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PURSUIT

Crohn's Disease linked Polymorphisms associated with Autophagy contribute to Th17 cell induction through down regulating Il-10 and up regulating IL-1 and TNF-α

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Genome wide association studies (GWAS) have linked polymorphisms in autophagy genes to Crohn's Disease (CD). Interestingly, recent studies have shown that defective autophagy leads to increased levels of the cytokines IL-1 and TNF-α, which play a critical role in Th17 cell differentiation. This is significant because CD is marked by a Th17 cell mediated inflammatory response. Through a unique synthesis of the current CD literature, this review examines the manners in which defective expression of autophagy linked proteins indirectly amplify and sustain Th17 cell induction through increasing the production of Th17 positively differentiating cytokines (IL-1 and TNF-α) and decreasing the production of the Th17 down regulating cytokine IL-10.

Introduction

In the western hemisphere over 2.5 million people have inflammatory bowel diseases (IBD) [1]. The two most prevalent varieties of IBD are Ulcerative Colitis (UC) and Crohn's Disease (CD), which are both marked by aberrant inflammation in the intestinal tract. Although previously thought to primarily affect Western Europe and North America, new evidence shows strikingly increased prevalence rates across the globe especially in the Middle East, India, China, and Japan [2]. In light of this increased prevalence, the need for effective treatment is urgent.

This paper will focus on Crohn's Disease (CD), which is marked by chronic intestinal inflammation primarily in the terminal ileum. Symptoms include abdominal pain, fever, intestinal blockage and diarrhea. Severe cases are characterized by intestinal stricturing and intra-abdominal or perianal fistulae. CD results from genetically and environmentally driven defects in intestinal immune cells, which allows the intestinal micro biota to improperly stimulate the intestinal immune system [3]. This continual and improper stimulation results in excessive intestinal inflammation.

Th17 Cells

The culprit for much of the CD inflammation is the IL-17 producing helper T cells (Th17). Th17 cells are highly inflammatory CD4+ T cells divergent from CD4+ T helper cells and are categorized as a distinct Th cell lineage [4, 5]. Th17 cells are constitutively present in

CD inflammation is strongly associated with increased Th17 cell levels. Compared to normal intestinal mucosa, the mucosa of CD patients contains increased levels of Th17 cells [5]. The increased numbers of Th17 cells in active CD, is logical as high levels of both the cytokines needed for Th17 induction (IL-1, IL-6, TNF-α) and stabilization (IL-23) and the effector cytokines produced by Th17 cells (IL-17, IL-21, and IL-22) are found in the inflamed mucosa of CD patients.

Genome Wide Association Studies (GWAS) have further strengthened the link between Th17 cells and CD by linking single nucleotide polymorphisms (SNPs) in components of the Th17 pathway, including p40, jak2, ccr6, and most significantly the IL-23 receptor (IL-23R), to IBD [6]. The most common SNP for the IL-23R is the second most frequent polymorphism linked to CD [7]. Th17 cells are specifically marked by the expression of $CD161^+$, the presence of the retinoic acid receptor-related orphan nuclear receptor gamma transcription factor (ROR-γt) and high levels of IL-23R [8].

Th17 Effector cytokines lead to CD inflammation

Increased Th17 cell levels lead to CD inflammation. Th17 cells promote a hyper immune response through the secretion of highly inflammatory cytokines. Th17 predominantly produce the effector cytokines IL-17, IL-21, and IL-22. Likewise, high levels of IL-17 [9], Il-21 [10], and IL-22 [11, 12] are present in the inflamed mucosa of CD patients. Thus the primary effector cytokines produced by Th17 cells are found in abundance in the intestine of CD patients.

Th17 cell derived cytokines contribute to the hyperactive and damaging immune response associated with CD in a number of ways. IL-17 [13], IL-21 [14] and IL-22 [15] activate colonic fibroblasts to produce enzymes known as matrix metalloproteinases (MMPs) that degrade the extracellular matrix. The extracellular matrix between cells is made up of proteins, like collagen, that help to maintain the integrity and strength of intestinal walls. Thus, high levels of MMPs damage the intestinal wall. The abnormally high levels of MMPs are likely to be responsible for much of the intestinal damage (ulceration and fibrosis-scarring) of CD [16]. Indeed, in mouse models of CD, antibodies designed to inhibit MMPs have shown a therapeutic ability to reduce intestinal damage and inflammation [17]. Thus, effector cytokines of Th17 cells contribute to CD symptoms partially through the activation of MMPs.

Besides activating MMPs, Th17 effector cytokines contribute to the hyper immune response associated with CD in a plethora of other ways. IL-17 binds to receptors on endothelial and myeloid cells, where it induces the expression of key chemokines involved in neutrophil recruitment and of pro-inflammatory cytokines including IL-1β, tumor necrosis factor-α (TNF-α) and IL-6 [18]. IL-21 induces the expression of the T-cell chemoattractant macrophage inflammatory protein-3α (MIP-3α) from intestinal epithelial cells [19], and endows $CD4^+$ T cells with resistance to Treg-mediated suppression [20]. IL-22, up regulates -defensin production [21], TNF-α and IL-6 mRNA expression [12], intestinal epithelial cell migration [12], and levels of acute-phase reactants [22].Thus, through the activation of various factors, including MMPs, the effector cytokines produced by Th17 cells are responsible for much of the severe inflammation associated with CD.

Th17 Polarization

Interestingly, Th17 cells are closely related to inducible T regulatory (iTreg) cells, another sub population of $CD4^+$ T cells. In contrast to the damaging properties of Th17 cells, Treg cells, including both peripherally generated induced Treg (iTreg) and thymus-derived natural Treg (nTreg), actively suppress a broad range of immune cell responses [23, 24]. Decreased levels of functional Treg cells have been found in CD patients [25], this is fitting as Treg cells suppress inflammation.

Th17 and iTreg cells both arise from the differentiation of naïve $CD4^+$ Th cells. Once naive $CD4^+$ T cells have developed and matured in the thymus, they exit and begin to circulate through the lymph system and lymph nodes (LN). (Naive $CD4^+$ T cells are T cells that have not

encountered the antigen that they are designed to react to). In the LN, these naive $CD4^+$ T cells are activated by antigen-presenting cells (APCs) through T-cell receptor ligation (presentation of a specific antigen on the APC's major histocompatibility complex II to the naive $CD4^+$ T cell's T cell receptor) and costimulation. The specific antigens that the APCs are using to activate naive $CD4^+$ T cells in CD remain unclear. The final phenotype of a naive $CD4^+$ T cell is then determined by the cytokine environment. Thus the factors that alter the cytokine environment have a major role in determining the terminal lineage commitment of naive $CD4^+$ T cells.

Indeed the fate of a naïve $CD4^+$ T cell is critically influenced by which cytokines it comes into contact with. In the LN, naïve $CD4^+$ T cells are likely to come into contact with stimulatory cytokines. These cytokines can be either anti-inflammatory, including IL-10, or pro-inflammatory, including IL-1, TNF- and IL-23.

Treg cell (both iTreg and nTreg) induction largely depends upon the activation of the Treg transcription factor, Foxp3 (forkhead box protein 3) [26-28]. In contrast Th17 cell induction largely depends upon the activation of Th17 transcription factor, ROR-γt [29]. Without the interference of inflammatory cytokines, Foxp3 normally binds to and down regulates RORγt [30]. Thus, the induction of iTreg cells is naturally favored over the induction of Th17 cells in non-inflammatory conditions. Yet, the inflammatory cytokines IL-23 and TNF-, prevent the Foxp3 down regulation of RORγt by deactivating Foxp3 [30-32]. Once Foxp3 is deactivated, RORγt is no longer inhibited and if additionally activated by IL-1 will begin polarizing the naïve $CD4^+$ T cell towards a Th17 phenotype [29, 33]. Thus, high levels of IL-1, IL-23 and TNF- can strongly bias the differentiation of naïve $CD4^+$ T cells into Th17 cells.

