1$^{Fl/Fl}$ littermates were sacrificed and intestinal tissues harvested for analyses. **(a)** Representative photomicrographs of H&E stained sections of SI (jejunum), proximal colon and mid-colon, scale bar 150μm. **(b)** SI lengths. **(c)** Representative immunofluorescence images of SI (jejunum), proximal colon and mid-colon, stained for CD3 (red), β-catenin (green) and DAPI (blue). **d)** Frequencies and **(e)** total numbers of cLP CD4$^+$ TCRβ$^+$ T cells. **(f)** Representative FACS plots and **(g)** frequencies of CD44 and CD62L expression by CD4$^+$ T cells from cLP and mLN (gated on CD4$^+$ TCRβ$^+$ Foxp3$^-$ T cells).

Data are representative of three independent experiments with 2 to 5 mice per group (a,c,f), or are combined from three or four independent experiments with 2 to 5 mice per group (b,d,e,g). Each dot represents an individual mouse and horizontal bars denote means. Numbers indicate percentage of cells in quadrants. Statistical significance was determined using the Mann Whitney test, * $p<0.05$; ** $p<0.01$; *** $p<0.001$.


a
SI (jejunum)
Prox colon
Mid colon
Atg16l1fl/fl
Atg16l1ΔFoxp3
b
SI length [cm]
50
40
30
20
***
Atg16l1fl/fl
Atg16l1ΔFoxp3
c
SI (jejunum)
Prox colon
Mid colon
Atg16l1fl/fl
Atg16l1ΔFoxp3
CD3
bcat
DAPI
d
colonic CD4+ T cells (%)
30
20
10
0
**
e
colonic CD4+ T cells (x10^6)
2.0
1.5
1.0
0.5
0
**
f
mLN
cLP
Atg16l1fl/fl
Atg16l1ΔFoxp3
89.7
3
2.29
4.99
15.9
0.895
6.51
76.7
33.7
4.97
8.39
52.9
0.061
0.081
2.84
97
CD62L
CD44
g
CD44+CD62L- CD4+ T cells (%)
100
80
60
40
20
0
**
*
mLN
cLP
Atg16l1fl/fl
Atg16l1ΔFoxp3

4.7 Discussion

Although our initial characterisation of the intestinal $CD4^+$ T cell compartment in *Atg16l1*$^{\Delta CD4}$ mice showed decreased frequencies and numbers of total $CD4^+$ T cells, more detailed examination of intestinal Th subsets revealed that Atg16l1-deficiency differentially affected distinct subsets of $CD4^+$ T cells in the gut. The block of autophagy most drastically affected $Foxp3^+$ Treg cells, and, although Th1 and Th17 subsets did not show altered frequencies, their overall numbers were also decreased. Conversely, the lack of autophagy resulted in a selective expansion of the Th2 cell population within the lamina propria. The decrease in Treg cells and increase in Th2 cells were most pronounced in the intestinal mucosa, suggesting different requirements for autophagy for the accumulation and survival of distinct subsets of $CD4^+$ T cells within the intestinal environment.

The role of autophagy in $Foxp3^+$ Treg cells is not well defined. One study, using mice with T cell specific ablation of *Vps34,* which encodes a class III PI3K that promotes autophagy, reported decreased frequencies of Treg cells in the thymus, spleen and lymph nodes (Parekh *et al.*, 2013). This group also reported weight loss and signs of intestinal pathology that developed in aged mice lacking Vps34 in the T cell compartment (Parekh *et al.*, 2013). However, as Vps34 also has autophagy independent functions (Backer, 2008), it was unclear as to what extent these changes were due to impaired autophagy. Indeed, an independent study where Vps34 kinase was selectively deleted in T cells using Lck-driven Cre expression reported that increased death of Vps34-deficent $CD4^+$ and $CD8^+$ T cells was not due to impaired autophagy, but was rather a consequence of impaired IL-7R expression and signalling in Vps34-deficent T cells (McLeod *et al.*, 2011). Whether the same

deficiency in IL-7 signalling occurs in Vps34-deficent Treg cells remains to be established, although Tregs are known to rely predominantly on IL-2 signalling for survival, and less so on IL-7 signalling (Ma *et al.*, 2006). Nevertheless, these results suggest that in addition to defective autophagy, Vps34-deficient T cells have defects that are autophagy unrelated.

The importance of autophagy for Foxp3$^+$ Treg cell homeostasis was supported by our observation that Treg cells isolated from the mLN and colonic LP had increased levels of autophagy compared to conventional CD4$^+$ T cells. Indeed, our data revealed a crucial, cell-intrinsic role of autophagy for the homeostasis and function of Foxp3$^+$ Treg cells, particularly within the intestine, as *Atg16l1*$^{\Delta CD4}$ mice exhibited a drastic reduction in Treg populations in the cLP and SI LP. The cell-intrinsic role of autophagy in intestinal Foxp3$^+$ Treg cell homeostasis was confirmed in two experimental *in vivo* settings. First, generation of mixed bone marrow chimeras revealed that *Atg16l1*-deficient CD4$^+$ T cells were unable to generate Foxp3$^+$ Treg cells when competing with WT CD4$^+$ T cells. Second, generation of *Atg16l1*$^{\Delta Foxp3}$ mice, in which *Atg16l1* was selectively ablated only in Foxp3$^+$ Treg cells, confirmed that cell-intrinsic autophagy was critical to maintain intestinal Treg cells. Further analyses revealed that *Atg16l1*$^{\Delta Foxp3}$ mice developed a late-onset multi-organ inflammatory disease, including severe inflammation of the stomach, small intestine and colon, demonstrating that Treg cell-intrinsic autophagy is necessary for the control of excessive inflammatory responses within the gastrointestinal tract. It is interesting to notice that rapamycin, which is a known autophagy inducer through its inhibitory activity on mTOR, has been shown to promote expansion of Treg cells *in vitro* and *in vivo* (Pollizzi and Powell, 2015). More recently, several small-molecule inducers of autophagy were shown to promote development of Treg cells, but not other CD4$^+$ T cells, *in vitro* (Shaw *et al.*, 2013). Conversely, an adipokine leptin, which reduces autophagy in CD4$^+$ T cells during TCR engagement (Cassano *et al.*,

2014), has also been reported to inhibit TCR-induced proliferation in Treg cells (De Rosa *et al.*, 2007; Zeng and Chi, 2015), but to increase proliferation of effector $CD4^+$ T cells (Lord *et al.*, 1998). Together with our results, these studies support the notion that Treg cells could be particularly reliant on autophagy.

The small population of intestinal Treg cells that persisted in *Atg16l1*$^{\Delta CD4}$ mice exhibited phenotypic and functional alterations, including increased cell cycling and aberrant expression of Th effector cytokines. Whether *Atg16l1*-deficiency had any effect on the suppressive capacity of Treg cells on the cellular level remains to be determined. We could not address this question directly because the classic *in vitro* inhibitory assay and *in vivo* co-transfer models that are used to measure suppression by Treg cells (Powrie *et al.*, 1993; Takahashi *et al.*, 1998; Thornton and Shevach, 1998) cannot easily distinguish between defective Treg survival or accumulation versus changes in their inhibitory capacity. In future analysis it will be useful to verify if autophagy deficient Treg cells are capable of secreting IL-10 and TGF-β_1, as production of these suppressive cytokines is crucial for the regulatory function of these cells within the intestine (Harrison and Powrie, 2013). Nevertheless, we established that autophagy deficient Treg cells had normal or increased expression of key inhibitory molecules, such as CTLA4 and ICOS, and the late-onset of clinical disease (around 4-5 months) in both *Atg16l1*$^{\Delta CD4}$ and *Atg16l1*$^{\Delta Foxp3}$ mice suggests that *Atg16l1*-deficient Treg cells possess some regulatory capacity. Thus, it is plausible that the inflammatory responses that eventually manifest in these strains are primarily due to the gradual decrease in Treg cell numbers rather than to diminished suppressive capacity.

It is well appreciated that Treg cells are not a uniform population, and, based on the site of Foxp3 induction, they are divided into thymic (tTreg) or peripherally induced (pTreg) Treg cells (Harrison and Powrie, 2013). It remains difficult to distinguish these two populations due to the lack of definitive markers but the transcription

factor Helios and the cell-surface receptor Neuropilin-1 have been reported to be preferentially expressed by tTreg (Thornton *et al.*, 2010; Weiss *et al.*, 2012; Yadav *et al.*, 2012). We found that *Atg16l1*-deficiency did not significantly change the balance between tTregs and pTreg cells within the lamina propria of *Atg16l1*$^{\Delta CD4}$ mice, suggesting that autophagy is essential for maintaining both tTreg and pTreg cells. This may also suggest that the local environment, rather than the site of Treg induction, primarily dictates the requirement for autophagy in Treg cells.

Despite the relatively late onset of clinical symptoms, we found that young *Atg16l1*$^{\Delta CD4}$ mice already exhibited a reduction in peripheral Treg cells and increased Th2 cell responses that preceded the onset of overt pathology. The delayed disease onset could therefore reflect our findings that thymic production of Treg cells was unimpaired in *Atg16l1*$^{\Delta CD4}$ mice and, as noted above, *Atg16l1*-deficient Treg cells continued to express high levels of Foxp3 and other key Treg cell effector molecules. This may allow them to maintain some regulatory activity, even though their number in the periphery is compromised. However, as thymic involution occurs in older mice, the central output of Treg and conventional T cells is decreased, meaning that the peripheral Treg cell compartments cannot be fully replenished, suggesting that autophagy might be important for maintenance and self-renewal of Treg cells (Rubtsov *et al.*, 2010).

Paralleling decreased Treg responses in *Atg16l1*$^{\Delta CD4}$ mice, we observed a selective expansion of Th2 cells in the colon and SI lamina propria that was already present in young mice. This increase could have arisen due to the lack of cell extrinsic regulation from autophagy deficient Treg cells or could have been a consequence of a cell intrinsic effect of autophagy on Th2 cells. We have explored both hypotheses in more detail and the results of these experiments are presented and discussed in Chapter 5. Increase in Th2 cell numbers at the intestinal mucosa was accompanied

by increased frequencies of eosinophils in the mLN and spleen, as well as increased levels of MCPT-1 in the serum of *Atg16l1*$^{\Delta CD4}$ mice. These result suggest that innate type 2 responses are also elevated in *Atg16l1*$^{\Delta CD4}$ mice, however future analysis should include more detailed characterisation of the innate type 2 cell compartment within the intestinal mucosa, including mast cells, ILC2 and eosinophils. Additionally, it will be informative to establish if the levels of Th2 cytokines are increased within the intestinal tissue of *Atg16l1*$^{\Delta CD4}$ mice.

Importantly, the increase in Th2 cells in *Atg16l1*$^{\Delta CD4}$ mice led to dysregulated humoral responses towards benign luminal antigens. We showed that serum IgG_1 and IgA antibodies from *Atg16l1*$^{\Delta CD4}$ mice recognized dietary protein antigens such as soy, as well as commensal microbiota antigens, and that these responses gradually increased with age. We also showed that *Atg16l1*$^{\Delta CD4}$ mice had very high levels of circulating IgE, and we established that these IgE antibodies could be induced against novel dietary antigens, as they were elicited by feeding soluble OVA, although we could not detect soy-specific IgE. The IgE responses might therefore be directed towards less abundant antigens present at low concentrations in the food (Berin and Sampson, 2013). Another possibility is that high levels of soy-specific IgG_1 and IgA are acting as an inhibitory response, masking epitopes that could be recognized by IgE antibodies, as IgG_1 (or IgG_4 in humans) antibodies have been shown to limit responses mediated by more pro-inflammatory isotypes (Nouri-Aria *et al.*, 2004; Berin and Sampson, 2013; Strait *et al.*, 2015).

Fulminant disease manifested in the gut, a site of continuous antigen exposure and immune activation, where responses to environmental antigens must be tightly regulated. This gradual loss of tolerance to intestinal antigens paralleled the development of chronic intestinal pathology, suggesting that these responses were a consequence of a loss of intestinal Treg cells as well as expansion of Th2 cells. However, it would be important to formally prove that enhanced Th2 responses are

the primary cause of intestinal pathology in *Atg16l1*$^{\Delta CD4}$ mice. This could be achieved by performing long-term blockade of Th2 type cytokines in *Atg16l1*$^{\Delta CD4}$ mice using monoclonal antibodies, or by crossing the *Atg16l1*$^{\Delta CD4}$ mice with strains lacking key cytokines, such as IL-4 or IL-13. Indeed, it can be argued that Th2 responses may in fact have a protective role, as evidence from early studies show beneficial effects of type 2 immune responses in some chronic diseases, owing to the ability to inhibit type 1 responses (West *et al.*, 1996; Lubberts *et al.*, 2000b). Altogether, the contribution of enhanced type 2 responses for the intestinal pathology in *Atg16l1*$^{\Delta CD4}$ mice requires further investigation.

One important limitation of this study is that we have not examined Tfh responses in *Atg16l1*$^{\Delta CD4}$ mice. As Tfh cells are crucial regulators of humoral responses in germinal centres (Linterman *et al.*, 2012), it will be interesting to assess how *Atg16l1*-deficiency affects this subset of Th cells. Indeed, it will be important to establish whether changes in the Tfh compartment may contribute to the dysregulated humoral responses in *Atg16l1*$^{\Delta CD4}$ mice, as both Tfh and Th2 cells have been implicated in mediating IgG_1 and IgE switch (Reinhardt *et al.*, 2009; Turqueti-Neves *et al.*, 2014; He *et al.*, 2015). Additionally, it is important to point out that the characterisation of humoral responses in *Atg16l1*$^{\Delta CD4}$ mice in our study was limited to serum antibodies. It would be informative to extend this analysis to the local mucosal production. In particular, it will be interesting to investigate how *Atg16l1* deficiency affects sIgA and IgE responses in the intestinal lamina propria. Nevertheless, the results presented in this chapter strongly suggest that tolerance towards luminal antigens is not properly maintained in mice lacking autophagy in the T cell compartment.

Chapter 5. Role for autophagy in cell intrinsic and cell extrinsic (Treg-mediated) regulation of the intestinal Th2 compartment

5.1 Autophagy in Treg cells is necessary to control intestinal Th2 responses

Generation of *Atg16l1*$^{\Delta Foxp3}$ mice made it possible to examine the relationship between increased Th2 and decreased Treg frequencies originally found in the intestines of *Atg16l1*$^{\Delta CD4}$ mice. Therefore we determined whether selective ablation of *Atg16l1* within Foxp3^{+} Treg cells led to aberrant Th2 responses in *Atg16l1*$^{\Delta Foxp3}$ mice. Indeed, we observed significantly increased frequencies of cLP Gata3^{+} CD4^{+} T cells in inflamed colons of aged *Atg16l1*$^{\Delta Foxp3}$ mice when compared to *Atg16l1*$^{fl/fl}$ littermates, as well as significantly increased frequencies of IL-13^{+} CD4^{+} T cells (**Figure 27a-c**). In contrast, we did not detect any significant increase in the frequencies of IFN-γ or IL-17A producing CD4^{+} T cells in the cLP of aged *Atg16l1*$^{\Delta Foxp3}$ mice (**Figure 27c**). However, these increased Th2 responses were only present in aged *Atg16l1*$^{\Delta Foxp3}$ mice, as we did not detect increased Th2 frequencies in young *Atg16l1*$^{\Delta Foxp3}$ mice (**Figure 27d**). Thus, selective ablation of *Atg16l1* in Foxp3^{+} Treg cells led to increase frequencies of Th2 cells in the intestinal mucosa, but the kinetic of this increase was delayed when compared to *Atg16l1*$^{\Delta CD4}$ mice (see **Figure 11** for comparison).

However, it is important to point out that the intestinal inflammatory disease in *Atg16l1*$^{\Delta Foxp3}$ had a distinct Th cell signature from that observed in *Atg16l1*$^{\Delta CD4}$ mice. Indeed, the massive cLP infiltrate in aged *Atg16l1*$^{\Delta Foxp3}$ mice contained a mixed

population of Th1, Th17 and Th2 effector cells (**Figure 26e**), which was not unexpected, since in these mice all $CD4^+$ T cell subsets apart from Treg cells have an intact autophagy pathway. In addition, we observed a strong increase in IFN-γ^+ $CD4^+$ Th1 frequencies in the mLN of inflamed *Atg16l1*$^{\Delta Foxp3}$ mice (**Figure 27f**).

