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Towards therapeutic disease control in inflammatory bowel diseases

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Chapter three

Dendritic cell autophagy attenuates adaptive immune responses by destabilization of the immunological synapse

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Abstract

Autophagy-related gene variants are associated with the development of Crohn's disease, an aberrant inflammatory response to the intestinal flora. How autophagy is linked to the loss of immune tolerance has not been established. We demonstrate that autophagy regulates the stability of the immunological synapse in dendritic cells (DC). Autophagy is induced upon formation of the immunological synapse, and autophagosomes preferentially localize to the site of interaction. Autophagosomes engulf synaptic components such as MHCII and ICAM-1, targeting them for degradation. Knockdown of the autophagy-related genes *ATG16L1* and *IRGM* in DC results in hyperstable DC-T cell interactions, leading to increased T cell signaling and Th17 differentiation. Similar aberrations were observed in DC obtained from patients with Crohn's disease carrying the disease associated polymorphisms in *ATG16L1*. Our findings show that autophagy is induced upon formation of the immunological synapse and acts as a negative regulator of T cell activation and Th17 differentiation.

Introduction

A number of genome-wide association studies have associated autophagy-related genes *ATG16L1* and *IRGM* with the development of Crohn's disease, an inflammatory bowel disease.¹³ During autophagy, the cell envelops damaged or excess proteins with a lipid bilayer, thus creating an autophagosome. The autophagosome then fuses with a lysosome, resulting in the degradation of the protein and the release of free amino acids. Although the pathway was initially described as a cell survival mechanism during nutrient depletion,⁴ more recent studies show that the process is also involved in the proper function of various immune cells, including pathogen degradation and antigen processing by phagocytes.⁵⁻⁷ The Crohn's disease risk alleles result in decreased autophagic function, suggesting that autophagy is required to limit the intestinal immune response. Mice expressing a hypomorphic variant of *ATG16L1* display aberrant Paneth cell morphology and decreased secretion of anti-microbial proteins into the intestinal lumen.⁸ Additionally, autophagy is involved in the killing of intracellular pathogens by phagocytes, including *Salmonella enterica* and *Mycobacterium tuberculosis*. This suggested that the effects of autophagy on intestinal inflammation are secondary, as impaired autophagy leads to hampered innate immune responses and microbial overgrowth, which in turn results in hyperstimulation of the intestinal immune system. However, we hypothesized that autophagy also has a direct effect in the regulation of immunity.

Dendritic cells (DC) are key players in the maintenance of immune homeostasis, as they link the innate and the adaptive immune system and can drive both immunogenic and tolerogenic responses. The mechanisms by which DC can dampen immune responses include secretion of cytokines which induce Foxp3+ regulatory T cells and skew the T cell response to a less damaging subtype.^{9,10} Additionally, a lack of costimulatory molecules or expression of inhibitory proteins such as IDO and PD-L1 can lead to T cell anergy and thus also limit immunogenic responses.^{11,12} In contrast to the volume of data on these topics, the involvement of the immunological synapse in the regulation of immune responses by DC has been studied to a limited degree.

The immunological synapse is the site of contact between DC and T cells and is characterized by a highly organized structure containing molecules involved in antigen recognition such as MHC class II and the T cell receptor and adhesion molecules ICAM-1 and LFA-1.¹³ The proper formation is important for the efficacy and outcome of the ensuing immune response, and synaptic destabilization has been shown to result in decreased T cell signaling.¹⁴ Although the formation of the immunological synapse as well as the signaling pathways involved have been described in some detail from the T cell side, the regulation of synapse formation in DC has remained largely unexplored.

Given the pivotal role of DC and DC-T cell interactions in immunity and the role of autophagy in the control of intestinal inflammation, we studied the role of autophagy in DC, in particular during cell-cell interactions. We report here that autophagy is induced in DC upon formation of an immunological synapse. Autophagosomes localize preferentially towards the site of interaction and engulf components of the synapse. DC in which expression of the autophagy-related genes *ATG16L1* or *IRGM* is decreased show increased stability of the immunological synapse, enhanced stimulation of T cell proliferation and increased Th17 activation. Our data suggest that autophagy acts as a negative feedback mechanism in

DC-T cell interactions and that a loss of this negative feedback mechanism may be a contributing factor in the pathogenesis of Crohn's disease.