Th17 Inducing Cytokines

IL-1β

Increased levels of IL-1β are consistently found in patients with CD [34-37]. Indeed, much of CD inflammation is the result of heightened IL-1 levels. The potent anti-inflammatory power of anti-TNF antibodies is partially attributed to the ability of anti-TNF antibodies to down regulate IL-1β secretion [38]. In mice with increased IL-1 levels, anti-IL-1 antibodies tapered the severe intestinal inflammation that resulted from DSS treatment [39], suggesting the primary role IL-1 plays in intestinal inflammation.

The IL-1 receptor (IL-1R) is found not only on leukocytes, but also on numerous other cells including hepatocytes, endothelial and epithelial cells [40]. This broad expression of IL-1R helps account for the broad and potent inflammatory potential of IL-1. Yet, the primary reason that IL-1 plays such an important role in intestinal CD inflammation is because it plays a pivotal role in activating Th17 cells [29, 31, 33, 34, 41-44].

IL-1 activates and sustains Th17 populations. In the gut, resident macrophages (CD68 +) are the main source of IL-1 [29, 45]. The removal of these IL-1 producing macrophages impaired the Th17 response and subsequently reduced intestinal inflammation [29, 45]. Th17 (CD161+) lymphocytes express unusually higher levels of IL-1 receptors than CD161 negative lymphocytes [8]. This shows the critical role IL-1 plays in driving differentiation of Th17 cells, as CD161 is a marker of cells committed to the Th17 phenotype [8]. Studies have shown that disruption of the IL-1R strongly impairs Th17 differentiation and accumulation as well as intestinal inflammation [34].

Like IL-23 and TNF-α, IL-1 plays a critical role in polarizing the Th17 phenotype from the Treg phenotype. While IL-1 has not yet directly been shown to inhibit the Foxp3 inhibition of RORγt, it does significantly up regulate RORγt [29, 31, 40]. In addition to up regulating RORγt, IL-1 up regulates Interferon regulatory factor 4 (IRF4), which has been shown to be essential for Th17 differentiation [46].

Besides polarizing the Th17 phenotype, IL-1 has a key role in maintaining the activated state and growth of Th17 cells. In vitro experiments showed IL-1's ability to activate IL-17

production and Th17 expansion in an environment devoid of TCR stimulation [33, 43]. Therefore, through driving the polarization and expansion of the Th17 phenotype, IL-1 increases intestinal inflammation.

IL-23

IL-23 plays a major role in the stabilization and maintenance of Th17 cells and is implicated in CD inflammation. In the intestine it is mainly produced by stimulated macrophages and dendritic cells (DCs) [47]. Polymorphisms increasing the activation and expansion of IL-23R are linked to heightened risks of developing CD, while polymorphisms decreasing the activation and expansion IL-23R are linked to increased protection against developing CD [48]. Indeed, in the inflamed gut mucosa of CD patients, high levels of IL-23 and high numbers of cells with IL-23 receptors are found [49, 50]. Likewise, anti-IL-23 antibodies have displayed benefits in CD patients [51]. IL-23 plays a key role in inhibiting IL-10 production [52], activating RORγt [30], up regulating the expression of IL-1R [53], and sustaining the expansion of Th17 cells [54]. All of these factors lead to the induction of Th17 cells and thus the inflammatory conditions of CD.

High levels of IL-23 specifically inhibit the production of IL-10 by lamina propria mononuclear cells and $CD4^+$ T cells [52]. IL-10 is a potent anti-inflammatory cytokine that plays a vital role in the regulation of inflammatory responses [55]. IL-10 has the capability to influence the differentiation of naïve $CD4^+$ T cells. IL-10 helps persuade naïve $CD4^+$ T cells to differentiate into iTreg cells [56]. High levels of IL-10 can persuade naïve $CD4^+$ T cells in vitro to become specialized Tr1 ($Foxp3^-$) cells that in turn secrete increased levels of IL-10 [57]. Thus inhibition of IL-10 would lead to decreased Treg cell induction and increased inflammatory conditions that would favor Th17 development. Studies continue to show that the polarization of the Th17 phenotype is strongly dependent on inflammatory conditions in the surrounding in vivo microenvironment [58].

The addition of IL-23 to naïve $CD4^+$ T cells decreases the inhibition of the Th17 transcription factor RORγt by Foxp3 and accordingly leads to the increased polarization of the Th17 phenotype [30]. Besides polarizing the Th17 phenotype, studies have shown that IL-23 works with IL-1 to support the maintenance and expansion of the Th17 population. Indeed IL-23 signals for the increased display of IL-1 receptors on Th17 cells [34, 53]. IL-1β and IL-23 work in synchrony to drive the differentiation of the Th17 phenotype and to maintain the homeostasis and expansion of Th17 cells [34]. Therefore, increased levels of either or both IL-1β and IL-23 would lead to large populations of mature Th17 cells.

TNF-α

TNF-α is a potent pro-inflammatory cytokine that is found at elevated levels in the intestinal mucosa of CD patients [25, 36]. The relief of CD symptoms by anti-TNF-α antibodies testifies to the central role TNF-α plays in CD inflammation. TNF-α is primarily produced by activated macrophages, T lymphocytes and monocytes [25]. TNF-α inactivates Foxp3 function [32] and down regulates Foxp3 expression in $CD4^+$ T cells [59]. Thus, TNF-α strongly polarizes naïve $CD4^+$ T cells towards the pro-inflammatory Th17 phenotype and inhibits functional Treg cell induction. Thus any genetic mutation that would lead to increased levels of IL-1β, IL-23 or TNF-α, would result in increased Th17 induction.

The presence of certain cytokines has an enormous effect on dictating whether the immune response will be regulated or will become excessive. Because the up-regulation of Th17 cells simultaneously down regulates Treg cells, the induction of Th17 cells simultaneously removes the primary cell associated with immune regulation. This results in an increase in the ratio of Th17 cells to Treg cells. Thus, the compound effect pro-inflammatory cytokines have of unleashing Th17 cells at the cost of Treg cells, results in severe and unchecked inflammation. Therefore, any cellular process that leads to

abnormally high levels of the specific cytokines needed for Th17 cell differentiation could potentially be linked to increased Th17 induction. Fascinatingly, both TNF-α and IL-1 secretion is up regulated when a cellular process known as autophagy is defective. The remainder of this paper will attempt to trace the manners in which TNF-α and IL-1 are up regulated by the defective autophagy.

Autophagy

Autophagy refers to the cellular response to stress that results in the degradation/recycling of cytoplasmic components. This catabolic pathway responds to a wide variety of cellular stress such as nutrient deprivation, hypoxia, DNA damage, mechanical injury, ROS, misfolded protein/ER stress, organelle dysfunction and pathogen (viruses, bacteria, or parasites) infection [60-63].

Xenophagy is the form of autophagy when the cytoplasmic component being degraded is a pathogen. In other words, xenophagy is the autophagic engulfment and breakdown of intracellular bacteria [64]. Xenophagy has been shown to occur in fibroblasts [65], intestinal epithelial cells [66], non-intestinal epithelial cells [67], monocytes [68], and macrophages [68, 69].

Autophagy (and thus xenophagy) functions through the engulfment of cytoplasmic components by an autophagosome, which then fuses with a lysosome, resulting in the degradation of the components [70]. The autophagosome is a double membrane vesicle that is assembled by autophagy related proteins (Atgs) [70]. Proper autophagosome induction is a complex process involving many steps and is dependent on many different proteins [70]. Interestingly three of these proteins, ATG16L1, NOD2 and IRGM, have been genetically linked to CD.