Expression of the pleiotropic transcription factor IRF4 in Treg cells was reported to be essential for the ability of Treg cells to control Th2 responses (Zheng *et al.*, 2009). We therefore examined the expression of IRF4 in *Atg16l1*-deficent Treg cells in aged *Atg16l1*$^{\Delta Foxp3}$ mice. However, we did not see evidence of defective expression of this transcription factor, in contrast, expression of IRF4 was significantly increased in Treg cells isolated from aged *Atg16l1*$^{\Delta Foxp3}$ mice compared to Treg cells from aged *Atg16l1*$^{fl/fl}$ littermates (**Figure 27g**).

We also characterised serum antibody responses in *Atg16l1*$^{\Delta Foxp3}$ mice. Selective deletion of *Atg16l1* in $Foxp3^+$ Treg cells resulted in significantly elevated levels of circulating IgE and IgA antibodies (**Figure 28a,b**). However, levels of IgG_1 were significantly decreased in aged *Atg16l1*$^{\Delta Foxp3}$ mice compared to those present in *Atg16l1*$^{fl/fl}$ littermates (**Figure 28b**). Levels of isotypes not associated with Th2 responses (i.e. IgG_{2b} and IgG_{2c}) were similar in aged *Atg16l1*$^{\Delta Foxp3}$ mice and *Atg16l1*$^{fl/fl}$ littermates (**Figure 28b**). More detailed analyses of the specificity of humoral responses revealed significantly elevated levels of soy-specific and CBir-specific IgA antibodies in the sera of *Atg16l1*$^{\Delta Foxp3}$ mice compared to *Atg16l1*$^{fl/fl}$ littermates (**Figure 28c,d**), whereas *Atg16l1*$^{\Delta Foxp3}$ mice did not show elevated levels of soy-specific or CBir-specific IgG_1 (or other IgG classes) (**Figure 28c,d**).

In summary, deletion of *Atg16l1* in $Foxp3^+$ Treg cells led to intestinal inflammation that was characterized by accumulation of all Th effector types, with a disproportionate increase in Th2 responses in the lamina propria. Importantly though, the magnitude of this Th2 response was less pronounced compared to what we previously observed in *Atg16l1*$^{\Delta CD4}$ mice and it was only detectable in aged,

inflamed mice. Additionally, *Atg16l1*$^{\Delta Foxp3}$ mice only partially recapitulated dysregulation in humoral responses observed in *Atg16l1*$^{\Delta CD4}$ mice, as they did not exhibit the excessive IgG_1 responses towards intestinal luminal antigens. Therefore, we concluded that although lack of sufficient Treg cell control contributes to the elevated Th2 phenotype, it could not fully explain the more striking enhancement in Th2 responses observed in *Atg16l1*$^{\Delta CD4}$ mice.

Figure 27 Aged *Atg16l1*$^{\Delta Foxp3}$ mice exhibit elevated intestinal Th2 responses

Cohorts of aged (>5 months) *Atg16l1*$^{\Delta FoxP3}$ and *Atg16l1*$^{fl/fl}$ littermates were sacrificed and analysed by FACS. **(a)** Frequencies of Gata3$^+$ CD4$^+$ T cells in spleen, mLN and cLP (gated on CD4$^+$ TCRβ^+ Foxp3$^-$ T cells). **(b)** Representative FACS plots of Gata3 and IL-13 expression in cLP CD4$^+$ T cells (gated on CD4$^+$ TCRβ^+ Foxp3$^-$ T cells). **(c,d)** Frequencies of T_H1 (IFN-γ^+), T_H17 (IL-17A$^+$), T_H2 (IL-13$^+$) T cells in the cLP of **(c)** aged and **(d)** young (8-12 weeks) *Atg16l1*$^{\Delta Foxp3}$ and *Atg16l1*$^{fl/fl}$ littermates (gated on CD4$^+$ TCRβ^+ Foxp3$^-$ T cells). **(e)** Numbers of T_H1 (IFN-γ^+), T_H17 (IL-17A$^+$) and T_H2 (IL-13$^+$) cells in the cLP of aged *Atg16l1*$^{\Delta Foxp3}$ and *Atg16l1*$^{fl/fl}$ littermates (gated on CD4$^+$ TCRβ^+ Foxp3$^-$ T cells). **(f)** Frequencies of T_H1 (IFN-γ^+), T_H17 (IL-17A$^+$), T_H2 (IL-13$^+$) in the mLN of aged *Atg16l1*$^{\Delta Foxp3}$ and *Atg16l1*$^{fl/fl}$ littermates (gated on CD4$^+$ TCRβ^+ Foxp3$^-$ T cells). (**g**) Frequencies of IRF4$^+$ Treg cells in spleen, mLN and cLP of aged *Atg16l1*$^{\Delta Foxp3}$ and *Atg16l1*$^{fl/fl}$ littermates (gated on CD4$^+$ TCRβ^+ Foxp3$^+$ T cells).

Data are combined from two to five independent experiments with 2 to 5 mice per group (a,c-f), are representative of three independent experiments with 2 to 5 mice per group (b) or are from one experiment (g). Each dot represents an individual mouse and horizontal bars denote means. Numbers indicate percentage of cells in gates. Statistical significance was determined using the Mann Whitney test, * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

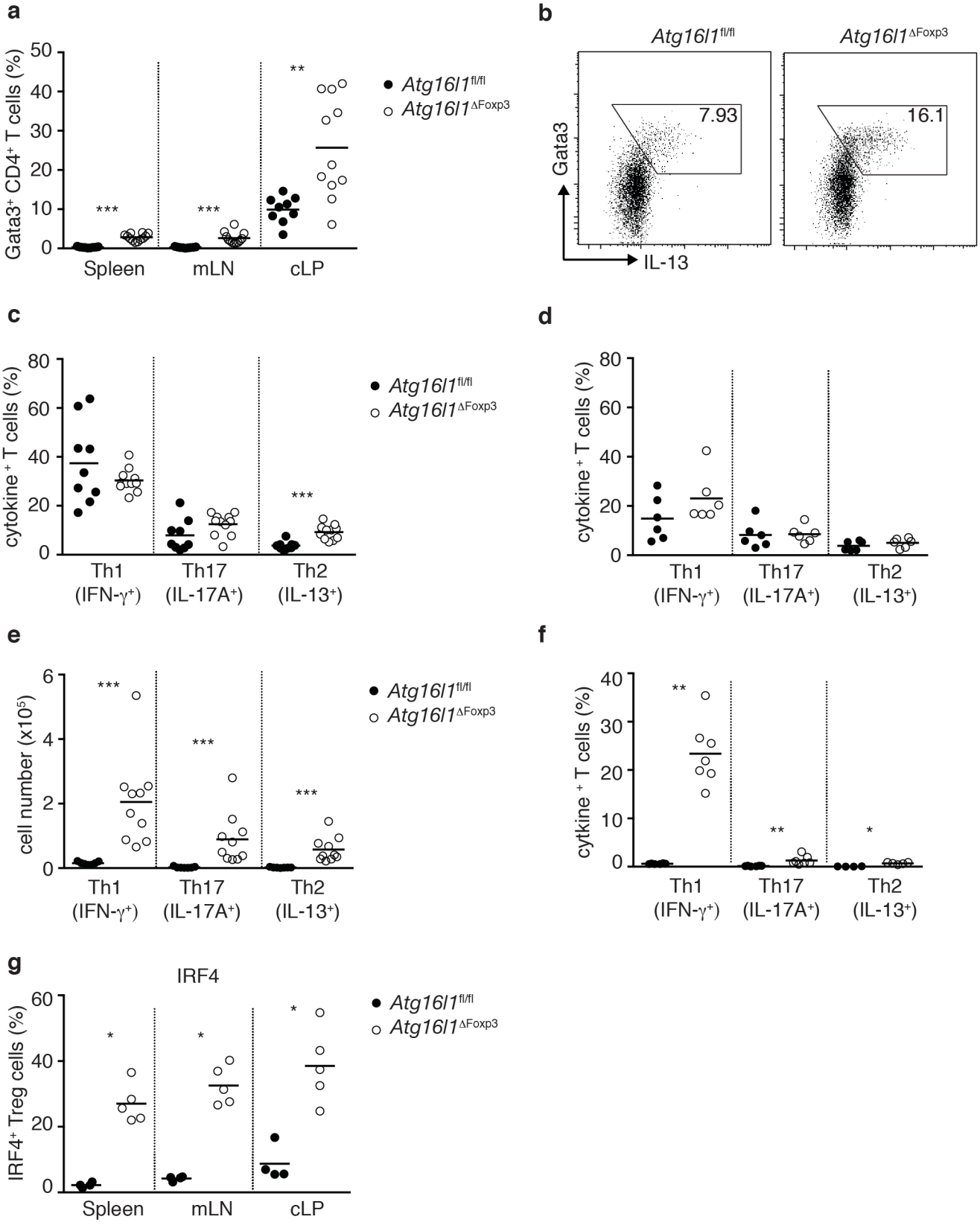
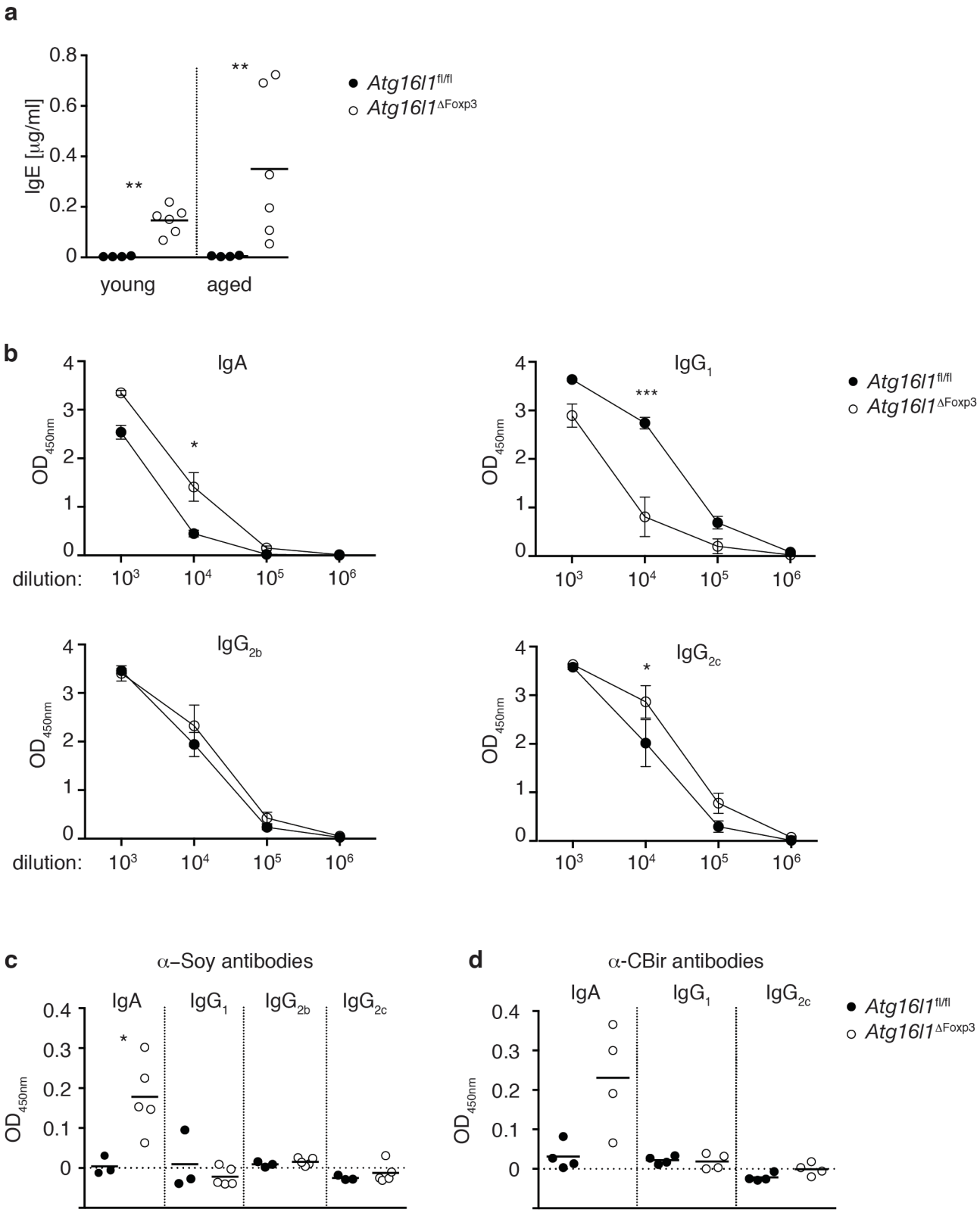

a
Gata3+ CD4+ T cells (%)
50
40
30
20
10
0
***
***
**
Spleen
mLN
cLP
Atg16l1fl/fl
Atg16l1ΔFoxp3
b
Atg16l1fl/fl
Atg16l1ΔFoxp3
7.93
16.1
Gata3
IL-13
c
cytokine+ T cells (%)
80
60
40
20
0
***
Th1
(IFN-γ+)
Th17
(IL-17A+)
Th2
(IL-13+)
Atg16l1fl/fl
Atg16l1ΔFoxp3
d
cytokine+ T cells (%)
80
60
40
20
0
Th1
(IFN-γ+)
Th17
(IL-17A+)
Th2
(IL-13+)
e
cell number (x10^5)
6
4
2
0
***
***
***
Th1
(IFN-γ+)
Th17
(IL-17A+)
Th2
(IL-13+)
Atg16l1fl/fl
Atg16l1ΔFoxp3
f
cytkine+ T cells (%)
40
30
20
10
0
**
**
*
Th1
(IFN-γ+)
Th17
(IL-17A+)
Th2
(IL-13+)
g
IRF4
IRF4+ Treg cells (%)
60
40
20
0
*
*
*
Spleen
mLN
cLP
Atg16l1fl/fl
Atg16l1ΔFoxp3

Figure 28 Aberrant humoral responses in *Atg16l1*$^{\Delta Foxp3}$ mice

Cohorts of young (8-12 weeks) and aged (>5 months) *Atg16l1*$^{\Delta Foxp3}$ and *Atg16l1*$^{fl/fl}$ littermates were bled and serum antibody levels assayed by ELISA. **(a)** Serum IgE concentrations. **(b)** Serum antibody IgA, IgG_1, IgG_{2b}, IgG_{2c} isotype levels in aged *Atg16l1*$^{\Delta Foxp3}$ and *Atg16l1*$^{fl/fl}$ littermates. **(c,d)** Levels of **(c)** Soy-specific (sera diluted 100x pre-assay) and **(d)** CBir1-specific (sera diluted 50x pre-assay) IgA, IgG_1, IgG_{2b}, IgG_{2c} antibodies in sera of aged *Atg16l1*$^{\Delta Foxp3}$ and *Atg16l1*$^{fl/fl}$ littermates.

Data are from one experiment (c), or combined from two to three independent experiments with 2 to 6 mice per group (a,b,d). Each dot represents an individual mouse and horizontal bars denote means (a,c,d). Serum isotype levels are shown as mean ± s.e.m (b). Statistical significance was determined using the Mann Whitney test (a,c,d) or two-way analysis of variance (ANOVA) with Bonferroni's correction for multiple comparisons (b), * $p<0.05$; ** $p<0.01$; *** $p<0.001$.


a
IgE [µg/ml]
0.8
0.6
0.4
0.2
0
**
**
young
aged
Atg16l1fl/fl
Atg16l1ΔFoxp3
b
IgA
IgG1
IgG2b
IgG2c
OD450nm
dilution:
10³
10⁴
10⁵
10⁶
*
***
*
*
c
α–Soy antibodies
IgA
IgG1
IgG2b
IgG2c
d
α-CBir antibodies
IgA
IgG1
IgG2c

5.2 Autophagy also regulates Th2 responses in a cell-intrinsic manner

As pTreg cells are required to control Th2 responses at mucosal sites (Mucida *et al.*, 2005; Curotto de Lafaille *et al.*, 2008; Josefowicz *et al.*, 2012b) we designed an experimental approach to restore the pTreg population in *Atg16l1*$^{\Delta CD4}$ mice, through adoptive transfer of congenic WT naïve CD4$^+$ T cells (CD45.1$^+$) (**Figure 29a**). We used *Atg16l1*$^{\Delta CD4}$ mice at the age of 10-12 weeks, before the onset of intestinal pathology, and kept control groups of untreated *Atg16l1*$^{\Delta CD4}$ and *Atg16l1*$^{fl/fl}$ littermates. Three months after reconstitution, when untreated *Atg16l1*$^{\Delta CD4}$ mice had developed clear signs of intestinal inflammation, all animals were euthanized and examined. We detected CD45.1$^+$ donor CD4$^+$ T cells in all adoptively transferred *Atg16l1*$^{\Delta CD4}$ mice, but the level of donor CD4$^+$ T cell reconstitution varied by the organ examined (**Figure 29b,c**). In peripheral lymphoid tissues donor WT CD4$^+$ T cells accounted for 37±5% in spleen and 18±2% in mLN, whereas in the cLP donor WT CD4$^+$ T cells represented 56±4% of total CD4$^+$ T cells of *Atg16l1*$^{\Delta CD4}$ recipients (**Figure 29c)**. These results demonstrated that autophagy-deficient CD4$^+$ T cells had a strong survival disadvantage when compared to WT CD4$^+$ T cells, especially in the intestine.

When we examined Foxp3$^+$ Treg cells, the survival advantage conferred by autophagy was even more apparent, with around 50% of the donor WT CD45.1$^+$ T cells developing into Foxp3$^+$ pTreg cells in spleen, mLN and cLP of *Atg16l1*$^{\Delta CD4}$ recipients (**Figure 29b)**. As a result, the majority of Foxp3$^+$ Treg cells were of WT donor origin (67±5% in spleen, 59±4% in mLN and 80±5% in cLP) (**Figure 29d**). Overall, adoptive transfer of WT naive CD4$^+$ T cells resulted in efficient reconstitution of Foxp3$^+$ pTreg cells in *Atg16l1*$^{\Delta CD4}$ mice. As such, we could utilize this system to determine whether

excessive Th2 cell accumulation in *Atg16l1*$^{\Delta CD4}$ mice was due to impaired mucosal pTreg cells or due to a cell-intrinsic effect of *Atg16l1*-deficiency in Th2 cells.

When we analysed the frequencies of Th2 cells in the cLP of pTreg-reconstituted *Atg16l1*$^{\Delta CD4}$ mice, we observed significantly higher frequencies of Gata3^{+} IL-13^{+} Th2 cells among *Atg16l1*-deficient CD45.2^{+} CD4^{+} T cells when compared to WT CD45.1^{+} CD4^{+} T cells (**Figure 30a-c**). Indeed, frequencies of IL-13^{+} *Atg16l1*-deficient CD4^{+} T cells in pTreg-reconstituted mice were comparable to those found in untreated *Atg16l1*$^{\Delta CD4}$ littermates (**Figure 30a**). In contrast, there was no difference in Th17 cell frequencies between *Atg16l1*-deficient CD45.2^{+} and WT CD4^{+} T cells, and there was a significant decrease in Th1 frequencies among *Atg16l1*-deficient CD4^{+} T cells (**Figure 30a,b**). Accumulation of Th2 cells was still largely limited to the intestinal lamina propria (**Figure 30c**). Thus, provision of WT pTreg cells did not ameliorate the increased Th2 phenotype of *Atg16l1*-deficient CD4^{+} T cells, suggesting that autophagy regulates Th2 cells through a cell-intrinsic mechanism. Consistent with this finding, *Atg16l1*$^{\Delta CD4}$ mice that were reconstituted with WT pTreg cells still developed intestinal pathology and elevated serum IgE levels that were comparable to those present in untreated *Atg16l1*$^{\Delta CD4}$ littermates (**Figure 31a-c**).

Figure 29 Efficient reconstitution of pTreg compartments in *Atg16l1*$^{\Delta CD4}$ mice by adoptive transfer with WT naïve CD4$^+$ T cells

Young (12 weeks) *Atg16l1*$^{\Delta CD4}$ mice (CD45.2$^+$) were adoptively transferred with 4-5x10^6 naïve WT CD4$^+$ T cells (CD45.1$^+$) by *i.v.* injection and analysed three months later. **(a)** Schematic representation of adoptive transfer. **(b)** Representative FACS plots showing Foxp3 expression by *Atg16l1*-deficient (CD45.2$^+$) and donor WT (CD45.2$^-$) CD4$^+$ T cells in spleen, mLN and cLP (gated on CD4$^+$ TCRβ^+ T cells). **(c)** Frequencies of WT (CD45.1$^+$) and *Atg16l1*-deficient (CD45.2$^+$) CD4$^+$ T cells in the spleen, mLN and cLP. **(d)** Frequencies of WT (CD45.1$^+$) and *Atg16l1*-deficient (CD45.2$^+$) Foxp3$^+$ Treg cells in the spleen, mLN and cLP (gated on CD4$^+$ TCRβ^+ T cells).

Data are representative of two independent experiments with at least 4 mice per group (b-d). Each dot represents the proportion of cells derived from either donor or host T cells within an individual recipient mouse and horizontal bars denote group means. Numbers indicate percentage of cells in quadrants. Statistical significance was determined using the Mann Whitney test, ** $p<0.01$; *** $p<0.001$.

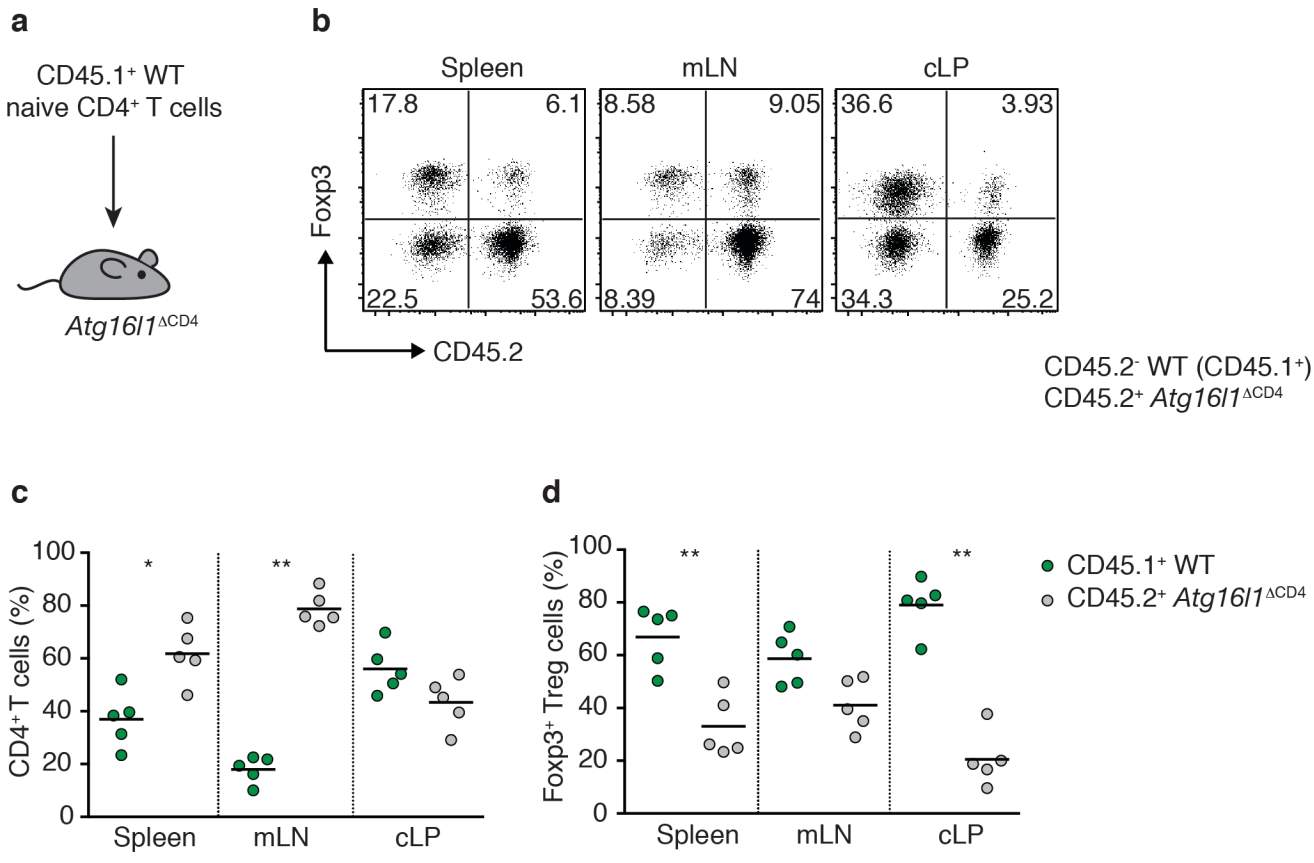

a
CD45.1+ WT
naive CD4+ T cells
Atg16l1ΔCD4
b
Spleen
mLN
cLP
Foxp3
CD45.2
17.8
6.1
22.5
53.6
8.58
9.05
8.39
74
36.6
3.93
34.3
25.2
CD45.2- WT (CD45.1+)
CD45.2+ Atg16l1ΔCD4
c
CD4+ T cells (%)
100
80
60
40
20
0
*
**
Spleen
mLN
cLP
d
Foxp3+ Treg cells (%)
100
80
60
40
20
0
**
**
Spleen
mLN
cLP
CD45.1+ WT
CD45.2+ Atg16l1ΔCD4

Figure 30 Autophagy regulates Th2 responses in a cell-intrinsic manner

Young (12 weeks) *Atg16l1*$^{\Delta CD4}$ mice (CD45.2$^+$) were adoptively transferred with 4-5x10^6 naïve WT CD4$^+$ T cells (CD45.1$^+$) by *i.v.* injection and analysed three months later. **(a)** Upper panel: representative FACS plots showing gating of WT (CD45.1$^+$) and *Atg16l1*-deficient (CD45.1$^-$) CD4$^+$ T cells and expression of IL-13 (Th2), IFN-γ (Th1) and IL-17A (Th17) in the cLP. Lower panels: cLP from control untreated *Atg16l1*$^{\Delta CD4}$ and *Atg16l1*$^{fl/fl}$ littermates are shown for comparison. All plots gated on CD4$^+$ TCRβ$^+$ Foxp3$^-$ T cells. **(b)** Frequencies of WT (CD45.1$^+$) and *Atg16l1*-deficient (CD45.2$^+$) Th2 (IL-13$^+$), Th1 (IFN-γ$^+$) and Th17 (IL-17A$^+$) cells in the cLP (gated on CD4$^+$ TCRβ$^+$ Foxp3$^-$ T cells). **(c)** Frequencies of WT (CD45.1$^+$) and *Atg16l1*-deficient (CD45.2$^+$) Gata3$^+$ CD4$^+$ T cells in the spleen, mLN and cLP (gated on CD4$^+$ TCRβ$^+$ Foxp3$^-$ T cells).

Data are representative of two independent experiments with at least 4 mice per group. Each dot represents the proportion of cells derived from either donor or host T cells within an individual recipient mouse and horizontal bars denote group means (b,c). Numbers indicate percentage of cells in gates (a). Statistical significance was determined using the Mann Whitney test, * $p<0.05$; ** $p<0.01$.

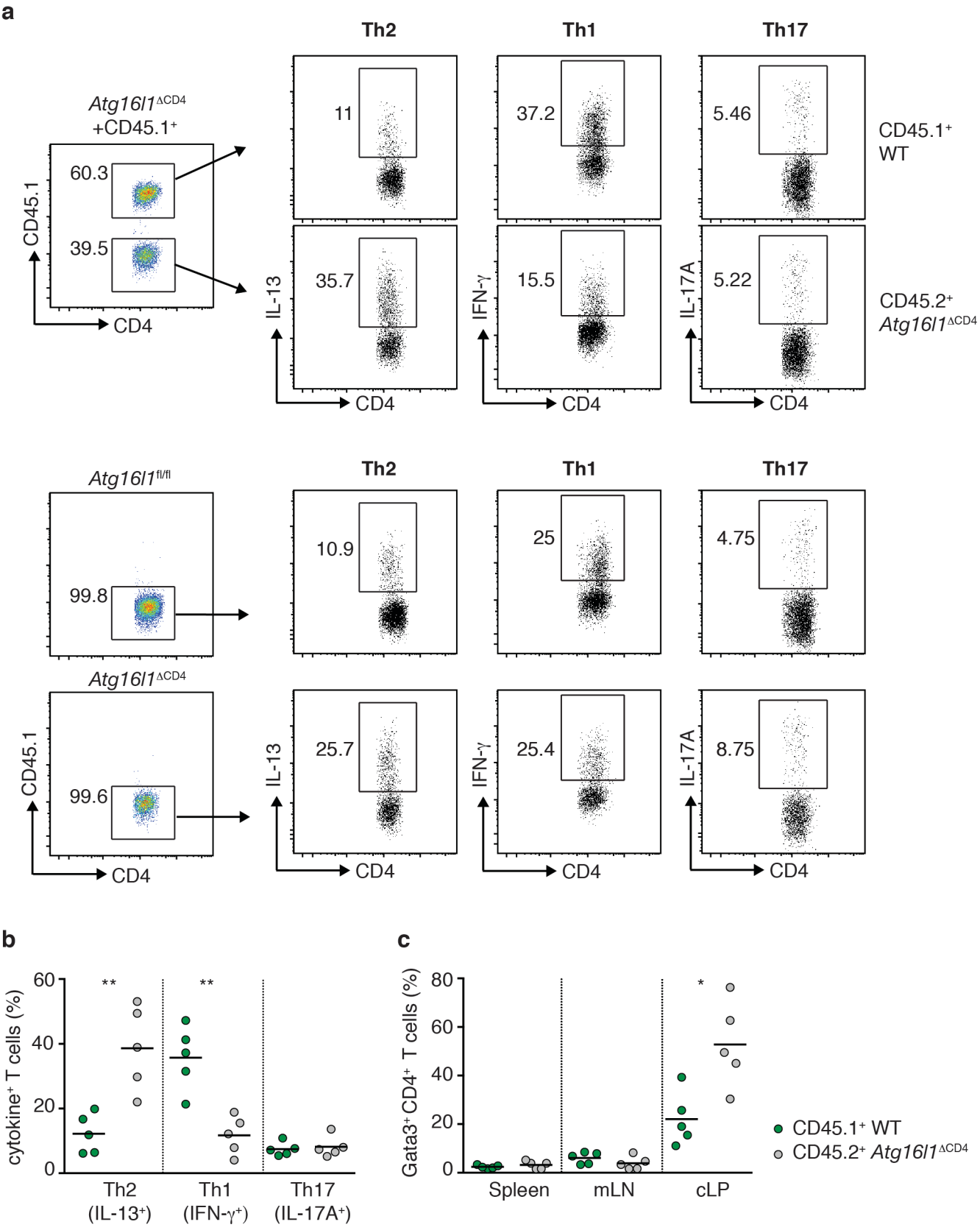
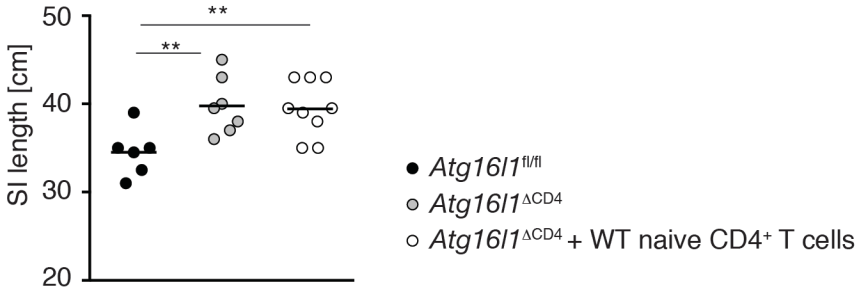

a
Th2
Th1
Th17
Atg16l1ΔCD4 +CD45.1+
60.3
39.5
CD45.1
CD4
11
37.2
5.46
CD45.1+ WT
IL-13
IFN-γ
IL-17A
35.7
15.5
5.22
CD45.2+ Atg16l1ΔCD4
Atg16l1fl/fl
99.8
10.9
25
4.75
Atg16l1ΔCD4
99.6
25.7
25.4
8.75
b
cytokine+ T cells (%)
60
40
20
0
**
**
Th2 (IL-13+)
Th1 (IFN-γ+)
Th17 (IL-17A+)
c
Gata3+CD4+ T cells (%)
80
60
40
20
0
*
Spleen
mLN
cLP
CD45.1+ WT
CD45.2+ Atg16l1ΔCD4

Figure 31 Reconstitution of *Atg16l1*$^{\Delta CD4}$ mice with WT pT$_{reg}$ cells does not prevent intestinal inflammation

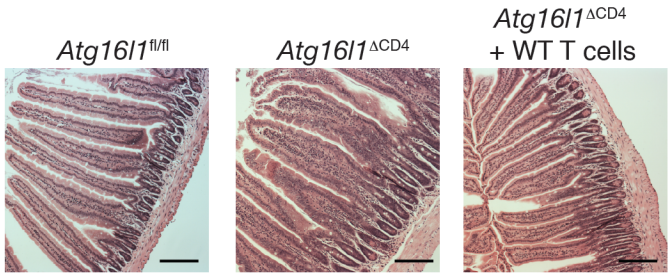
Young (12 weeks) *Atg16l1*$^{\Delta CD4}$ mice (CD45.2^{+}) were adoptively transferred with 4-5x10^{6} naïve WT CD4^{+} T cells (CD45.1^{+}) by i.v. injection and analysed three months later. **(a)** SI lengths, **(b)** representative photomicrographs of jejunum, and **(c)** serum IgE concentrations of control untreated *Atg16l1*$^{fl/fl}$ or *Atg16l1*$^{\Delta CD4}$ littermates and of reconstituted *Atg16l1*$^{\Delta CD4}$ mice (*Atg16l1*$^{\Delta CD4}$ + WT naïve CD4^{+} T cells), scale bar 150μm.