Genetics (GWAS Genome Wide Association Studies)

In an attempt to understand the pathogenesis of CD, researchers have utilized new technology in attempt to find genes that correlate with CD. In the last decade genotyping, a once expensive and laborious process, has become significantly more affordable and efficient [71]. This improvement in genotyping has led to the arrival of Genome Wide Association Studies (GWAS). In GWAS, thousands of genome wide single nucleotide polymorphisms (SNPs) in controls and cases are compared. So far, this process has resulted in the labeling of 71 risk loci for CD [6, 72]. Intriguingly, three of the genes most strongly linked to CD code for proteins (ATG16L1, NOD2 and IRGM) that are involved in autophagy [72]. These studies thus suggested a key role of autophagy in the pathogenesis of CD.

CD linked Single Nucleotide Polymorphisms (SNPs) associated with autophagy

NOD2

Pattern recognition receptors (PRRs), including Toll like receptors (TLRs) and Nod like receptors (NLRs) recognize components of viruses and bacteria. NOD2 (nucleotide-binding oligomerization domain 2) is a NLR intracellular sensor of muramyl dipeptide (MDP), a bacterial cell peptidoglycan [73]. Upon recognition of MDP, NOD2 activates the production of various pro-inflammatory and anti-inflammatory cytokines through the NFB pathway [73]. Because NOD2 is an intracellular receptor it only recognizes cytoplasmic MDP. Thus, NOD2 principally recognizes bacteria inside the cell. Consequently as NOD2 is strongly linked to CD, intracellular bacteria have the potential to play a major role in CD.

Three specific mutations in the NOD2 genes in humans have been strongly linked to CD. Both the two SNPs, substitution of positively charged arginine for neutrally charged glycine at amino acid residue 908 (Gly908Arg) and substitution of neutrally charged tryptophan for positively charged arginine at amino acid residue 702 (Arg702Trp), and the frameshift mutation (Leu1007fs) are found in the leucine-rich-repeat (LRR) domain in NOD2. The LRR domain is the microbe-associated molecular pattern recognition region of NOD2 [73]. Thus these mutations affect the microbial recognition ability of NOD2. The frame shift mutation (Leu1007fs) is the product of a cytosine insertion (3020insC) that truncates the terminal LRR domain of NOD2 protein by 33 amino acids. As the frameshift mutation cleaves off part of the LRR domain it severely inhibits NOD2 function. Accordingly a severe form of CD, often requiring surgery, is found in patients homozygous for the (L100fsinsC) frame shift mutation [74].

Stimulation of NOD2 by MDP results in the activation of xenophagy [75], the autophagic engulfment and breakdown of intracellular bacteria. Functional xenophagy depends upon the complete engulfment of intracellular pathogens and thus depends on the correct induction of autophagosomes at the sites of pathogen entry. In turn this induction depends upon the recruitment of ATG16L1 to the plasma membrane, where the sites of pathogen entry are located [76].

The recruitment of ATG16L1 to the sites of bacterial entry was found to be completely dependent upon NOD2 [76]. NOD2 binds to ATG16L1 and positions it in the appropriate place around the plasma membrane. Indeed, the physical interaction between MDP-stimulated NOD2 and ATG16L1 resulted in the proper recruitment of ATG16L1 to the sites of bacterial entry on the plasma membrane [76]. Human macrophages homozygous for the (L100fsinsC) frameshift mutation were unable to recruit ATG16L1 to the sites of bacterial entry on the plasma membrane [76]. Consequently xenophagy was defective because the autophagosomes failed to sufficiently engulf the invading bacteria.

This landmark study directly linked NOD2 with the autophagy pathway. This study also strengthened the linkage between autophagy and CD, as defects in NOD2 are strongly linked to CD. Later experiments confirmed the critical role NOD2 plays in functional xenophagy in DCs, epithelial cells and macrophages [77]. Thus, functional xenophagy depends upon the correct orientation of ATG16L1, which in turn depends on the proper recruitment by NOD2.

ATG16L1

In 2007, a GWAS linked the substitution of a polar threonine with a nonpolar alanine on the ATG16L1 gene to CD [78]. This SNP (Thr300Ala) is specifically connected to CD in the ileum [78]. The mutation is found in the carboxyl terminal WD-repeat domain, which is implicated in protein-membrane/protein-protein interactions [78].

ATG16L1 combines with Atg5 and Atg12 to form a multimeric protein complex that is essential for correct autophagosome induction [79]. Dysfunctional ATG16L1 leads

to dysfunctional autophagosome induction and hence, dysfunctional autophagy. Thus, dysfunctional ATG16L1 would also impair xenophagy. Indeed, severely weakened clearance of the intracellular pathogen *Salmonella typhimurium* was observed in human epithelial cells with the CD-linked ATG16L1 mutation [80]. Other studies suggest that impaired xenophagy (cellular clearance of intracellular bacteria) leads to the activation of Th17 cells.

IRGM

The third of the primary CD linked autophagy proteins is IRGM (Immunity-related GTPase family M). Polymorphisms in both the coding and promoter regions of the IRGM gene have been repeatedly linked to CD [6, 72, 81]. IRGM is thought to play a crucial role in the early activation of xenophagy [65]. Defective expression of IRGM leads to defects in xenophagy, and as a result the failure to clear intracellular bacterial [66, 82].

Functional autophagy (and thus xenophagy) depends on the proper expression of ATG16L1, NOD2 and IRGM. Accordingly polymorphisms in any of these three genes lead to dysfunctional autophagy (xenophagy). Dysfunctional expression of autophagic (xenophagic) proteins leads to the activation of Th17 cells through the up regulation of IL-1, IL-23 and TNF- and possibly through the down regulation of IL-10.

Increased secretion of IL-1

Recent studies have shown that autophagy plays an integral role in the production of various cytokines. Specifically it has been found that the production of IL-1 is closely linked to autophagy. Studies in human monocytes and murine macrophages have found that impaired autophagy leads to increased secretion of IL-1 [39, 64, 83].

A common method to impair autophagy is the application of 3-methyladenine, which blocks the Beclin-1 complex, which is required for the initiation of the induction of the autophagosome [39, 84, 85]. Numerous studies have shown increased IL-1 production in the presence of 3-methyladenine [39, 83, 86].

Various murine studies in macrophages have specifically studied the effect of the disturbance of key autophagy proteins on IL-1 production. Impaired expression of Beclin 1[84, 87], LC3B [87] or Atg 7[88], all of which are required for proper autophagosome induction, results in increased IL-1 production. Likewise impaired expression of ATG16L1, additionally required for autophagosome induction, also led to heightened IL-1 production [39].

Because autophagy negatively regulates IL-1 production, defective autophagy would lead to IL-1 secretion in response to TLR ligands that would normally fail to stimulate IL-1 secretion. Saitoh et al., found higher secretion of IL-1 in response to LPS as well as *Eneterobacter aerogenes, E. coli* and *Klebsiella pneumoniae (*non-invasive gram negative commensal bacteria) in murine macrophages lacking ATG16L1 compared to controls [39]. These indicate that defective autophagy could be implicated in a hyperactive immune response to commensal bacteria. Thus, defective autophagy could lead to a potentially harmful immune response to non-pathogenic bacteria.

In order to understand how impaired autopahgy controls IL-1 production, it is necessary to look at the IL-1 production pathway. The production of IL-1 is a complex process that testifies to the potent inflammatory potential of active IL-1 and the host's attempts to monitor it. IL-1 production begins with the induction of the transcription of the cytokine gene. TLR ligands, primarily TLR-4 ligands, are the most common stimulator, though NLR ligands have also been implicated as inducers [89, 90]. Stimulation of TLRs activates NF-κB, a cytokine transcription regulating protein (factor), which initiates gene transcription [91].