Data are representative of two independent experiments with at least 4 mice per group (b) or combined from two independent experiments (a,c). Each dot represents an individual recipient mouse and horizontal bars denote means (a,c). Numbers indicate percentage of cells in quadrants. Statistical significance was determined using the Mann Whitney test, ** $p<0.01$; *** $p<0.001$.

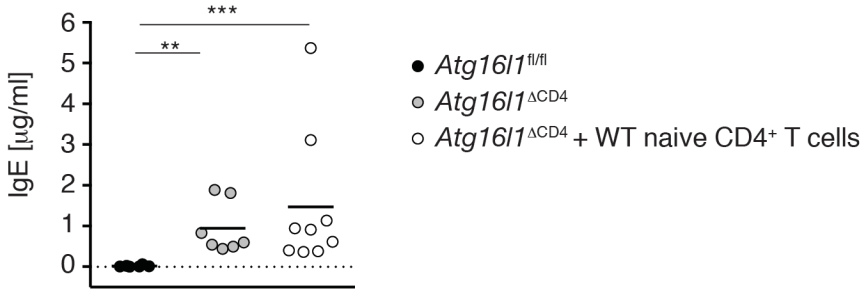
a


50
40
30
20
SI length [cm]
**
**
Atg16l1fl/fl
Atg16l1ΔCD4
Atg16l1ΔCD4 + WT naive CD4+ T cells

b


Atg16l1fl/fl
Atg16l1ΔCD4
Atg16l1ΔCD4
+ WT T cells

c


6
5
4
3
2
1
0
IgE [µg/ml]
***
**
Atg16l1fl/fl
Atg16l1ΔCD4
Atg16l1ΔCD4 + WT naive CD4+ T cells

5.3 Atg16l1 does not affect differentiation towards the Th2 or Treg lineage but has opposing effects on their survival

To further characterize the differential effects of autophagy on Th2 and Treg cells, we performed *in vitro* $CD4^+$ T cell differentiation assays. When stimulated under Th2 or Treg polarizing conditions, we observed comparable differentiation of naïve $CD4^+$ T cells isolated from *Atg16l1*$^{\Delta CD4}$ or *Atg16l1*$^{fl/fl}$ littermates towards the $Gata3^+$ Th2 or $Foxp3^+$ Treg cell phenotype (**Figure 32a-c**).

As Th2 cytokines can negatively affect Treg differentiation and stability (Dardalhon *et al.*, 2008; Feng *et al.*, 2014a), it was possible that instability of the Treg phenotype may have contributed to the loss of intestinal Treg in *Atg16l1*$^{\Delta CD4}$ mice. We therefore isolated $Foxp3^+$ Treg cells from *Atg16l1*$^{\Delta CD4}$ and *Atg16l1*$^{fl/fl}$ littermates, activated them for 48h with anti-CD3 and anti-CD28 antibodies and then cultured them for a further 5 days in the presence of IL-4 and IL-13. We saw a marked decrease in the survival of *Atg16l1*-deficient Treg cells during prolonged *in vitro* culture (**Figure 32d**). However, we did not find any evidence of Treg instability, as expression of Foxp3 and CD25 remained equally high in *Atg16l1*-deficient and WT Treg cells (**Figure 32d**). Similarly, expression of Foxp3 and CD25 was unaltered in *Atg16l1*-deficient Treg cells that were cultured without addition of Th2 cytokines (data not shown).

As we observed marked decrease in the *Atg16l1*-deficient Treg survival during prolonged *in vitro* culture (**Figure 32d**), we further examined how autophagy deficiency influenced the survival of $Foxp3^+$ Treg cells versus Th2 cells. Thus, naïve $CD4^+$ T cells isolated from *Atg16l1*$^{\Delta CD4}$ or *Atg16l1*$^{fl/fl}$ littermates were activated for 48h with anti-CD3 and anti-CD28 antibodies and then rested for 5 days. Cells were kept in Treg or Th2 polarizing conditions throughout the experiment. Following activation

with anti-CD3 antibody, Atg16l1-deficient Th2 cells exhibited comparable or increased survival to WT Th2 cells (**Figure 33a,b**). In contrast, there was a 50-75% decrease in survival of Atg16l1-deficient Treg cells when compared to Atg16l1-sufficient Treg cells (**Figure 33a,b**).

To establish if we could detect similar differences in the survival of Treg and Th2 cells *in vivo,* $CD4^+$ T cells isolated from cLP of young *Atg16l1*$^{\Delta CD4}$ or *Atg16l1*$^{fl/fl}$ littermates were stained with a viability dye, as well as Annexin V to detect apoptotic cells. In line with the *in vitro* culture results, increased proportions of *Atg16l1*-deficient intestinal Treg cells exhibited an apoptotic or dead phenotype when compared to WT Treg cells (**Figure 33c**). In contrast, *Atg16l1*-deficiency had no effect on the survival of intestinal Th2 cells, which were comparably healthy to those present in the cLP of WT controls (**Figure 33c**). Thus, autophagy differentially regulates the survival of mucosal Treg and Th2 cells *in vivo*. In addition to their impaired survival phenotype, Treg cells from the cLP of young *Atg16l1*$^{\Delta CD4}$ mice showed decreased expression of $CD25^+$, $CD69^+$ and $KLRG1^+$ when compared to cLP Treg cells from *Atg16l1*$^{fl/fl}$ littermates (**Figure 33d**). As high expression of CD25, CD69 and KLRG-1 is associated with an activated phenotype, and KLRG-1 has been proposed as a marker of highly activated, terminally differentiated Treg cells (Cheng *et al.*, 2012), this result suggests that autophagy is particularly important for the survival of highly activated Treg cells in the intestinal lamina propria.

Previous studies on autophagy deficient T cells indicated changes in the expression levels of pro- and anti-apoptotic proteins, although the relation between autophagy and apoptosis in T cells remains unclear (He *et al.*, 2012). To verify if decreased survival of *Atg16l1*-deficient cLP T_{reg} cells was associated with increased expression of pro-apoptotic genes we took advantage of Fluidigm gene expression analysis (Biomark Fluidigm). This system allows to measure changes in gene expression using very small cell populations (200 cells in our case) and expression levels of up to 48

distinct genes can be verified during one assay. This was important in our study, as the cell number of *Atg16l1*-deficient Treg cells that could be sorted from the intestinal lamina propria was a limiting factor. Cells were sorted from the colon of *Atg16l1*$^{\Delta Foxp3}$ and Foxp3-YFP-Cre mice, as this allowed us to sort purify Treg cells based on YFP expression, which corresponds to Foxp3 expression in these cells. Autophagy deficient Treg cells did not show increase in pro-apoptotic gene expression (*Bim*, *Bax*) or decrease expression of anti-apoptotic Bcl-*2* (**Figure 34a**) compared to control Treg cells. In fact, expression of Bcl-2 was slightly increased in *Atg16l1*-deficient Treg cells (**Figure 34b,c**). However, further analysis of apoptotic pathways in *Atg16l1*-deficient Treg cells would be useful, as another anti-apoptotic protein Mcl-1 was recently shown to specifically regulate apoptosis in peripheral Treg cells (Pierson *et al.*, 2013).

As tissue resident Treg cell populations have been reported to exhibit specialized metabolic adaptations (Burzyn *et al.*, 2013a), we compared the expression of metabolic genes by WT and *Atg16l1*-deficient intestinal Treg cells. For that we again used the Fluidigm gene expression system, as described above. Analyses of genes involved in glycolysis, fatty acid synthesis (FAS) and fatty acid oxidation (FAO) revealed that *Atg16l1*-deficient Treg cells had markedly higher expression of many glycolytic genes, including *Glut1, Slc16ac* (*MCT4*), *Tpi1*, *LDH-*α and *Aldo-*α, *Pgk1* and *Gpi1*, than WT Treg cells (**Figure 35a**). Strikingly, this augmented glycolytic signature was much more pronounced in *Atg16l1*-deficient Treg cells isolated from cLP versus those from the spleen (**Figure 35a**).

Conversely, expression of many key genes involved in FAS/FAO, including *Acc1, Acc2, Srebf1, Srebf2, Fabp1, Fabp4*, *Fabp5* and *Fdft1*, was markedly decreased in *Atg16l1*-deficient Treg cells (**Figure 35b**). Again, these differences were most pronounced in the intestine; WT cLP Treg cells showed increased FAS/FAO gene expression compared to their spleen counterparts, whereas *Atg16l1*-deficient cLP Treg cells

were not able to up-regulate the expression of FAS/FAO genes (**Figure 35b**). Thus, *Atg16l1*-deficiency dramatically changed the metabolic profile of intestinal Treg cells, with an altered balance of glycolytic and FAS/FAO gene expression.

Together, these results suggest that *Atg16l1*-deficiency does not impair the differentiation or stability of Treg cells *in vitro* and does not promote differentiation towards the Th2 lineage. However, autophagy-deficient Treg cells exhibited impaired survival compared to their WT counterparts, whereas autophagy-deficient Th2 cells had no survival defect. Decreased survival of *Atg16l1*-deficient Treg cells was associated with an altered metabolic profile, suggesting that autophagy plays an integral role in facilitating the metabolic adaptations required for long-term Treg cell survival in the intestine.

Figure 32 Normal differentiation towards Treg and Th2 phenotypes by *Atg16l1*-deficient CD4$^+$ T cells

(a,b) Sorted splenic naïve CD4$^+$ T cells from *Atg16l1*$^{\Delta CD4}$ or *Atg16l1*$^{fl/fl}$ mice were cultured in Th0, Treg, or Th2 polarizing conditions for 48h and analysed by FACS. Representative FACS plots show **(a)** Foxp3 and **(b)** Gata3 expression (gated on CD4$^+$ TCRβ^+ T cells). **(c)** Frequencies of Treg cells (Foxp3$^+$) and Th2 cells (Gata3$^+$) arising from *Atg16l1*$^{\Delta CD4}$ or *Atg16l1*$^{fl/fl}$ naïve CD4$^+$ T cells cultured in Treg or Th2 polarizing conditions for 5 days. **(d)** *Atg16l1*$^{\Delta CD4}$ or *Atg16l1*$^{fl/fl}$ Treg cells were cultured with anti-CD3 (3μg/ml) and anti-CD28 (1μg/ml) for 48h, then maintained in the presence of IL-4 and IL-13 for further 5 days before FACS analysis. Histograms of live/dead staining of CD4$^+$ T cells showing gate and frequency of live cells (left panel) and FACS plots of Foxp3 and CD25 expression by live CD4$^+$ T cells (right panels).

Data are representative from 2 (d) or 3 independent experiments (a,b) with two technical replicates, or are combined from three independent experiments (c). Annotations represent percentages of cells in the indicated gates (mean ± s.e.m), or each dot represents an individual cell culture (c).

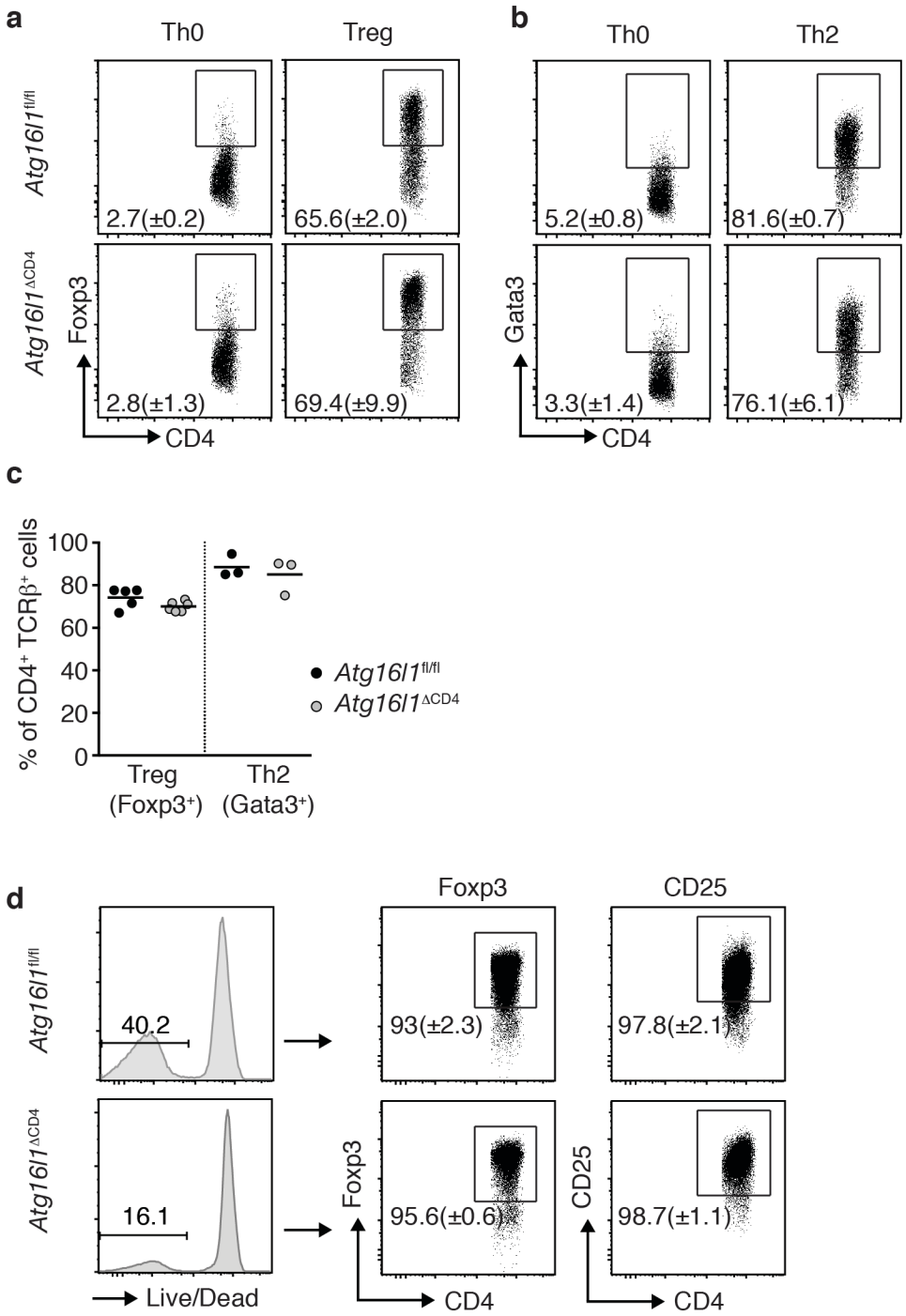

a
Th0
Treg
Atg16l1fl/fl
Atg16l1ΔCD4
2.7(±0.2)
65.6(±2.0)
2.8(±1.3)
69.4(±9.9)
Foxp3
CD4
b
Th0
Th2
5.2(±0.8)
81.6(±0.7)
3.3(±1.4)
76.1(±6.1)
Gata3
CD4
c
% of CD4+ TCRβ+ cells
100
80
60
40
20
0
Atg16l1fl/fl
Atg16l1ΔCD4
Treg
(Foxp3+)
Th2
(Gata3+)
d
Atg16l1fl/fl
Atg16l1ΔCD4
40.2
16.1
Live/Dead
Foxp3
93(±2.3)
95.6(±0.6)
CD4
CD25
97.8(±2.1)
98.7(±1.1)
CD4

Figure 33 *Atg16l1* promotes survival of Treg cells but limits Th2 cell survival

(**a,b**) Sorted splenic naïve $CD4^+$ T cells from *Atg16l1*$^{\Delta CD4}$ or *Atg16l1*$^{fl/fl}$ mice were cultured with **(a)** 1μg/ml or **(b)** 5μg/ml anti-CD3 plus anti-CD28 (1μg/ml) for 48h in Treg or Th2 polarizing conditions, then maintained in polarizing conditions for a further 5 days before FACS analysis. Histograms show gates and frequencies of live $CD4^+$ T cells. **(c)** Representative FACS plots of viability dye and Annexin V staining of $Foxp3^+$ Treg cells and $Gata3^+$ Th2 cells from the cLP of young (8-12 weeks) *Atg16l1*$^{\Delta CD4}$ and *Atg16l1*$^{fl/fl}$ littermates (gated on $Foxp3^+$ $CD4^+$ $TCR\beta^+$ T cells). (**d**) Expression of CD25, CD69 and KLRG1 by cLP $Foxp3^+$ Treg cells from young *Atg16l1*$^{\Delta CD4}$ and *Atg16l1*$^{fl/fl}$ littermates (gated on $Foxp3^+$ $CD4^+$ $TCR\beta^+$ T cells).

Data are representative from 2 independent experiments with at least two technical replicates (a,b) or at least 4 mice per group (c,d). Annotations represent percentages of cells in the indicated gates (mean ± s.e.m).

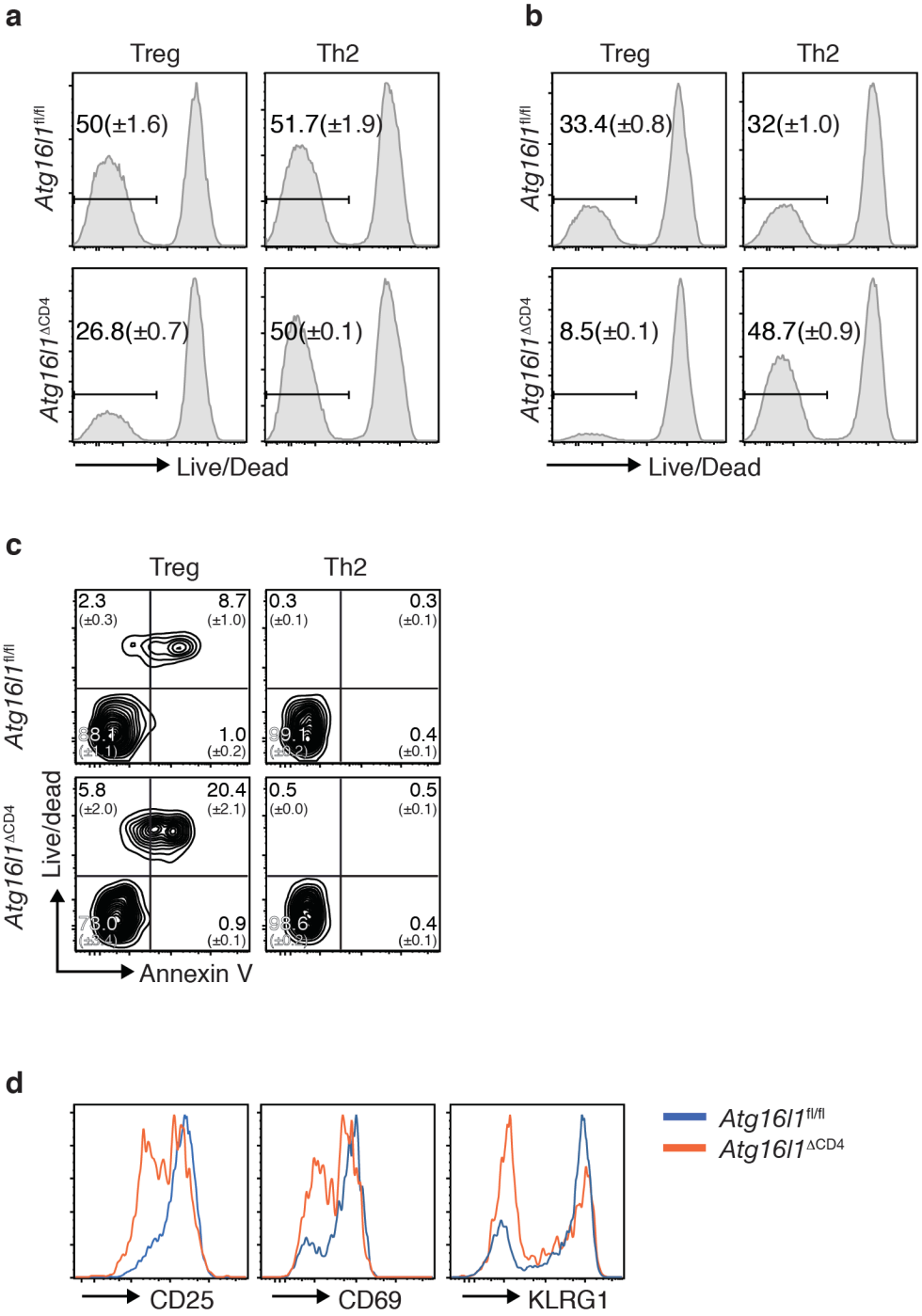

a
Treg
Th2
Atg16l1fl/fl
50(±1.6)
51.7(±1.9)
Atg16l1ΔCD4
26.8(±0.7)
50(±0.1)
Live/Dead
b
Treg
Th2
Atg16l1fl/fl
33.4(±0.8)
32(±1.0)
Atg16l1ΔCD4
8.5(±0.1)
48.7(±0.9)
Live/Dead
c
Treg
Th2
Atg16l1fl/fl
2.3 (±0.3)
8.7 (±1.0)
0.3 (±0.1)
0.3 (±0.1)
1.0 (±0.2)
0.4 (±0.1)
Atg16l1ΔCD4
5.8 (±2.0)
20.4 (±2.1)
0.5 (±0.0)
0.5 (±0.1)
0.9 (±0.1)
0.4 (±0.1)
Live/dead
Annexin V
d
CD25
CD69
KLRG1
Atg16l1fl/fl
Atg16l1ΔCD4

Figure 34 *Atg16l1*-deficiency does not increase pro-apoptotic gene expression in Treg cells

(**a**) Treg cells were sorted as YFP$^+$ CD4$^+$ TCRß$^+$ cells from the spleen and cLP of young (8-12 weeks) *Atg16l1*$^{\Delta Foxp3\text{-}YFP}$ and Foxp3-YFP-Cre mice and expression of *Bcl-2*, *Bim* and *Bax* genes was analysed by Fluidigm gene expression system, as described in the Material and Methods section. (**b,c**) Quantification (**b**), and representative FACS staining (**c**), of Bcl-2 expression in Foxp3$^+$ Treg cells from cLP of young (8-12weeks) *Atg16l1*$^{\Delta Foxp3}$ and *Atg16l1*$^{fl/fl}$ littermates (gated on Foxp3$^+$ CD4$^+$ TCRβ^+ T cells).

Data are from one experiment and represent means ± s.e.m, n=3 (a), or each dot represents an individual mouse and horizontal bars denote means (b). Statistical significance was determined using the Mann Whitney test (b) ** $p<0.01$.

Analysis in (a) was done in collaboration with Dr Katja Simon and Thomas Riffelmacher (University of Oxford).

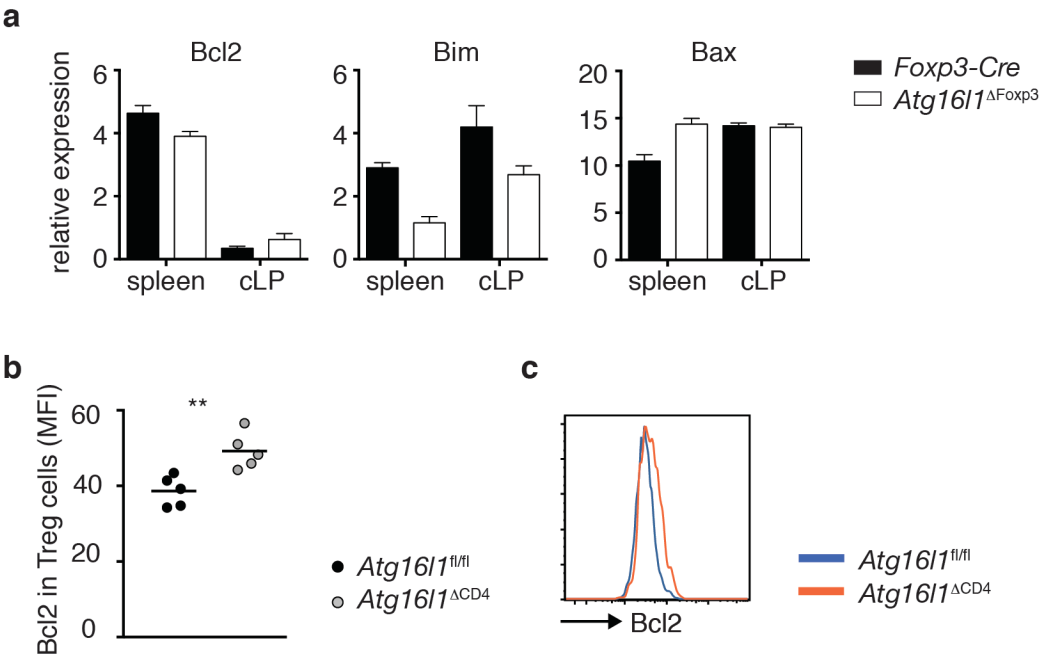

a
Bcl2
Bim
Bax
relative expression
6
4
2
0
20
15
10
5
spleen
cLP
Foxp3-Cre
Atg16l1ΔFoxp3
b
Bcl2 in Treg cells (MFI)
60
40
20
0
**
Atg16l1fl/fl
Atg16l1ΔCD4
c
Bcl2
Atg16l1fl/fl
Atg16l1ΔCD4

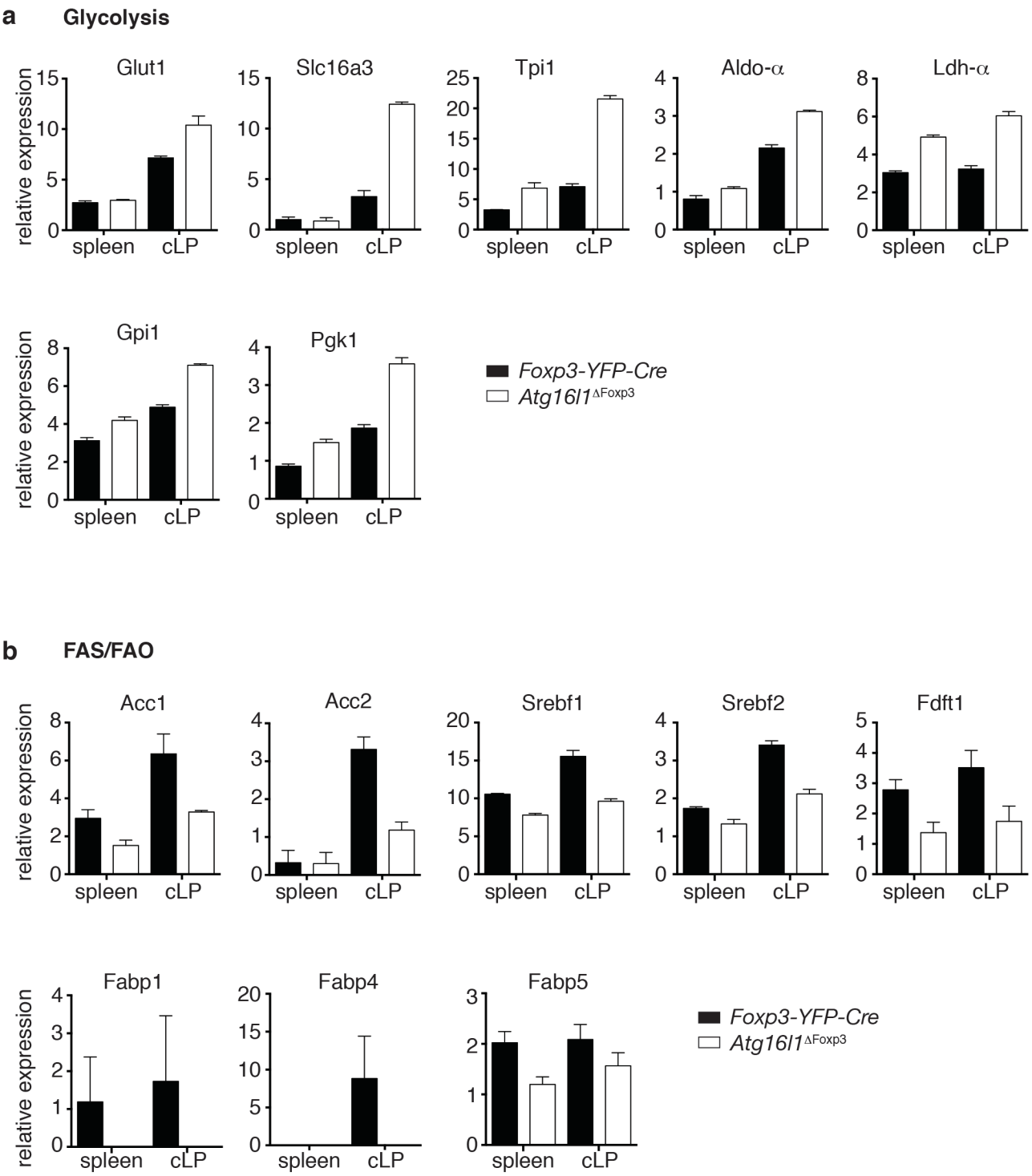
Figure 35 *Atg16l1*-deficiency alters the metabolic profile of colonic Treg cells

Treg cells were sorted as YFP^+ $CD4^+$ $TCR\beta^+$ cells from the spleen and cLP of young (8-12 weeks) *Atg16l1*$^{\Delta Foxp3\text{-}YFP}$ and Foxp3-YFP-Cre mice and expression of genes involved in glycolysis, fatty acid synthesis (FAS) and fatty acid oxidation (FAO) was analysed by Fluidigm gene expression system, as described in the Material and Methods section. **(a)** Analyses of glycolytic gene expression levels. **(b)** Analyses of FAS and FAO gene expression levels.

Glut1: glucose transporter 1, Slc16ac: solute carrier family 16 member 3 (lactic acid and pyruvate transporter), Tpi1: triosephosphate isomerase 1, Aldo -α: aldolase α, Ldh- α: lactate dehydrogenase α, Gpi1: glucose phosphate isomerase 1, Pgk1: phosphoglycerate kinase 1, Acc1: acetyl-CoA carboxylase 1, Acc2: acetyl-CoA carboxylase 2, Srebf1: sterol regulatory element binding transcription factor 1, Srebf2: sterol regulatory element binding transcription factor 2. Fdft1: farnesyl-diphosphate farnesyltransferase 1, Fabp1: fatty acid-binding protein 1, Fabp4: fatty acid-binding protein 4, Fabp1: fatty acid-binding protein 5.

Data are representative of two independent Fluidigm experiments, performed as described in the Material and Methods section. Data are shown as mean ± s.e.m. of three technical replicates. Data were analysed using the $2^{-\Delta Ct}$ method and results were normalized to actin or HPRT expression.