After transcription, translation creates inactive precursor IL-1 which must be

proteolytically cleaved by the cysteine protease Caspase 1 to become activated IL-1β [92, 93]. Caspase 1 itself exists in an inactivate form, pro-caspase 1, until it is activated by the cytosolic multiprotein complex, NLPR3 (Nucleotide-binding-and-oligomerization domain (Nod) and leucine-rich-repeat–containing) inflammasome [87, 94]. Thus the secretion of mature IL-1β is dependent upon NLRP3 inflammasome induction.

Autophagic Regulation of IL-1β production dependent on the NLRP3 inflammasome

Why did the removal of autophagic proteins lead to increased NLRP3 inflammasome mediated cytokine production? Recently, mitochondrial homeostasis was linked to functional autophagy through studies that showed dysfunctional autophagy disrupted mitochondrial homeostasis [87, 94]. Because autophagy is responsible for organelle recycling, defective autophagy impairs mitochondrial homeostasis. Disruption of mitochondrial homeostasis was marked by increased mitochondrial membrane permeability, increased production of mitochondrial reactive oxygen species (ROS) and release of mitochondrial DNA into the cytosol [87, 94, 95]. Both the increase in mitochondrial ROS and the cytosolic translocation of mitochondrial DNA leads to the induction of the NLRP3 inflammasome [87, 94].

Further studies showed that blocking ROS in macrophages with impaired autophagy, led to decreased NLRP3 inflammasome activation [83]. Thus through maintaining mitochondrial homeostasis, autophagic proteins regulate NLRP3 inflammasome induction which in turn regulates the production of IL-1β.

The key role ROS plays in NLRP3 inflammasome activation, links IL-1β to the potent cytokine, TNF-α. This inflammatory cytokine is one of the primary cytokines produced by macrophages in response to TLR ligands [96]. TNF-α has been shown to disrupt mitochondrial homeostasis, which in turn heightens ROS production that ultimately can lead to the increased secretion of mature IL-1β [97-99]. Indeed, as anti-TNF-α antibodies leads to reduced secretion of IL-1β [38]. TLR ligands stimulate both IL-1β and TNF-α production [99]. In cells with defective autophagy, the TNF-α produced from this stimulation will substantially disrupt mitochondrial homeostasis, leading to increased ROS production and NLRP3 inflammasome induction and subsequently, to highly increased IL-1β secretion.

In summary defective autophagy leads to mitochondrial stress, which leads to increased numbers of ROS, which in turn up regulates the NLRP3 inflammasome, which in turn activates Caspase 1, which cleaves precursor IL-1β into mature IL-1β ready for secretion. Thus autophagy can monitor IL-1β secretion in a NLRP3 inflammasome dependent manner.

Autophagic Regulation of IL-1β production independent of the NLRP3 inflammasome

Autophagy has been also shown to monitor IL-1β secretion in manners independent of the NLRP3 inflammasome. The NLRP3 inflammasome plays a major but not comprehensive role in the autophagic monitoring of IL-1β production.

Studies have shown that autophagy targets precursor IL-1β for degradation. Harris, et al. using bone marrow-derived macrophages (iBMM) and DCs (BMDC) from mice, showed that in addition to the monitoring of the NLRP3 inflammasome, autophagy regulates IL-1β by targeting precursor IL-1β for engulfment by autophagosomes and thus for subsequent lysosomal degradation [83]. This concept that autophagy monitors precursor IL-1β was partially supported by experiments that showed in ATG16L1 deficient murine macrophages, the forced induction of autophagy by rapamycin, leads to decreased levels of precursor IL-1β [83].

Autophagy has been shown to regulate IL-1β on the transcriptional level. Murine models have shown that impaired expression of Atg7 can be linked to increased levels of IL-1β mRNA [88]. Plantinga and Crisan showed that in human peripheral blood monocytes (PBMCs), autophagy regulates IL-1β in a manner independent of the NLRP3 inflammasome pathway and

dependent on gene transcription [64, 86, 90]. Their data shows increased IL-1β mRNA when autophagy is blocked and decreased IL-1β mRNA when autophagy is stimulated.

Plantinga et al., also showed that in response to the Nod2 ligand MDP, human peripheral blood mononuclear cells with ATG16L1 polymorphisms had increased levels of IL-1β mRNA expression [90]. These levels were shown to be independent of NLRP3 inflammasome activation, as no affect in the processing of precursor IL-1β by caspase-1 activation was found [90].

The actual mechanism of how autophagy regulates IL-1β transcription remains to be discovered. Though it is thought that this mechanism is likely linked to the blocking of p38 mitogen activated protein kinase (MAPK) phosphorylation [86]. Thus autophagy monitors IL-1β production through regulating: IL-1β transcription, precursor IL-1β levels and the processing of precursor IL-1β into its mature form. Accordingly defective autophagy leads to increased IL-1β production and ultimately increased activation of Th17 cells.

Defective autophagy heightens Il-1β signal transduction

Besides initiating autophagosome induction in autophagy and thus down regulating IL-1 production, ATG16L1 was recently found to negatively regulate IL-1 signaling. Lee et al showed that ATG16L1 deficient murine embryonic fibroblasts exhibited heightened IL-1 signal transduction cascades [100]. Functional ATG16L1 promotes the degradation of Nucleoporin 62 (p62), a protein complex in the nuclear envelope [101], through both autolysosomal and Cul-3-mediated proteasomal pathways [100]. This regulation of p62 is crucial to regulating the IL-1 signal transduction cascade, as higher numbers of p62 are directly linked to increased oligomerization and activation of the signal transducer, TRAF (TNF receptor associated factor) 6 [100].

In mouse embryonic fibroblasts the loss of ATG16L1 lead to the heightened activation of TRAF 6, which in turn lead to the increased activation of NF-kB and MAPK signaling in response to IL-1 [100]. This results in an increased inflammatory response that is the consequence of heightened IL-1 signal transduction. Because the signal transduction cascade is amplified in mouse embryonic fibroblasts with impaired expression of ATG16L1, significantly lower amounts of IL-1 would be needed to stimulate an inflammatory response than those without impaired expression of ATG16L1. [100] This implicates the ATG16L1 gene as responsible for an excessive inflammatory response to signals that would typically trigger a much smaller inflammatory response or fail to trigger any inflammatory response at all. As previously discussed IL-1 is potent activator of Th17 cells, and thus amplification of the IL-1 signal transduction cascade up regulates Th17 differentiation. Thus, defective expression of a key autophagy protein amplifies the IL-1 signal transduction cascade and potentially increases the up regulation of Th17 cells.

Defective autophagy leads to impaired clearance of Adherent-Invasive E. coli (AIEC), which results in increased production of TNF-α

Xenophagy plays a central role in the clearance of intracellular pathogens. Hence polymorphisms in xenophagy genes have been linked to defective clearance of intracellular pathogens. Numerous studies have reported high levels of a unique strain of *E. coli* in CD patients [102, 103]. This strain is marked by strong adherence and invasive properties and hence is referred to as adherent-invasive *E. coli* (AIEC).

AIEC are able to survive and replicate inside intestinal epithelial cells and macrophages [82, 104]. This replication is normally negated by functional xenophagy, as xenophagy works to degrade and remove intracellular pathogens. Indeed, the intracellular presence of AIEC

stimulates the association of xenophagic components around the point of endocytosis where the AIEC enter [82]. Impaired xenophagy leads to increased AIEC proliferation. Lapaquette et al., showed that dysfunctional expression of any of the three primary xenophagy genes linked to CD (NOD2, ATG16L1, or IRGM), leads to increased concentration of AIEC [82]. These experiments also showed that forced induction of xenophagy decreased the concentration of AIEC [82].

AIEC invade intestinal epithelial cells via a macropinocytosis-like process [105]. Once inside cells, AIEC begin to replicate inside acidic vacuolar phagolysosomes despite the presence of cathepsin D [104]. AIEC are able to survive and replicate without causing host cell death [102]. In fact, AIEC infections inhibit macrophages from undergoing programmed apoptosis [103, 106]. Interestingly AIEC proliferation up regulates the expression of TNF-α, which in turn activates Th17 cells.

As the intracellular concentration of AIEC in macrophages rises, the secretion of TNF-α increases [82, 102, 107]. The increased secretion of TNF-α correlates with increased replication of AIEC. The reasons why and the mechanism for how AIEC induces TNF-α secretion remains unclear. Yet it was found that the secretion of TNF-α was found to be primarily induced by AIEC in order to provide conditions favorable for their replication instead of by the host cell in an attempt to clear the AIEC [106].

Regardless of the reasons why and the mechanisms how, substantial evidence points to increased secretion of TNF-α by AIEC infected macrophages. This increase in TNF-α up regulates Th17 cell induction specifically through increasing claudin-2 levels and through the deactivation of the Foxp3 transcription factor.

Increased Claudin-2

Impaired clearance of AIEC results in the specific degradation of the intestinal barrier. Dysfunctional xenophagy of AIEC would lead to increased claudin-2 levels as the AIEC population up regulates TNF-α secretion. Increased claudin-2 expression amplifies intestinal permeability. Thus high levels of TNF-α increase intestinal permeability, which in turn leads to the induction of Th17 cells (and also allows for fecal-oral transmission of AIEC).

Defects in the intestinal barrier lead to increased Th17 cell induction. This results due to the increased contact of commensal and pathogenic microorganisms in the gut lumen with immune cells in the lamina propria. Increased T cell activation would result from the increased number of antigens antigen-presenting cells (APCs) would encounter. Likewise increased stimulation of immune cells, principally macrophages and Dendritic cells (DCs), would lead to secretion of pro-inflammatory cytokines, including TNF-α, IL-1 and IL-23, that would create a microenvironment that favors inflammation and promotes Th17 cell induction. This effect would be compounded by any genetic factors that would predispose the microenvironment to increased inflammation (example: ATG16L1 related defective regulation of IL-1 production or signaling). Thus defects in the intestinal barrier can lead to increased Th17 induction.

Increased polarization of Th17 cells through specific inhibition of Treg cell induction

As already noted, in addition to weakening the intestinal barrier, TNF- specifically deactivates the Treg transcription factor, Foxp3. TNF- is a crucial driver of Th17 polarization and increased levels of TNF-α would lead to increased levels of Th17 cells and decreased levels of Treg cells. Thus increased levels of AIEC leads to increased levels of TNF-, which in turn leads to increased levels of Th17 cells.

High levels of AIEC are linked to defective autophagy and lead to elevated levels of TNF-α. TNF-α promotes Th17 cell induction through degrading the intestinal barrier and through deactivating the Foxp3 transcription factor. Thus in the presence of AIEC, dysfunctional expression of autophagy genes leads to the specific activation of Th17 cells.

IL-10

Polymorphisms in the autophagy-associated gene NOD2, have the potential to activate Th17 cells in manners relatively independent of autophagy. Specifically, a certain polymorphism in NOD2 has been shown to down regulate IL-10 and, thus, to indirectly activate Th17 cells. IL-10 is pleiotropic cytokine with multiple regulatory effects [52]. Treg cells secrete IL-10 as one of their key effectors [108]. While monocytes, dendritic cells (DCs), mast cells, and macrophages secrete IL-10 upon stimulation of their pattern PRRs, including TLRs and NLRs. It was purported in 2009 that the 3020insC frameshift mutation in the NOD2 gene, down regulates IL-10 transcription through impeding the activation (phosphorylation) of the nuclear ribonucleoprotein hnRNP-A1 [3].

The study by Noguchi et al., is significant because it directly links a common CD mutation with down regulation of a key anti-inflammatory cytokine IL-10. Yet this study is not watertight. Clearly, this experiment showed that the NOD2 frame shift mutation results in decreased secretion of IL-10. Yet it failed to specify the levels of the secretion of other cytokines. Other studies have shown that defects in NOD2 lead to reduced secretion of various cytokines; less total production of all cytokines both pro-inflammatory and anti-inflammatory [73]. Thus further research is needed to implicate the NOD2 frame shift mutation with the specific impairment of IL-10 production. If the NOD2 frame shift mutation can be shown to specifically inhibit IL-10 then this autophagy related gene has the potential to drive Th17 induction through inhibiting IL-10.

IL-10 regulates CD inflammation

IL-10 plays an important role in intestinal homeostasis. Down regulation of IL-10 leads to reduced inhibition of inflammation and consequently, increased inflammation [55]. Accordingly reduced levels of IL-10 are found in CD patients [109]. Specifically very low levels of IL-10 correlate to severe manifestations of CD that often require multiple surgeries [109]. In addition, GWAS have linked both IL-10 promoter and IL-10 R polymorphisms to CD [110].

IL-10 maintains intestinal barrier through activation of IgA

IL-10 stimulates the production of the immunoglobulin A (IgA) antibody by plasma cells [52, 111]. IgA is present in the mucus layers of the gut and plays a critical role in maintaining the intestinal barrier [52]. Indeed, polymorphisms in IgA genes have been linked to CD [6]. Likewise, patients with active CD have low levels of both IL-10 and IgA [52, 109]. Thus, the NOD2 mediated down regulation of IL-10 results in decreased levels of IgA, which would negatively effect the intestinal barrier.

IL-10 enhances Treg induction

IL-10 also skews the differentiation of naïve $CD4^+$ T cells into Treg cells [56]. High levels of IL-10 lead to the differentiation of naïve $CD4^+$ T cells, in vitro, into specialized Treg1 (Foxp3 -) cells, which secrete very high levels of IL-10 [57]. Thus inhibition of IL-10 would lead to decreased Treg induction. Thus decreased levels of IL-10 leads to increased inflammatory conditions in general, defective intestinal barrier and specific impairment of Treg activation all of which favors Th17 cell activation.

Conclusion

This paper examined the ways autophagy linked proteins regulate IL-1 production and signaling, IL-10 production and intracellular bacterial levels. Autophagy monitors secretion of IL-1. Likewise defective autophagy leads to the increased levels of IL-1. The autophagic linked protein ATG16L1 monitors the IL-1 signal transduction cascade. Dysfunctional expression of ATG16L1 leads to the amplification of the IL-1 signal transduction cascade. The autophagic linked protein NOD2 monitors IL-10 production. Dysfunctional NOD2 has been purported to specifically down regulate IL-10. Autophagy, through xenophagy, controls intracellular bacterial levels. Defective autophagy leads to increased AIEC levels, which leads to the specific up regulation of TNF-α by AIEC.

Defective expression of autophagy-linked proteins can lead to the up regulation of the cytokines needed for Th17 induction (IL-1 and TNF-α) and the down regulation of a Th17 induction inhibiting cytokine, IL-10. In light of this, the specific linking of the autophagic proteins (ATG16L1, NOD2 and IRGM) to CD helps explain the deviant Th17 cell driven inflammation of CD.

This understanding should lead to the continued exploration and development of CD treatment that specifically restores defective autophagy. If defective autophagy can be restored Th17 induction would likely be reduced. Restored autophagy would down regulate IL-1 production, decrease the levels of the TNF-α inducing AIEC and reduce organelle stress. One known enhancer of autophagy is a compound called rapamycin [112]. Indeed, the administration of rapamycin analogues, including sirolimus and everolimus, has led to reduced CD symptoms [112-114]. By clarifying the manner in which defective autophagy induces Th17 cells, this paper encourages the continued exploration of potential CD treatment involving autophagy.