Analysis in (a) and (b) was done in collaboration with Dr Katja Simon and Thomas Riffelmacher (University of Oxford).


a
Glycolysis
Glut1
Slc16a3
Tpi1
Aldo-α
Ldh-α
Gpi1
Pgk1
relative expression
spleen
cLP
Foxp3-YFP-Cre
Atg16l1ΔFoxp3
b
FAS/FAO
Acc1
Acc2
Srebf1
Srebf2
Fdft1
Fabp1
Fabp4
Fabp5
relative expression
spleen
cLP
Foxp3-YFP-Cre
Atg16l1ΔFoxp3

5.4 Discussion

5.4.1 Role of autophagy for intestinal Treg cells

Our data suggest that autophagy is dispensable for the differentiation of Treg cells, as, when cultured under Treg polarising conditions *in vitro*, *Atg16l1*-deficient $CD4^+$ T cells were as capable of differentiating into $Foxp3^+$ T cells as WT $CD4^+$ T cells. This is consistent with our finding that thymic generation of Treg cells was not impaired in either *Atg16l1*$^{\Delta CD4}$ or *Atg16l1*$^{\Delta Foxp3}$ mice. However, additional experiments are needed to establish if autophagy deficiency does not affect the lineage commitment into Treg cells in sub-optimal conditions (for instance, with lower concentration of IL-2 or TGF-$ß_1$). *Atg16l1*-deficiency did not appear to affect Treg cell stability *in vitro*, however we observed increased Th effector cytokine production by *Atg16l1*-deficient intestinal Treg cells, therefore phenotypic instability *in vivo* in the absence of autophagy remains a possibility that requires further investigation.

An alternative explanation for the decrease numbers of Treg cells in the lamina propria of *Atg16l1*$^{\Delta CD4}$ and *Atg16l1*$^{\Delta Foxp3}$ mice arises from our *in vitro* experiments showing that *Atg16l1*-deficient Treg cells exhibited significantly decreased survival after activation when compared to WT Treg cells. The same trend was observed *in vivo,* as Treg cells isolated from the cLP of *Atg16l1*$^{\Delta CD4}$ mice also showed increased levels of apoptosis and cell death. As Treg cells are known to exhibit a highly activated phenotype within the intestinal environment (Izcue *et al.*, 2009; Harrison and Powrie, 2013), the decreased numbers of intestinal Treg cells present in *Atg16l1*$^{\Delta CD4}$ and *Atg16l1*$^{\Delta Foxp3}$ mice may reflect impaired survival of *Atg16l1*-deficient Treg cells upon antigen driven activation. This is consistent with our observation that the impaired survival of *Atg16l1*-deficient intestinal Treg cells correlated with decreased expression of the Treg cell activation markers CD25 and CD69 and the

terminal differentiation marker KLRG1. Additional evidence for autophagy playing an essential role in Treg cell survival was presented in the previous chapter, where we found that WT Foxp3$^+$ Treg cells contained more LC3$^+$ autophagosomes than conventional CD4$^+$ T cells, both during steady state and following TCR activation.

The molecular mechanisms through which autophagy enhances survival of Treg cells remain to be completely elucidated. However, based on recent evidence that Treg cells rely on a distinct metabolic program compared to other effector CD4$^+$ T cells (MacIver *et al.*, 2013), one attractive hypothesis is that autophagy is critical for regulating Treg metabolism and thereby their survival. Indeed, we found that *Atg16l1*-deficient Treg cells expressed a distinct metabolic profile to their WT counterparts, exhibiting increased expression of genes involved in glycolysis and reduced expression of genes involved in FAS/FAO. Fatty acid metabolism is emerging as a potent regulator of T cell responses; utilization of fatty acids as a fuel source seems to be a characteristic of long-lived T cells, such as CD8$^+$ memory T cells (Pearce *et al.*, 2009; O'Sullivan *et al.*, 2014), and preferential utilization of FAO has been linked to Treg cell induction (Lochner *et al.*, 2015). Furthermore, autophagy has been implicated in the regulation of fatty acid metabolism (Singh *et al.*, 2009; Liu and Czaja, 2013; Lizaso *et al.*, 2013; Kaur and Debnath, 2015) and recent studies found that autophagy plays a key role in the generation of CD8$^+$ memory T cells (Puleston *et al.*, 2014; Xu *et al.*, 2014), which are heavily dependent on FAO for survival (Pearce *et al.*, 2009; O'Sullivan *et al.*, 2014). Thus, it is tempting to speculate that autophagy could play a similar survival role in Treg cells, perhaps by facilitating the degradation of intracellular lipid stores to release FA that fuel FAO. Additionally, degrading excessive intracellular lipids by autophagy is a part of protective mechanisms to avoid lipotoxicity (Galluzzi *et al.*, 2014), therefore defective autophagy could lead to toxic build up of intracellular lipids in Treg cells.

It is interesting that genes that are downregulated in *Atg16l1*-deficient Treg cells, such as *Acc1, Acc2* and *Srebf1*, are primarily engaged in lipid biosynthesis. Lipid biosynthesis driven by SREBP was shown to be critical for effector $CD8^+$ T cell expansion (Kidani *et al.*, 2013). While a recent report indicated that *de novo* FAS was not required for $Foxp3^+$ Treg cell differentiation (Berod *et al.*, 2014) optimal *in vivo* Treg cell function was associated with intrinsic lipid synthesis (Zeng *et al.*, 2013). Therefore, the role of FAS in Treg development and function is not entirely clear and might be related to the availability of lipids in the surrounding environment (Buck *et al.*, 2015). It is important to note that whether decreased FAS gene expression actually reflects reduced FAS by *Atg16l1*-deficient Treg cells remains to be established. Indeed, a distinct scenario could be imagined where excessive biosynthesis of lipids led to a negative feedback response that decreased expression of FAS genes. In addition, the balance between FAS and FAO is regulated by signalling through the mTORC1 and AMPK pathways, and this point will be discussed in more detail in Chapter 6.

The increased glycolytic profile of *Atg16l1*-deficient Treg cells further emphasizes their dysregulated metabolic state and indeed, increased glycolysis when autophagy is impaired appears to be a general trend in immune cells (Stranks *et al.*, 2015). This is also in line with our previous results from the analysis of metabolic profiles in total $CD4^+$ T cells, where levels of glycolysis were high in *Atg16l1*-deficient $CD4^+$ T cells activated *in vitro*, but OXPHOS was undetectable (**Figure 6g**). Increased aerobic glycolysis is observed in activated and proliferating T cells (MacIver *et al.*, 2013; Buck *et al.*, 2015), which may be partially explained by the increased requirements for metabolic intermediates used as precursors for nucleotides or amino acid synthesis (Vander Heiden *et al.*, 2009; Wang *et al.*, 2011a). Therefore, it could be speculated that glycolysis might provide a compensatory mechanism to generate building blocks that would otherwise be provided by recycling through autophagy. On

the other hand, the imbalance between glycolysis and FAS/FAO observed in autophagy-deficient Treg cells could indicate that these cells have stalled in the activated/effector state and are unable to make the metabolic adaptations necessary for long-term survival. This is supported by our data showing that although a higher proportion of *Atg16l1*-deficient Treg cells appeared to be in cell cycle, they had reduced expression of terminal differentiation markers.

It is striking that autophagy deficiency had a more detrimental effect on intestinal Treg cells than on those found in secondary lymphoid organs. Recent evidence suggests that tissue-resident Treg cells undergo tissue-specific adaptations, and metabolic changes are emerging as an important part of such tissue specific reprogramming (Burzyn *et al.*, 2013a; Liston and Gray, 2014). For instance, transcription factor PPARγ expressed by visceral adipose tissue (VAT) Treg cells upregulates the expression of low-density lipoprotein transporter CD36, which suggest that VAT Treg cells co-opt local metabolic program for their function in the adipose tissue (Cipolletta *et al.*, 2012; Zeng and Chi, 2015). In terms of the gut environment, it is also interesting that metabolites present in the intestine can affect Treg cell development and function. Recently SCFA produced by commensal bacteria were shown to facilitate pTreg induction and to increase proliferation of Treg cells already present in the intestine (Arpaia *et al.*, 2013; Atarashi *et al.*, 2013; Furusawa *et al.*, 2013; Smith *et al.*, 2013). This effect was dependent on the expression of the free fatty acid receptor 2 (FFAR2, also known as GPR43) (Smith *et al.*, 2013). The mechanism through which SFCA influence intestinal Treg cells was attributed to regulation of Foxp3 expression by acetylation of the *Foxp3* locus (Arpaia *et al.*, 2013; Furusawa *et al.*, 2013). However, it is tempting to speculate that some of the beneficial effects of SCFA on intestinal Treg cells could be due to SCFA acting as a metabolic fuel, consistent with our observations that intestinal Treg might be more dependent on FAO metabolism compared to lymphoid Treg cells. Taken

together, we hypothesize that autophagy endows intestinal Treg cells with the metabolic flexibility required to survive in the gut tissue, where essential growth factors may be in short supply (Pearce *et al.*, 2013).

5.4.2 Regulation of Th2 cells by autophagy

By employing various complementary experimental approaches, we established that the enhanced Th2 phenotype in *Atg16l1*$^{\Delta CD4}$ mice arises due to two distinctive mechanisms; a cell-intrinsic effect of *Atg16l1*-deletion on Th2 cell survival and accumulation; and impaired Treg control due to the loss of autophagy-deficient Treg cells.

A link between defective Treg homeostasis and increased Th2 responses in the mucosa has previously been described (Mucida *et al.*, 2005; Curotto de Lafaille *et al.*, 2008; Zheng *et al.*, 2009; Josefowicz *et al.*, 2012b; Ulges *et al.*, 2015). While it is clear that a compromised Treg compartment can result in exacerbated Th2 responses, the mechanism through which Treg cells selectively inhibit mucosal Th2 cells is not well understood. The pleiotropic transcription factor IRF4 was initially implicated in differentiation of Treg cells that have the ability to supress Th2 cells (Zheng *et al.*, 2009). As such, it has been proposed that Treg cells specialize by engaging a transcriptional program corresponding to the Th subsets they antagonize; similar to IRF4 being important for Treg cells to control Th2 cells, T-bet and Stat3 expression by Treg cells was shown to facilitate suppression of Th1 and Th17 cells, respectively (Chaudhry *et al.*, 2009; Koch *et al.*, 2009). However, we found that *Atg16l1*-deficent Treg cells did not show impaired IRF4 expression; on the contrary, we saw significantly increased IRF4 expression in *Atg16l1*-deficient Treg cells. Upregulation of IRF4 expression could be due to increased pressure on Treg cells to control enhanced Th2 responses in *Atg16l1*$^{\Delta Foxp3}$ mice. However, other studies suggest

that IRF4 has a more complex role in Treg homeostasis, as it has been implicated in differentiation of effector Treg cells and in tissue-specific adaptations (Cretney *et al.*, 2011; Vasanthakumar *et al.*, 2015). Interestingly, IRF4 is induced upon TCR activation and plays a role in sustaining high levels of glycolysis in activated $CD8^+$ T cells (Man *et al.*, 2013). Thus, the functional relevance of increased IRF4 expression in *Atg16l1*-deficient Treg cells remains to be further investigated.

A recent study provided new insight into Treg-mediated suppression of Th2 cells (Ulges *et al.*, 2015). This group found that protein kinase CK2 was preferentially expressed by Treg cells upon activation and was necessary to control Th2 cell responses in the lungs, by regulating expression of the inhibitory receptor ILT3 on Treg cells (Ulges *et al.*, 2015). Whether autophagy-deficiency has any effect the CK2 kinase activity and ILT3 expression in Treg cells remains to be established.

Studies on functional specialization of tTreg and pTreg cells indicated that pTreg cells are critical in limiting mucosal Th2 responses and allergic inflammation (Curotto de Lafaille *et al.*, 2008; Josefowicz *et al.*, 2012b), however precise reasons why pTreg are more effective in suppressing Th2 cells are still not well understood (Josefowicz *et al.*, 2012b). In our experiments, we showed that reconstitution of *Atg16l1*$^{\Delta CD4}$ mice with WT pTreg cells was not sufficient to correct the enhanced Th2 responses. Of note, we also attempted to adoptively transfer *Atg16l1*$^{\Delta CD4}$ mice with WT tTreg cells, with the same result: WT tTreg cell were unable to prevent the exacerbated Th2 responses in *Atg16l1*$^{\Delta CD4}$ mice (data not shown). However, these experiments were technically challenging because it was very difficult to sort sufficient numbers of tTreg cells to achieve the same level of reconstitution in *Atg16l1*$^{\Delta CD4}$ mice as obtained with the WT pTreg cell experiments. Nevertheless, these results point towards a cell-intrinsic mechanism that allows autophagy-deficient Th2 cells to preferentially expand in the mucosa.

Interestingly, analysis of humoral responses in *Atg16l1*$^{\Delta Foxp3}$ mice revealed that although some dysregulated Th2-associated antibody responses observed in *Atg16l1*$^{\Delta CD4}$ mice were recapitulated when autophagy was selectively blocked in Treg cells, including increased IgE levels, others, such as IgG_1 responses, were not observed in *Atg16l1*$^{\Delta Foxp3}$ mice. This further supports the notion that the full phenotype of enhanced Th2 response in *Atg16l1*$^{\Delta CD4}$ mice is not only a consequence of impaired Treg cell function. Hyper IgE syndrome was previously observed in mice with defective Treg cell compartment (Lin *et al.*, 2005; Wing and Sakaguchi, 2014), and in line with these observations we also detected increase in IgE levels in Atg16l1$^{\Delta Foxp3}$ mice. This could indicate that a proportion of total IgE in the serum of Atg16l1$^{\Delta CD4}$ mice arises solely due to Treg cell defect in these mice. Furthermore, while *Atg16l1*$^{\Delta Foxp3}$ mice mounted similar IgA responses towards food and microbiota antigens as found in *Atg16l1*$^{\Delta CD4}$ mice, the IgG_1 response towards these luminal antigens was not conserved. Treg cells are known to control GC responses; in particular a Bcl-6-expressing subset of Foxp3$^+$ Treg cells, known as T follicular regulatory (Tfr) cells, was shown to be a crucial regulator of GC reactions (Chung *et al.*, 2011; Linterman *et al.*, 2011; Wollenberg *et al.*, 2011) and have recently been implicated in regulating IgA responses to microbiota antigens (Kawamoto *et al.*, 2014). Although we have not looked at Tfr cells in our experiments, it is likely that this Foxp3-expressing cell subset is also affected by the lack of autophagy in *Atg16l1*$^{\Delta Foxp3}$ mice and this could potentially contribute to dysregulated IgA responses towards luminal antigens.

In summary, we revealed a novel influence of autophagy on the Treg – Th2 regulatory axis. We found that Treg-specific deletion of *Atg16l1* in *Atg16l1*$^{\Delta Foxp3}$ mice led to increase in Th2 responses in the lamina propria and elevated serum IgE levels. The magnitude of these prototypic Th2 responses were less pronounced and arose later in *Atg16l1*$^{\Delta Foxp3}$ mice compared to *Atg16l1*$^{\Delta CD4}$ mice, emphasizing that both cell-intrinsic

and extrinsic mechanisms contributed to elevated mucosal Th2 responses when autophagy was blocked in $CD4^+$ T cells. Indeed, based on the evidence that increase in the frequencies in Th2 cells appears at the later stages of inflammation in *Atg16l1*$^{\Delta Foxp3}$ mice, it could be hypothesized that lack of sufficient Treg control over Th2 effector response could be a secondary event in *Atg16l1*$^{\Delta CD4}$ mice, where the initial increase in Th2 cells arises predominantly due to a cell-intrinsic effect of autophagy blockage in Th2 cells and is then further enhanced by the compromised suppression from autophagy deficient Treg cells.

How does autophagy intrinsically regulate Th2 cells? Although *Atg16l1*$^{\Delta CD4}$ mice contained equivalent frequencies of intestinal Th1 and Th17 cells as their WT counterparts, the T cell lymphopaenia in these mice meant that absolute numbers of these effector populations were significantly reduced. In contrast, we found that *Atg16l1*-deficiency selectively promoted expansion of intestinal Th2 cells *in vivo*, in a cell-intrinsic manner. The chronic accumulation of autophagy-deficient Th2 cells indicates that an alternative mechanism must account for their long-term survival.