1. Molodecky, N.A., et al., *Increasing incidence and prevalence of the inflammatory bowel diseases with time, based on systematic review*. Gastroenterology, 2012. **142**(1): p. 46-54 e42; quiz e30.
2. Lakatos, P.L., *Recent trends in the epidemiology of inflammatory bowel diseases: up or down?* World J Gastroenterol, 2006. **12**(38): p. 6102-8.
3. Noguchi, E., et al., *A Crohn's disease-associated NOD2 mutation suppresses transcription of human IL10 by inhibiting activity of the nuclear ribonucleoprotein hnRNP-A1*. Nat Immunol, 2009. **10**(5): p. 471-9.
4. Kastelein, R.A., C.A. Hunter, and D.J. Cua, *Discovery and biology of IL-23 and IL-27: related but functionally distinct regulators of inflammation*. Annu Rev Immunol, 2007. **25**: p. 221-42.
5. Annunziato, F., et al., *Phenotypic and functional features of human Th17 cells*. J Exp Med, 2007. **204**(8): p. 1849-61.
6. Franke, A., et al., *Genome-wide meta-analysis increases to 71 the number of confirmed Crohn's disease susceptibility loci*. Nat Genet, 2010. **42**(12): p. 1118-25.
7. Tremelling, M., et al., *IL23R variation determines susceptibility but not disease phenotype in inflammatory bowel disease*. Gastroenterology, 2007. **132**(5): p. 1657-64.
8. Kleinschek, M.A., et al., *Circulating and gut-resident human Th17 cells express CD161 and promote intestinal inflammation*. J Exp Med, 2009. **206**(3): p. 525-34.
9. Fujino, S., et al., *Increased expression of interleukin 17 in inflammatory bowel disease*. Gut, 2003. **52**(1): p. 65-70.
10. Monteleone, G., et al., *Interleukin-21 enhances T-helper cell type I signaling and interferon-gamma production in Crohn's disease*. Gastroenterology, 2005. **128**(3): p. 687-94.
11. Schmechel, S., et al., *Linking genetic susceptibility to Crohn's disease with Th17 cell function: IL-22 serum levels are increased in Crohn's disease and correlate with disease activity and IL23R genotype status*. Inflamm Bowel Dis, 2008. **14**(2): p. 204-12.
12. Brand, S., et al., *IL-22 is increased in active Crohn's disease and promotes proinflammatory gene expression and intestinal epithelial cell migration*. Am J Physiol Gastrointest Liver Physiol, 2006. **290**(4): p. G827-38.
13. Fossiez, F., et al., T cell interleukin-17 induces stromal cells to produce proinflammatory and hematopoietic cytokines. J Exp Med, 1996. **183**(6): p. 2593-603.
14. Monteleone, G., et al., Control of matrix metalloproteinase production in human intestinal fibroblasts by interleukin 21. Gut, 2006. **55**(12): p. 1774-80.
15. Andoh, A., et al., Interleukin-22, a member of the IL-10 subfamily, induces inflammatory responses in colonic subepithelial myofibroblasts. Gastroenterology, 2005. **129**(3): p. 969-84.
16. Altadill, A., et al., Comparative analysis of the expression of metalloproteases and their inhibitors in resected crohn's disease and complicated diverticular disease. Inflamm Bowel Dis, 2012. **18**(1): p. 120-30.
17. Sela-Passwell, N., et al., Antibodies targeting the catalytic zinc complex of activated matrix metalloproteinases show therapeutic potential. Nat Med, 2012. **18**(1): p. 143-7.
18. Iwakura, Y., et al., *Functional specialization of interleukin-17 family members*. Immunity, 2011. **34**(2): p. 149-62.
19. Caruso, R., et al., *A functional role for interleukin-21 in promoting the synthesis of the T-cell chemoattractant, MIP-3alpha, by gut epithelial cells*. Gastroenterology, 2007. **132**(1): p. 166-75.

20. Peluso, I., et al., *IL-21 counteracts the regulatory T cell-mediated suppression of human CD4+ T lymphocytes*. J Immunol, 2007. **178**(2): p. 732-9.
21. Wolk, K., et al., *IL-22 increases the innate immunity of tissues*. Immunity, 2004. **21**(2): p. 241-54.
22. Dumoutier, L., et al., *Human interleukin-10-related T cell-derived inducible factor: molecular cloning and functional characterization as an hepatocyte-stimulating factor.* Proc Natl Acad Sci U S A, 2000. **97**(18): p. 10144-9.
23. Shevach, E.M., *Regulatory T cells in autoimmmunity**. Annu Rev Immunol, 2000. **18**: p. 423-49.
24. Shevach, E.M., *Mechanisms of foxp3+ T regulatory cell-mediated suppression*. Immunity, 2009. **30**(5): p. 636-45.
25. Ricciardelli, I., et al., *Anti tumour necrosis-alpha therapy increases the number of FOXP3 regulatory T cells in children affected by Crohn's disease*. Immunology, 2008. **125**(2): p. 178-83.
26. Chen, W., et al., *Conversion of peripheral CD4+CD25- naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3*. J Exp Med, 2003. **198**(12): p. 1875-86.
27. Ivanov, II, et al., *The orphan nuclear receptor RORgammat directs the differentiation program of proinflammatory IL-17+ T helper cells*. Cell, 2006. **126**(6): p. 1121-33.
28. Veldhoen, M., et al., *TGFbeta in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells*. Immunity, 2006. **24**(2): p. 179-89.
29. Shaw, M.H., et al., *Microbiota-induced IL-1beta, but not IL-6, is critical for the development of steady-state TH17 cells in the intestine*. J Exp Med, 2012. **209**(2): p. 251-8.
30. Zhou, L., et al., *TGF-beta-induced Foxp3 inhibits T(H)17 cell differentiation by antagonizing RORgammat function*. Nature, 2008. **453**(7192): p. 236-40.
31. Chung, Y., et al., *Critical regulation of early Th17 cell differentiation by interleukin-1 signaling*. Immunity, 2009. **30**(4): p. 576-87.
32. Nie, H., et al., *Phosphorylation of FOXP3 controls regulatory T cell function and is inhibited by TNF-alpha in rheumatoid arthritis*. Nat Med, 2013. **19**(3): p. 322-8.
33. Sutton, C., et al., *A crucial role for interleukin (IL)-1 in the induction of IL-17-producing T cells that mediate autoimmune encephalomyelitis*. J Exp Med, 2006. **203**(7): p. 1685-91.
34. Coccia, M., et al., *IL-1beta mediates chronic intestinal inflammation by promoting the accumulation of IL-17A secreting innate lymphoid cells and CD4(+) Th17 cells*. J Exp Med, 2012. **209**(9): p. 1595-609.
35. Mahida, Y.R., K. Wu, and D.P. Jewell, *Enhanced production of interleukin 1-beta by mononuclear cells isolated from mucosa with active ulcerative colitis of Crohn's disease*. Gut, 1989. **30**(6): p. 835-8.
36. Reimund, J.M., et al., *Increased production of tumour necrosis factor-alpha interleukin-1 beta, and interleukin-6 by morphologically normal intestinal biopsies from patients with Crohn's disease*. Gut, 1996. **39**(5): p. 684-9.
37. Szczeklik, K., et al., *Proinflammatory cytokines in the saliva of patients with active and non-active Crohn's disease*. Pol Arch Med Wewn, 2012. **122**(5): p. 200-8.
38. Butler, D.M., et al., *Modulation of proinflammatory cytokine release in rheumatoid synovial membrane cell cultures. Comparison of monoclonal anti TNF-alpha antibody with the interleukin-1 receptor antagonist*. Eur Cytokine Netw, 1995. **6**(4): p. 225-30.
39. Saitoh, T., et al., *Loss of the autophagy protein Atg16L1 enhances endotoxin-induced IL-1beta production*. Nature, 2008. **456**(7219): p. 264-8.
40. Sims, J.E. and D.E. Smith, *The IL-1 family: regulators of immunity*. Nat Rev Immunol, 2010. **10**(2): p. 89-102.