We observed comparable or increased survival of *Atg16l1*-deficient $CD4^+$ T cells when maintained under Th2 polarizing conditions *in vitro*, confirming that the differentiation and survival of Th2 cells was independent of autophagy. These findings are consistent with a previous study which reported enhanced survival of Th2 cell line *in vitro* when autophagy was inhibited, and suggested that autophagy mediated death in Th2 cell line during growth-factor withdrawal (Li *et al.*, 2006). However, the same group more recently reported that genetic deletion of the autophagy inducer *Beclin-1* favoured Th17 cell survival over Th1 and Th2 cells during *in vitro* polarisation assay (Kovacs *et al.*, 2012). Earlier studies on cell death in $CD4^+$ T cell subsets reported differential apoptosis levels in Th1 and Th2 cells upon restimulation with antigen, where Th1, but not Th2 cells, undergo Fas/FasL –

mediated apoptosis (Zhang *et al.*, 1997b). This indicates that different Th subsets might be programed to use distinct cell death pathways. The involvement of autophagy in cell death remains controversial, mainly due to the technical challenge of differentiating between cell death accompanied by the block of autophagic flux from death mediated by autophagy. It appears however, that in particular circumstances autophagic cell death (ACD) can occur in mammalian cells (Marino *et al.*, 2014). Autophagy or components of autophagy machinery may also initiates apoptosis, for instance through caspase 8 activation (Marino *et al.*, 2014). Nevertheless, whether autophagy induction directly contributes to cell death of Th2 cells *in vivo* will require further investigation.

The precise mechanism that uniquely allows Th2 cells to survive independently of autophagy remains to be fully determined. However, one good candidate to be involved is Gata3, which, in addition to directing Th2 differentiation, can also promote peripheral T cell proliferation and maintenance (Tindemans *et al.*, 2014; Wan, 2014). This could be mediated through increased IL-7 signalling, as expression of IL-7Rα is controlled by Gata3, although this has been so far shown only for $CD8^+$ T cells (Wang *et al.*, 2013). Consistent with this hypothesis, we saw higher IL-7Rα expression in *Atg16l1*-deficient $CD4^+$ T cells upon activation (**Figure 5b**). In addition, Gata3 also regulates CD4 and TCR subunit expression (Wang *et al.*, 2008; Wei *et al.*, 2011; Tindemans *et al.*, 2014) and we have some preliminary evidence suggesting that *Atg16l1*-deficient T cells downregulated CD4 and TCRβ after activation to a higher extent than WT $CD4^+$ T cells (data not shown). Furthermore, Gata3 activation is also linked to induction of glycolysis after TCR activation, acting upstream of the critical regulator of the metabolic reprograming: c-Myc (Wang *et al.*, 2011a; Wang *et al.*, 2013; Wan, 2014). Distinguishing between these potential explanations would require a system that would allow us to sort large numbers of autophagy-deficient Th2 cells with high purity. However, we were not able to technically perform these

experiments due to the lack of a reliable surface marker for selective Th2 cell isolation. Alternatively, a Th2-reporter transgenic strain, such as IL-4eGFP mice (Mohrs *et al.*, 2001), could be used, but these would need to be extensively backcrossed onto the *Atg16l1*-deficient background beforehand, which is a time-consuming process. Overall, we speculate that Gata3 expression may provide Th2 cells with an autophagy-independent survival advantage in the periphery, but the mechanism needs further investigation.

Altogether, our study indicates that autophagy is a key pathway through which Th2 responses are restrained *in vivo*. Moreover, there may be an antagonistic relationship between autophagy and Th2 cell responses, as IL-4 and IL-13 can inhibit autophagy (Harris *et al.*, 2007), while IFN-γ promotes it (Levine *et al.*, 2011). Thus, the presence of Th2 cytokines could potentially enhance survival of Th2 cells by inhibiting autophagy, while at the same time limiting other effector Th responses, indicating that autophagy might be a specific intrinsic mechanism that regulates Th2 homeostasis in the gut.

Chapter 6. General discussion

6.1 Summary

The intestine is a specific site where the immune system encounters an enormous antigen load, therefore specialized multilayer mechanisms enforcing tolerance and a balanced immune response need to be in place to ensure homeostasis (Izcue *et al.*, 2009; Harrison and Powrie, 2013). An indispensable role for Treg cells in peripheral tolerance has been well documented and is highlighted by the fact that mutations in *Foxp3* lead to fatal autoimmunity with gastrointestinal manifestations (Wildin *et al.*, 2001). Although Treg cells have been shown to restrain Th2-driven pathologies (Mucida *et al.*, 2005; Curotto de Lafaille *et al.*, 2008; Zheng *et al.*, 2009; Josefowicz *et al.*, 2012b; Sekiya *et al.*, 2013), the precise mechanisms involved remain unclear. Changes in mucosal Treg and Th2 responses are implicated in intestinal diseases of increasing prevalence, including food allergies and IBD (Maloy and Powrie, 2011; Berin and Sampson, 2013). Therefore it is important to understand factors that control Treg and Th2 cells, and their reciprocal interactions in the periphery. Here we identified Atg16l1 and autophagy as a new crucial pathway regulating Treg and Th2 responses in the intestine.

Data presented in this thesis demonstrate that aged mice with T cell-specific deletion of the autophagy gene *Atg16l1* develop spontaneous intestinal inflammation. Inflammation was associated with a selective expansion of Th2 cells that was limited to the intestinal lamina propria. Furthermore, inflammation was preceded by the production of Th2-associated antibodies towards dietary and commensal antigens, the levels of which increased with age. Moreover, mice lacking autophagy in T cells showed a progressive loss of peripheral Treg cells, especially in the gut lamina propria. By generating mice with a Treg cell-specific deletion of *Atg16l1,* we demonstrated that cell intrinsic autophagy is indispensable for Treg cell

maintenance in the periphery and thus for the control of effector T cell responses, as these mice developed severe systemic and gastrointestinal inflammation. By employing two complementary *in vivo* approaches we were able to show that increase in mucosal Th2 responses observed in mice deficient in *Atg16l1* in the T cell compartment results from lack of sufficient Treg control, as well as from cell intrinsic dysregulation, as T cell intrinsic autophagy limits the expansion of Th2 cells. Finally, we presented evidence that autophagy differentially regulates survival of Treg cells and Th2 cells and that T cell intrinsic autophagy might facilitate the metabolic adaptations that are required for the survival of intestinal Treg cells (**Figure 36**). Overall, this study identifies a previously unrecognized role for Atg16l1 in the physiology of intestinal $CD4^+$ T cells, with unexpectedly distinct effects on Th2 and Treg cells, and provides new insights into the regulation of intestinal inflammation by autophagy. The aim of this chapter is to place these findings in the broader context of emerging themes in T cell biology, to postulate on how we intend to extend these studies, and to highlight potential implications for clinical applications.

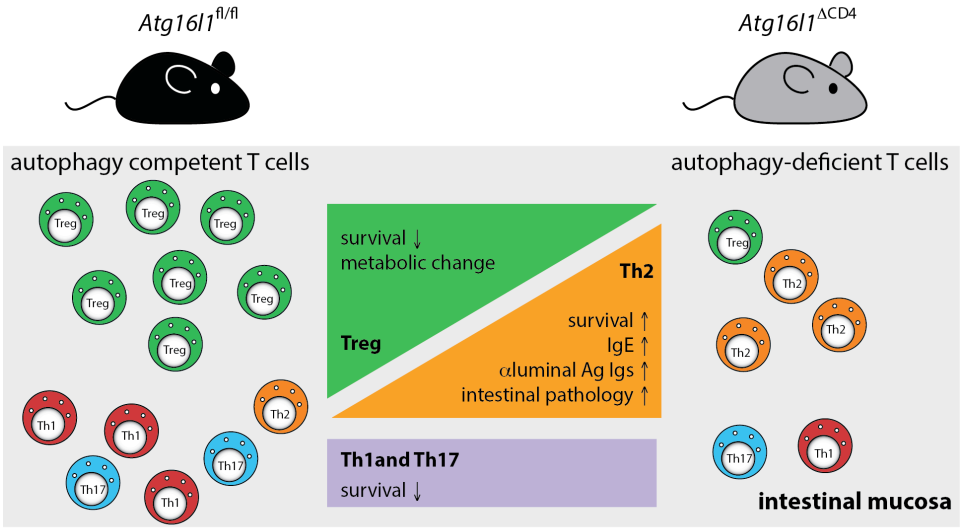


Figure 36 Summary of the main thesis findings

6.2 Autophagy in immunometabolic homeostasis of CD4$^+$ T cells

Recent years have seen a growing appreciation of the role of cellular metabolism in controlling immune responses, particularly in the field of T cell biology (Buck *et al.*, 2015). As autophagy is intimately linked with cellular metabolism (Galluzzi *et al.*, 2014), it is worth considering our findings in the broader context of metabolic regulation of CD4$^+$ T cells and speculate on the potential involvement of autophagy in metabolic reprograming of peripheral CD4$^+$ T cell subsets.

Data presented here demonstrate that autophagy deficiency primarily affects T cells that enter the periphery, and we established that autophagy is of particular importance for intestinal T cells. This was clearly shown in the setting of mixed bone marrow chimeras, where *Atg16l1*-deficient CD4$^+$ T cells failed to reconstitute the intestinal T cell compartment, despite efficient reconstitution of the thymus and spleen. Similar conclusions were drawn from experiments where naïve WT CD4$^+$ T cells were adoptively transferred into *Atg16l1*$^{\Delta CD4}$ mice: in this setting WT CD4$^+$ T cells had a striking survival advantage over autophagy-deficient CD4$^+$ T cells in the cLP. These results indicate that autophagy is crucial for the survival of CD4$^+$ T cells within the gut environment, suggesting that autophagy is a part of the adaptation program to operate within this tissue. As the intestinal LP is a site of constant antigen exposure and contains a high proportion of activated T cells, our findings suggest a more important role for autophagy in the homeostasis of antigen activated T cells, as opposed to naïve T cells.

As discussed briefly in Chapter 1, CD4$^+$ T cells undergo dramatic metabolic reprograming after antigen recognition in the periphery, switching from predominantly catabolic to anabolic metabolism (MacIver *et al.*, 2013; Buck *et al.*, 2015). This is indispensable to enable rapid proliferation and clonal expansion of effector T cells. Activated T cells upregulate both aerobic glycolysis and OXPHOS,

although it is the high rate of aerobic glycolysis that distinguishes effector T cells from naïve T cells, and glucose is the primary fuel choice for activated $CD4^+$ T cells (Frauwirth *et al.*, 2002; Pearce *et al.*, 2013). These metabolic changes are orchestrated by complicated network of signalling pathways downstream of TCR and cytokine receptors, with major roles for mTOR and AMPK signalling pathways. As might be expected, signals that drive differentiation of effector Th1, Th2, Th17 and Treg cells translate into distinct modes of mTORC1, mTORC2 and AMPK signalling and, ultimately, distinct metabolic profiles (**Figure 37)**(MacIver *et al.*, 2013; Buck *et al.*, 2015). Autophagy is directly regulated by both mTOR and AMPK (Alers *et al.*, 2012). Several studies in mammalian cells indicated that autophagy can in turn act as a feedback loop, regulating these signalling pathways (**Figure 38**)(Alers *et al.*, 2012). Here, we will focus on the regulation of Treg and Th2 cells by mTOR and AMPK signalling and potential role of autophagy in this network.

While mTORC1 activity is essential for Th2 differentiation (Yang *et al.*, 2013), other studies suggest that Th2 cells seem to be more reliant on mTORC2 signalling when compared to other Th subsets (Delgoffe *et al.*, 2011; MacIver *et al.*, 2013; Heikamp *et al.*, 2014). In addition, signalling downstream of mTORC2 operates in a different manner in Th2 cells versus Th1 cells, engaging PKCΘ and Gata3 instead of signalling through Akt (Lee *et al.*, 2010b). This is consistent with the notion that PKCΘ activity is required for Th2 effector responses (Marsland *et al.*, 2004; Salek-Ardakani *et al.*, 2004). Autophagy is one of the direct downstream targets of mTORC1, as active mTORC1 inhibits autophagy through negative regulation of the Ulk1/2 complex (Levine *et al.*, 2011). While this interaction is well established, less is known about the relationship between mTORC2 signalling and autophagy. However, there is evidence that mTORC2 can indirectly downregulate autophagy, as mTORC2 can decrease the activity of Foxo3a, which is known to promote autophagy pathway (Fullgrabe *et al.*, 2014). Additionally, mTORC1 can inhibit mTORC2, thereby

providing another potential link in the autophagy regulatory network (**Figure 38**)(Pollizzi and Powell, 2015). It would be interesting to explore potential links between autophagy and mTOR signalling in Th2 cells in comparison to other Th subsets. As both mTORC1 and mTORC2 are engaged in metabolic cell regulation, and mTORC2 has recently emerged as an important regulator of lipid metabolism (Duvel *et al.*, 2010; Lamming and Sabatini, 2013; Pollizzi and Powell, 2014), it is possible that distinct modes of mTOR signalling in Th2 cells are related to their specific metabolic adaptations. Surprisingly little is known regarding metabolic differences between Th2 cells and other $CD4^+$ T cell subsets (Pollizzi and Powell, 2014). Th2 cells are usually classified together with Th1 and Th17 cells in terms of their metabolic profiles (Michalek *et al.*, 2011a), but recent experimental evidence suggests that this might not be entirely correct. For instance, Th2 cells might be more reliant on reprograming towards glycolysis during very early time points of differentiation (Yang *et al.*, 2013). It has also been suggested that Th2 cells may not require sterol biosynthesis to the same extent as other effector Th cells, as inhibition of 3-hydroxy-3-methylgutaryl-coenzyme A (HMG-CoA) reductase, a crucial enzyme in the synthesis of cholesterol and isoprenoids, promotes bias towards Th2 cells (Youssef *et al.*, 2002). A recent study also suggested that Th2 cells might have a decreased requirement for glutamine and leucine uptake compared to Th1 and Th17 cells (Nakaya *et al.*, 2014). Lastly, a decrease in vitamin A metabolites seems to preferentially promote type 2 immunity (Spencer *et al.*, 2014). Further investigation of how autophagy impacts on the regulation of mTOR signalling and metabolic activity in Th2 cells represents an important avenue for future research.

Our current understanding of how mTOR signalling regulates Treg cells is incomplete. Early studies using selective deletion of mTOR in $CD4^+$ T cells found that this impaired their ability to differentiate into Th1, Th2 and Th17 effector subsets, but promoted their differentiation into Treg cells (Delgoffe *et al.*, 2009). This was

consistent with studies showing that rapamycin, an inhibitor of the mTORC1 complex, promotes Treg cell generation and expansion (Battaglia *et al.*, 2005; Kopf *et al.*, 2007; Kang *et al.*, 2008). These results indicated that mTOR signalling was dispensable for Treg cell differentiation, which is in line with the notion that Treg cells are much less glycolytic than effector $CD4^+$ T cell subsets, as mTORC1 signalling promotes glycolysis (Duvel *et al.*, 2010). However, detailed analysis of mTORC1 activity in mouse and human Treg cells suggested a more dynamic role for mTORC1 in Treg cell differentiation and proliferation (Procaccini *et al.*, 2010). Consistent with this hypothesis, mTORC1 activity was shown to be important for metabolic reprogramming of Treg cells, at least partially through its inhibitory effect on mTORC2 (Zeng *et al.*, 2013). An intriguing concept that could reconcile some of the discrepancies reported on mTOR signalling and Treg cells was recently put forward by Powell's group. They proposed that high mTORC1 activity would promote short-lived effector Treg cells, while low mTORC1 activity would be characteristic of long-lived, memory Treg cells, and that this could also explain the different metabolic profiles of these Treg cell subsets (Powell *et al.*, 2013; Pollizzi and Powell, 2015). Thus, distinct levels of mTORC1 signalling could reflect differential engagement of the autophagy pathway, which is consistent with our hypothesis that autophagy might be required for the transition from effector Treg cells into long-lived Treg cells. Additionally, autophagy may play a role in establishing the strength of mTOR signalling; results from other cell types showed that components of the autophagy pathway can block mTORC1 signalling, by interfering with S6K activation (Lee *et al.*, 2007b). In contrast, during prolonged starvation autophagy was required for reactivation of mTORC1 signalling (Yu *et al.*, 2010; Alers *et al.*, 2012). Weather the same regulation of mTORC1 by autophagy appears in Treg cells remains to be established.

While the precise role of mTOR signalling in Treg cells is still not entirely understood, experimental evidence suggests that AMPK is a crucial regulator of Treg cell metabolism (Michalek *et al.*, 2011a; MacIver *et al.*, 2013). AMPK acts in opposition to mTORC1 signalling, and can directly inhibit mTORC1 (Inoki *et al.*, 2003; Gwinn *et al.*, 2008). Activation of AMPK facilitates catabolic and inhibits anabolic processes in response to low ATP levels. In particular, AMPK activates autophagy by acting upstream of Ulk1 and Ulk2, activates mitochondria biogenesis, promotes FAO and inhibits FAS (Egan *et al.*, 2011; Kim *et al.*, 2011; Li *et al.*, 2011b; Mihaylova and Shaw, 2011). In turn, acting in negative feedback loop, autophagy can inhibit AMPK activity (Loffler *et al.*, 2011; Alers *et al.*, 2012). Treg cells show high AMPK activity and pharmacological activation of AMPK can promote Treg cells (Michalek *et al.*, 2011a). Interestingly, this requirement for high AMPK activation is shared with memory $CD8^+$ T cells, which, as already mentioned, show a similar metabolic profile to Treg cells (MacIver *et al.*, 2013). Of note, a recent study showed that AMPK activity is also important for allowing activated effector $CD4^+$ T cells to adapt to low glucose conditions (Blagih *et al.*, 2015). As activation of autophagy is one of the main downstream effects of AMPK, it further supports the notion that high levels of autophagy are important for Treg cell generation.

Modes of mTOR and AMPK signalling translate into distinct metabolic profiles and this in turn means that T cells can preferentially utilize distinct metabolites to fulfil their energetic and anabolic requirements. The requirement for amino acids increases in activated T cells and this is reflected by upregulated expression of amino acid transporters upon TCR engagement (Ramsay and Cantrell, 2015). Uptake of glutamine, leucine and arginine is increased upon activation and deficiency or depletion of these amino acids results in proliferation defects in effector T cells (Carr *et al.*, 2010; Hayashi *et al.*, 2013; Sinclair *et al.*, 2013; Nakaya *et al.*, 2014;

Fletcher *et al.*, 2015). Autophagy, together with the ubiquitin-proteasome system, serves as a cellular degradation system for proteins and protein aggregates, enabling amino acid recycling and amino acid levels negatively regulate autophagy by driving mTOR activation (Mizushima and Klionsky, 2007; Nicklin *et al.*, 2009). The functional importance of intracellular recycling of amino acids in activated $CD4^+$ T cells was recently demonstrated in cells with deletion of cytosolic protease tripeptidyl peptidase II (TPPII), which led to impaired cytokine production (Lu *et al.*, 2014). It could be speculated that autophagy also contributes to the supply of free amino acids in activated T cells, although the amino acid content in autophagy-deficient T cells remains to be investigated. In this context, it is interesting that Treg cells are resistant to amino acid depletion and can proliferate in an environment with low amino acids content, whereas effector $CD4^+$ T cells show decreased proliferation under such conditions (Cobbold *et al.*, 2009; Howie *et al.*, 2014). It would be of interest to evaluate if this resistance to low concentrations of external amino acids in Treg cells was attributable to their higher levels of autophagy compared to other $CD4^+$ T cell subsets, and therefore higher levels of intracellular amino acid turnover. If so, this could be another explanation for their increased reliance on autophagy.

The preferential requirements of Treg cells for lipid oxidation have already been discussed. Additionally, it is worth pointing out that utilizing this pathway makes Treg cells extremely reliant on mitochondrial respiration, as FAO takes place within the mitochondria compartment. In line with that, activation of AMPK drives mitophagy and mitochondria biogenesis, which ensures maintenance of healthy mitochondria (Mihaylova and Shaw, 2011). Therefore, autophagy-deficiency and impaired mitochondrial homeostasis could have a more detrimental effect on Treg cells than on other effector $CD4^+$ T cells, which are able to utilize glycolysis to a higher extent. Analysis of the mitochondrial compartment in intestinal Treg cells in

the presence and absence of a functional autophagy pathway will be informative to further assess this hypothesis.

While the discussion here focused on the reciprocal regulation of autophagy and mTOR and AMPK signalling pathways, it is perhaps important to point out that transcriptional control of autophagy is emerging as an important regulatory mechanism to maintain appropriate long term autophagic responses (discussed in more details in Chapter 1). As such, exploring how transcriptional control of autophagy differs between distinct $CD4^+$ T cell subsets would be of interest. For instance, activity of Foxo transcription factors, which are known to promote autophagy by increasing the expression of several autophagy genes (Mammucari *et al.*, 2007; Warr *et al.*, 2013), were shown to be important in promoting Treg cells differentiation and function, at least partially through their ability to regulate the expression of *Foxp3* and *CTLA4* (Harada *et al.*, 2010; Kerdiles *et al.*, 2010; Ouyang *et al.*, 2010). It would therefore be interesting to explore if the positive regulation by Foxo proteins in Treg cells also includes enhancement of autophagy pathway.

Altogether, autophagy can potentially play multiple roles in the biology of Treg cells and effector $CD4^+$ T cells, especially in establishing immunometabolic homeostasis. In particular, context-dependent interactions between mTOR, AMPK and autophagy pathways (schematically summarized in **Figure 38**) can ensure proper metabolic adaptations. Establishing to what degree autophagy participates in establishing the balance between mTORC1, mTORC2 and AMPK activity in the peripheral $CD4^+$ T cells will require detailed examination of signalling pathways and metabolic profiles in autophagy-deficient subsets of $CD4^+$ T cells. Nevertheless, it is likely that blocking the autophagy pathway could result in dramatic perturbations of these signalling networks, which would in turn result in dysregulated metabolic profiles. Since distinct subsets of $CD4^+$ T cells have different requirements for mTOR and AMPK

signalling (summarized in **Figure 37**), one could imagine that such perturbations could result in very different effects on their function and survival, and thus could affect Treg and Th2 cells in a different way. Investigating this hypothesis will be the focus of future studies.

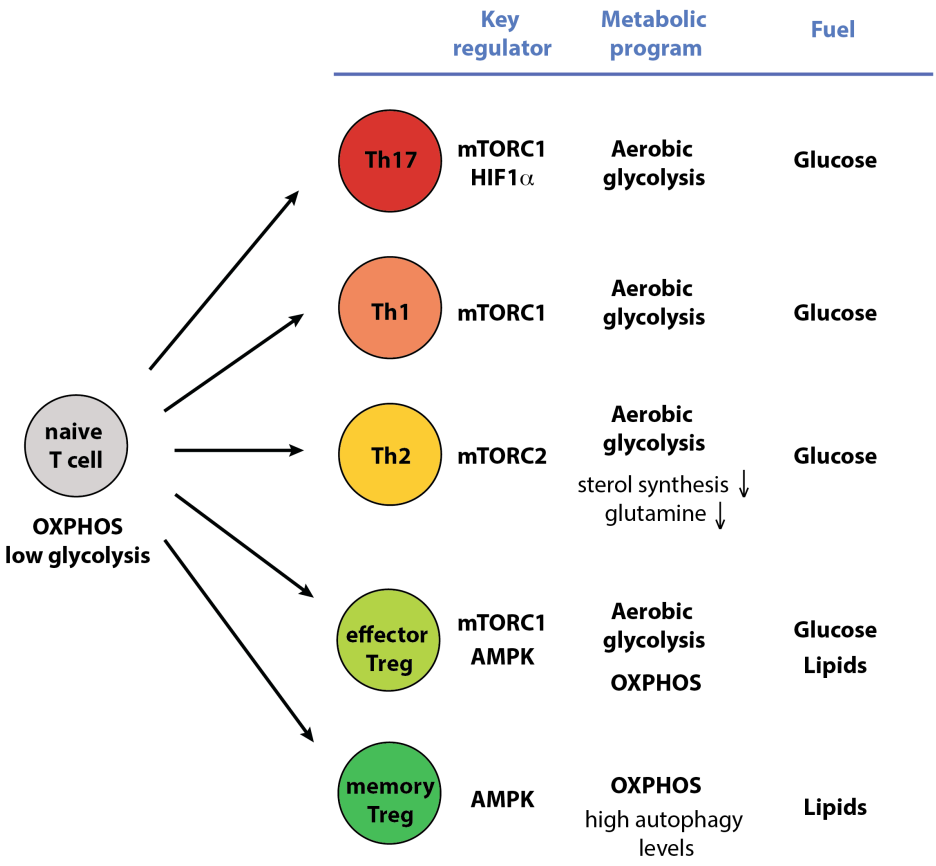


Figure 37 Metabolic profiles of specific T cell lineages are essential for their function and maintenance. Predominant metabolic profiles of Th1, Th2, Th17 and Treg cells are indicated, together with signalling regulators and main fuel choice.

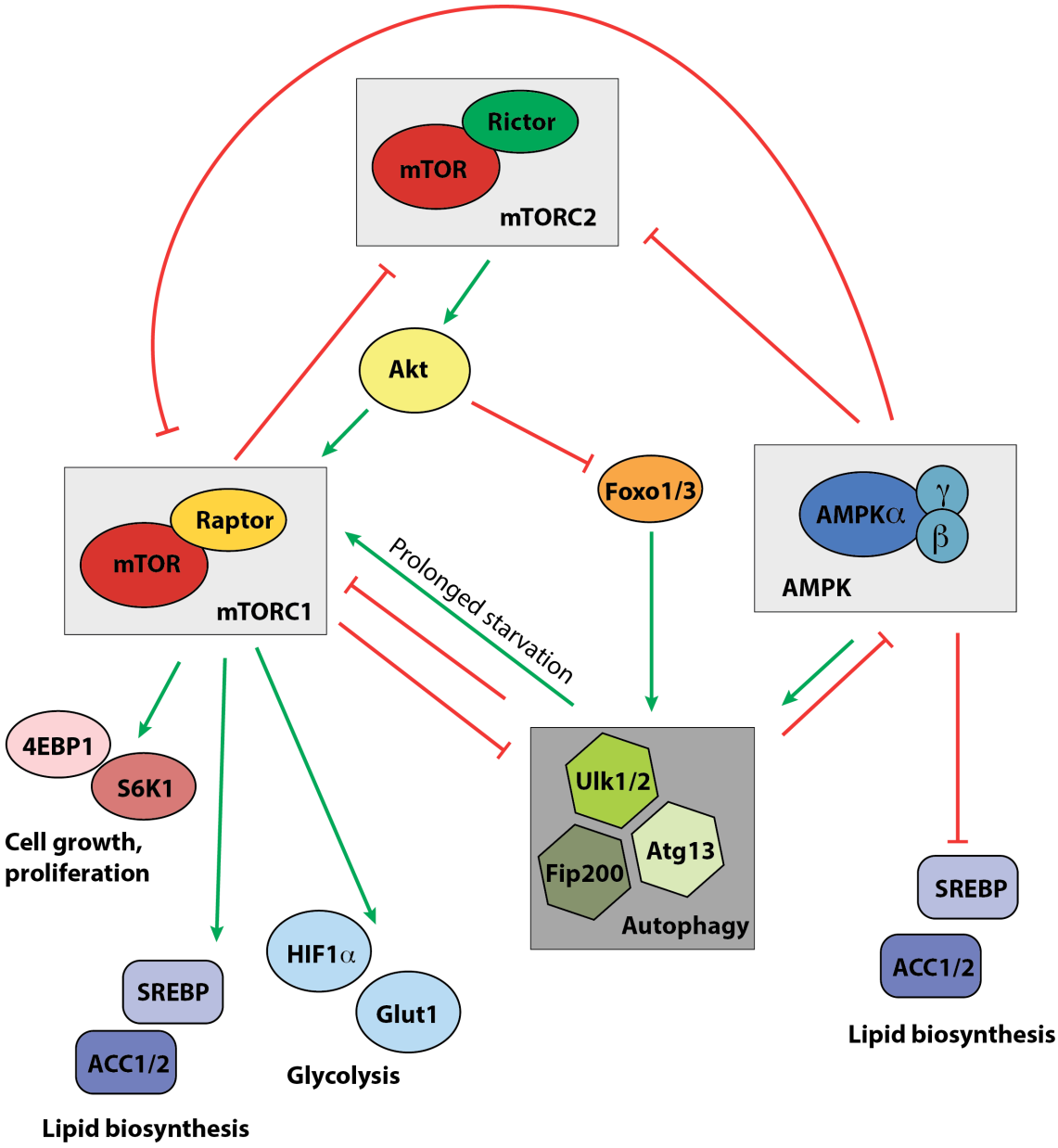


Figure 38 Crosstalk between AMPK-mTORC1/2-autophagy networks. Experimental evidence from studies in mammalian cells suggests that autophagy is directly linked to the mTORC1 and AMPK signalling pathways, and indirectly also with the mTORC2 pathway. Thus, autophagy block through deletion of *Atg16l1* could potentially result in perturbations to these signalling networks, and therefore have dramatic effects on distinct $CD4^+$ T cell subsets.

6.3 Potential implications

As polymorphisms in *ATG16L1* and other autophagy genes have been linked to IBD susceptibility, our results identify a potential novel mechanism that links genetic susceptibility in the autophagy pathway to intestinal inflammation through dysregulation of mucosal T cell responses. Both clinical studies and animal models provide strong evidence that defective Treg responses predispose to the development of IBD (Maloy and Powrie, 2011; Shale *et al.*, 2013). Here, we present evidence that in a mouse model autophagy deficiency selectively in Treg cells leads to a deficit in intestinal Treg cells and the development of severe intestinal pathology. It can therefore be speculated that mutations in autophagy genes that result in defective autophagy pathway could manifest through impaired Treg cell homeostasis in humans. As discussed in section Chapter 3, Crohn's disease associated polymorphism in *ATG16L1* leads to a decreased autophagy levels only in the presence of additional stress factor, such as starvation or infection. Therefore, it is possible that decreased fitness of Treg cells in patients bearing this mutation would only become apparent when additional environmental factors are also present.

As discussed in Chapter 1, contribution of the Th2 axis in IBD remains unclear and experimental and clinical evidence suggest that Th2 responses are less dominant compared to Th1 and Th17 responses (Strober *et al.*, 2002; Shale *et al.*, 2013). Nevertheless, polymorphisms in type 2 cytokines including IL-4, IL-5 and IL-13 have been implicated by GWAS in both CD and UC (Van Limbergen *et al.*, 2014). In addition, elevated levels of antibodies recognizing food and commensal antigens have been detected in IBD patients (Lodes *et al.*, 2004; Cai *et al.*, 2014), although the mechanisms behind this correlation remain unclear. Our study provides a new link between *Atg16l1*-deficiency in $CD4^+$ T cells and elevated Th2 responses, including Th2-associated antibodies recognizing intestinal luminal antigens,

highlighting a potential role of dysregulated Th2 responses in chronic intestinal inflammation.

Moreover, as defective Treg and increased Th2 responses at the mucosa are observed in food allergies and asthma, our findings might also have implications for these conditions. It is pertinent that epidemiological studies show overlap between IBD and Th2 driven diseases, like atopic dermatitis and asthma (Lees *et al.*, 2011). Furthermore, polymorphisms in the essential autophagy gene *ATG5* have recently been implicated in asthma susceptibility, however it remains unclear whether these mutations result in impaired autophagy (Martin *et al.*, 2012; Poon *et al.*, 2012).

Autophagy is an attractive therapeutic target and several autophagy modulating compounds are already in clinical trails for the treatment of various disorders (Jiang and Mizushima, 2014). For instance, the autophagy-enhancing drug carbamazepine has been shown to ameliorate hepatic fibrosis in the mouse model of α1-antitrypsin deficiency liver cirrhosis and this drug is currently in phase II of clinical trials (Hidvegi *et al.*, 2010; Jiang and Mizushima, 2014). Additionally, an autophagy-inducing agent has been shown to decrease pathology in a mouse model of chemically induced colitis (Eisenberg *et al.*, 2009; Moron *et al.*, 2013). The challenge however is to identify agents that can specifically induce autophagy, minimalizing side effects on other cellular processes. Indeed, considerable effort in the field of autophagy research is currently focused on finding small molecules that can induce autophagy in a very selective manner (Shaw *et al.*, 2013; Shoji-Kawata *et al.*, 2013). Interestingly, as some of the dietary derived compounds, including RA (Isakson *et al.*, 2010) and vitamin D (Yuk *et al.*, 2009) have been shown to enhance autophagy, it is tempting to speculate that the use of such natural inducers could proof beneficial for treatment of intestinal inflammatory disorders in patients bearing mutations in autophagy genes. Taken together with our results, these findings raise the possibility that activation of autophagy might have beneficial effects in disorders with a

signature of decreased Treg responses and elevated Th2 responses, including intestinal inflammation and various hypersensitivities. Therefore it will now be of vital importance to evaluate how our findings relate to human studies.

Chapter 7. References

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