41. Brereton, C.F., et al., *Escherichia coli heat-labile enterotoxin promotes protective Th17 responses against infection by driving innate IL-1 and IL-23 production*. J Immunol, 2011. **186**(10): p. 5896-906.

42. Gabay, C., C. Lamacchia, and G. Palmer, *IL-1 pathways in inflammation and human diseases*. Nat Rev Rheumatol, 2010. **6**(4): p. 232-41.

43. Sutton, C.E., et al., *Interleukin-1 and IL-23 induce innate IL-17 production from gammadelta T cells, amplifying Th17 responses and autoimmunity*. Immunity, 2009. **31**(2): p. 331-41.

44. van Beelen, A.J., et al., *Stimulation of the intracellular bacterial sensor NOD2 programs dendritic cells to promote interleukin-17 production in human memory T cells*. Immunity, 2007. **27**(4): p. 660-9.

45. Caprioli, F., et al., *Reduction of CD68+ Macrophages and Decreased IL-17 Expression in Intestinal Mucosa of Patients with Inflammatory Bowel Disease Strongly Correlate With Endoscopic Response and Mucosal Healing following Infliximab Therapy*. Inflamm Bowel Dis, 2013. **19**(4): p. 729-739.

46. Brustle, A., et al., *The development of inflammatory T(H)-17 cells requires interferon-regulatory factor 4*. Nat Immunol, 2007. **8**(9): p. 958-66.

47. McKenzie, B.S., R.A. Kastelein, and D.J. Cua, *Understanding the IL-23-IL-17 immune pathway*. Trends Immunol, 2006. **27**(1): p. 17-23.

48. Duerr, R.H., et al., *A genome-wide association study identifies IL23R as an inflammatory bowel disease gene*. Science, 2006. **314**(5804): p. 1461-3.

49. Schmidt, C., et al., *Expression of interleukin-12-related cytokine transcripts in inflammatory bowel disease: elevated interleukin-23p19 and interleukin-27p28 in Crohn's disease but not in ulcerative colitis*. Inflamm Bowel Dis, 2005. **11**(1): p. 16-23.

50. Liu, Z., et al., *The increased expression of IL-23 in inflammatory bowel disease promotes intraepithelial and lamina propria lymphocyte inflammatory responses and cytotoxicity*. J Leukoc Biol, 2011. **89**(4): p. 597-606.

51. Mannon, P.J., et al., *Anti-interleukin-12 antibody for active Crohn's disease*. N Engl J Med, 2004. **351**(20): p. 2069-79.

52. Liu, Z., et al., *Interleukin (IL)-23 suppresses IL-10 in inflammatory bowel disease*. J Biol Chem, 2012. **287**(5): p. 3591-7.

53. Doisne, J.M., et al., *Cutting edge: crucial role of IL-1 and IL-23 in the innate IL-17 response of peripheral lymph node NK1.1- invariant NKT cells to bacteria*. J Immunol, 2011. **186**(2): p. 662-6.

54. Siakavellas, S.I. and G. Bamias, *Role of the IL-23/IL-17 axis in Crohn's disease*. Discov Med, 2012. **14**(77): p. 253-62.

55. Bazzoni, F., et al., *Understanding the molecular mechanisms of the multifaceted IL-10-mediated anti-inflammatory response: lessons from neutrophils*. Eur J Immunol, 2010. **40**(9): p. 2360-8.

56. Fiorentino, D.F., M.W. Bond, and T.R. Mosmann, *Two types of mouse T helper cell. IV. Th2 clones secrete a factor that inhibits cytokine production by Th1 clones*. J Exp Med, 1989. **170**(6): p. 2081-95.

57. Roncarolo, M.G., et al., *Interleukin-10-secreting type 1 regulatory T cells in rodents and humans*. Immunol Rev, 2006. **212**: p. 28-50.

58. Hirota, K., et al., *Fate mapping of IL-17-producing T cells in inflammatory responses*. Nat Immunol, 2011. **12**(3): p. 255-63.

59. Valencia, X., et al., *TNF downmodulates the function of human CD4+CD25hi T-regulatory cells*. Blood, 2006. **108**(1): p. 253-61.

60. Yorimitsu, T., et al., *Endoplasmic reticulum stress triggers autophagy*. J Biol Chem, 2006. **281**(40): p. 30299-304.

61. King, J.S., D.M. Veltman, and R.H. Insall, *The induction of autophagy by mechanical stress*.

Autophagy, 2011. **7**(12): p. 1490-9.

62. Papandreou, I., et al., *Hypoxia signals autophagy in tumor cells via AMPK activity, independent of HIF-1, BNIP3, and BNIP3L*. Cell Death Differ, 2008. **15**(10): p. 1572-81.
63. Onodera, J. and Y. Ohsumi, *Autophagy is required for maintenance of amino acid levels and protein synthesis under nitrogen starvation*. J Biol Chem, 2005. **280**(36): p. 31582-6.
64. Plantinga, T.S., et al., *Modulation of inflammation by autophagy: consequences for Crohn's disease*. Curr Opin Pharmacol, 2012. **12**(4): p. 497-502.
65. Singh, S.B., et al., *Human IRGM induces autophagy to eliminate intracellular mycobacteria*. Science, 2006. **313**(5792): p. 1438-41.
66. Brest, P., et al., *A synonymous variant in IRGM alters a binding site for miR-196 and causes deregulation of IRGM-dependent xenophagy in Crohn's disease*. Nat Genet, 2011. **43**(3): p. 242-5.
67. Ogawa, M., et al., *Escape of intracellular Shigella from autophagy*. Science, 2005. **307**(5710): p. 727-31.
68. Biswas, D., et al., *ATP-induced autophagy is associated with rapid killing of intracellular mycobacteria within human monocytes/macrophages*. BMC Immunol, 2008. **9**: p. 35.
69. Alonso, S., et al., *Lysosomal killing of Mycobacterium mediated by ubiquitin-derived peptides is enhanced by autophagy*. Proc Natl Acad Sci U S A, 2007. **104**(14): p. 6031-6.
70. Klionsky, D.J., *Autophagy: from phenomenology to molecular understanding in less than a decade*. Nat Rev Mol Cell Biol, 2007. **8**(11): p. 931-7.
71. Baumgart, D.C. and W.J. Sandborn, *Crohn's disease*. Lancet, 2012. **380**(9853): p. 1590-605.
72. Jostins, L., et al., *Host-microbe interactions have shaped the genetic architecture of inflammatory bowel disease*. Nature, 2012. **491**(7422): p. 119-24.
73. Strober, W. and T. Watanabe, *NOD2, an intracellular innate immune sensor involved in host defense and Crohn's disease*. Mucosal Immunol, 2011. **4**(5): p. 484-95.
74. Seiderer, J., et al., *Predictive value of the CARD15 variant 1007fs for the diagnosis of intestinal stenoses and the need for surgery in Crohn's disease in clinical practice: results of a prospective study*. Inflamm Bowel Dis, 2006. **12**(12): p. 1114-21.
75. Cooney, R., et al., *NOD2 stimulation induces autophagy in dendritic cells influencing bacterial handling and antigen presentation*. Nat Med, 2010. **16**(1): p. 90-7.
76. Travassos, L.H., et al., *Nod1 and Nod2 direct autophagy by recruiting ATG16L1 to the plasma membrane at the site of bacterial entry*. Nat Immunol, 2010. **11**(1): p. 55-62.
77. Homer, C.R., et al., *ATG16L1 and NOD2 interact in an autophagy-dependent antibacterial pathway implicated in Crohn's disease pathogenesis*. Gastroenterology, 2010. **139**(5): p. 1630-41, 1641 e1-2.
78. Hampe, J., et al., *A genome-wide association scan of nonsynonymous SNPs identifies a susceptibility variant for Crohn disease in ATG16L1*. Nat Genet, 2007. **39**(2): p. 207-11.
79. Fujita, N., et al., *The Atg16L complex specifies the site of LC3 lipidation for membrane biogenesis in autophagy*. Mol Biol Cell, 2008. **19**(5): p. 2092-100.
80. Kuballa, P., et al., *Impaired autophagy of an intracellular pathogen induced by a Crohn's disease associated ATG16L1 variant*. PLoS One, 2008. **3**(10): p. e3391.
81. Glas, J., et al., *IRGM variants and susceptibility to inflammatory bowel disease in the German population*. PLoS One, 2013. **8**(1): p. e54338.
82. Lapaquette, P., M.A. Bringer, and A. Darfeuille-Michaud, *Defects in autophagy favour adherent-invasive Escherichia coli persistence within macrophages leading to increased pro-inflammatory response*. Cell Microbiol, 2012. **14**(6): p. 791-807.
83. Harris, J., et al., *Autophagy controls IL-1beta secretion by targeting pro-IL-1beta for degradation*. J Biol Chem, 2011. **286**(11): p. 9587-97.
84. He, C. and B. Levine, *The Beclin 1 interactome*. Curr Opin Cell Biol, 2010. **22**(2): p. 140-9.

85. Wu, Y., et al., *Synthesis and screening of 3-MA derivatives for autophagy inhibitors*. Autophagy, 2013. **9**(4).

86. Crisan, T.O., et al., *Inflammasome-independent modulation of cytokine response by autophagy in human cells*. PLoS One, 2011. **6**(4): p. e18666.

87. Nakahira, K., et al., *Autophagy proteins regulate innate immune responses by inhibiting the release of mitochondrial DNA mediated by the NALP3 inflammasome*. Nat Immunol, 2011. **12**(3): p. 222-30.

88. Fujishima, Y., et al., *Autophagy in the intestinal epithelium reduces endotoxin-induced inflammatory responses by inhibiting NF-kappaB activation*. Arch Biochem Biophys, 2011. **506**(2): p. 223-35.

89. Netea, M.G., et al., *The role of NLRs and TLRs in the activation of the inflammasome*. Expert Opin Biol Ther, 2008. **8**(12): p. 1867-72.

90. Plantinga, T.S., et al., *Crohn's disease-associated ATG16L1 polymorphism modulates pro-inflammatory cytokine responses selectively upon activation of NOD2*. Gut, 2011. **60**(9): p. 1229-35.

91. Baeuerle, P.A. and T. Henkel, *Function and activation of NF-kappa B in the immune system*. Annu Rev Immunol, 1994. **12**: p. 141-79.

92. Siegmund, B., et al., *IL-1 beta -converting enzyme (caspase-1) in intestinal inflammation*. Proc Natl Acad Sci U S A, 2001. **98**(23): p. 13249-54.

93. Thornberry, N.A., et al., *A novel heterodimeric cysteine protease is required for interleukin-1 beta processing in monocytes*. Nature, 1992. **356**(6372): p. 768-74.

94. Zhou, R., et al., *A role for mitochondria in NLRP3 inflammasome activation*. Nature, 2011. **469**(7329): p. 221-5.

95. Hornung, V., et al., *Silica crystals and aluminum salts activate the NALP3 inflammasome through phagosomal destabilization*. Nat Immunol, 2008. **9**(8): p. 847-56.

96. Zorzi, F., et al., *Distinct profiles of effector cytokines mark the different phases of Crohn's disease*. PLoS One, 2013. **8**(1): p. e54562.

97. Schulze-Osthoff, K., et al., *Cytotoxic activity of tumor necrosis factor is mediated by early damage of mitochondrial functions. Evidence for the involvement of mitochondrial radical generation*. J Biol Chem, 1992. **267**(8): p. 5317-23.

98. Baregamian, N., et al., *Tumor necrosis factor-alpha and apoptosis signal-regulating kinase 1 control reactive oxygen species release, mitochondrial autophagy, and c-Jun N-terminal kinase/p38 phosphorylation during necrotizing enterocolitis*. Oxid Med Cell Longev, 2009. **2**(5): p. 297-306.

99. Xue, X., et al., *Tumor necrosis factor alpha (TNFalpha) induces the unfolded protein response (UPR) in a reactive oxygen species (ROS)-dependent fashion, and the UPR counteracts ROS accumulation by TNFalpha*. J Biol Chem, 2005. **280**(40): p. 33917-25.

100. Lee, J., et al., *Autophagy suppresses interleukin-1beta (IL-1beta) signaling by activation of p62 degradation via lysosomal and proteasomal pathways*. J Biol Chem, 2012. **287**(6): p. 4033-40.

101. Sanz, L., et al., *The atypical PKC-interacting protein p62 channels NF-kappaB activation by the IL-1-TRAF6 pathway*. EMBO J, 2000. **19**(7): p. 1576-86.

102. Rolhion, N. and A. Darfeuille-Michaud, *Adherent-invasive Escherichia coli in inflammatory bowel disease*. Inflamm Bowel Dis, 2007. **13**(10): p. 1277-83.

103. Glasser, A.L., et al., *Adherent invasive Escherichia coli strains from patients with Crohn's disease survive and replicate within macrophages without inducing host cell death*. Infect Immun, 2001. **69**(9): p. 5529-37.

104. Bringer, M.A., et al., *The Crohn's disease-associated adherent-invasive Escherichia coli strain LF82 replicates in mature phagolysosomes within J774 macrophages*. Cell Microbiol, 2006. **8**(3): p. 471-84.

105. Darfeuille-Michaud, A., *Adherent-invasive Escherichia coli: a putative new E. coli*

pathotype associated with Crohn's disease. Int J Med Microbiol, 2002. **292**(3-4): p. 185-93.

106. Bringer, M.A., et al., *Replication of Crohn's disease-associated AIEC within macrophages is dependent on TNF-alpha secretion*. Lab Invest, 2012. **92**(3): p. 411-9.
107. Negroni, A., et al., *Characterization of adherent-invasive Escherichia coli isolated from pediatric patients with inflammatory bowel disease*. Inflamm Bowel Dis, 2012. **18**(5): p. 913-24.
108. Asseman, C., et al., *An essential role for interleukin 10 in the function of regulatory T cells that inhibit intestinal inflammation*. J Exp Med, 1999. **190**(7): p. 995-1004.
109. Correa, I., et al., *Defective IL-10 production in severe phenotypes of Crohn's disease*. J Leukoc Biol, 2009. **85**(5): p. 896-903.
110. Glocker, E.O., et al., *Inflammatory bowel disease and mutations affecting the interleukin-10 receptor.* N Engl J Med, 2009. **361**(21): p. 2033-45.
111. Napimoga, M.H., et al., *Possible involvement of IL-21 and IL-10 on salivary IgA levels in chronic periodontitis subjects*. Scand J Immunol, 2011. **74**(6): p. 596-602.
112. Massey, D.C., F. Bredin, and M. Parkes, *Use of sirolimus (rapamycin) to treat refractory Crohn's disease*. Gut, 2008. **57**(9): p. 1294-6.
113. Dumortier, J., et al., *Everolimus for refractory Crohn's disease: a case report*. Inflamm Bowel Dis, 2008. **14**(6): p. 874-7.
114. Nguyen, H.T., et al., *Autophagy and Crohn's Disease*. J Innate Immun, 2013.