Material and methods

Mice

C57BL/6-Tg(TcraTcrb)₄₂₅Cbn/J (OT-II) mice carrying the OVA₃₂₃₋₃₃₉ specific T cell receptor were obtained from Jackson Laboratories (Bar Harbor, ME) and supplied with water and standard chow ad libitum. Experimental procedures were approved by the local Animal Ethics Committee in accordance with national guidelines.

Antibodies, immunofluorescence and flow cytometry

Anti-IFN γ , anti-IL-17 and anti-IL-4 were obtained from Miltenyi Biotec (Bergisch Gladbach, Germany). Anti-CD40, CD80, CD83, CD86, CD209, anti-HLA-DR-FITC and isotype controls were obtained from BD Biosciences (San Jose, CA). Anti-HLR-DRbeta and anti-LAMP2 were obtained from Abcam (Cambridge, MA), anti-GFP, anti-mouse AlexaFluor 546, anti-rat AlexaFluor 568 and anti-rabbit-AlexaFluor 488 from Invitrogen (Carlsbad, CA). For immunofluorescence, cells were adhered to poly-L-lysine (Sigma Aldrich, Deisenhofen, Germany) coated coverslips, fixed in 4% paraformaldehyde, stained in permeabilisation buffer (PBS containing 0.05% Triton X-100) and embedded in SlowFade Gold (Invitrogen). Images were obtained on a Leica TCS SP2 confocal system equipped with 488 nm argon and 543 HeNe lasers (Leica, Mannheim, Germany) and processed using ImageJ software. For flow cytometry of surface markers, cells were washed and stained in PBS/0.5% BSA, washed again and fixed in 2% PFA. For cytokine staining, cells were washed in PBS, fixed in 2% PFA, permeabilized and stained in 0.5% saponin and washed in PBS/0.5% BSA. All samples were analyzed using a FACSCalibur (BD Bioscience) and FlowJo Software (Tree-star, Ashland, OR).

For immunoblotting the following antibodies were used: anti-human p62 lck ligand (BD), anti-GFP serum (Invitrogen), anti-pLAT Tyr191 (Cell Signaling, Beverly, MA).

Cell isolation and generation of dendritic cells

Monocytes and lymphocytes were isolated from buffy coats using Ficoll and Percoll density gradients according to a previously described protocol.¹⁵ For generation of human DC, monocytes were then cultured for 6-8 days in AIM-V culture medium (Invitrogen) in the presence of recombinant human GM-CSF and IL-4 (both 100 ng/ml, RnD Systems, Inc. Minneapolis, MN).

For patient studies, Crohn's disease patients were genotyped for rs_2241880 (ATG16L1) and rs_5743293 (NOD2) using polymerase chain reaction restriction fragment length polymorphisms. Inclusion criteria were homozygosity for either the risk or wild type allele of ATG16L1 and homozygosity for the wild type allele of NOD2. Venous heparinized blood was obtained and monocytes were isolated using Ficoll density gradient followed by magnetic bead isolation (Miltenyi). Monocytes were then used for further analysis and culture. All patients gave informed consent and the study was approved by the Ethical Review Committee.

For generation of mouse dendritic cells (BMDC), bone marrow was isolated from femur and cultured in RPMI 1640 culture medium (Invitrogen), in the presence of 10% FCS and 20 ng/ml recombinant mouse GM-CSF (RnD Systems Inc.) for 7-9 days.

Transfection of dendritic cells

ON-TargetPlus ATG16L1, IRGM and NOD2 specific siRNA pools were obtained from Dharmacon (Epsom, United Kingdom), and control non-specific siRNA from Ambion (Austin, Texas). All were transfected using Dharmafect 4 (Dharmacon) reagent according to the manufacturer's protocol. Plasmid encoding eGFP-LC3 fusion protein was described previously (Addgene plasmid 11546¹⁶). To generate the plasmid encoding the SNAP-ICAM-1 fusion protein, human ICAM-1 was amplified by PCR from Addgene plasmid 8632¹⁷ using targeted primers (forward CAGGCGCGCCAGCTCCCAGCAGCCCCCGG, reverse CAGGATCCTCAGGGAGGCGTGGCTTG) containing BamHI and ASCI restriction sites and cloned into the pSNAP-Tag vector (New England Biolabs, Ipswich, MA). All plasmids were transfected using Lipofectamine 2000 reagent (Invitrogen). Surface expression of SNAP-ICAM was visualized by staining of transfected DC with SNAP-Surface 549 cell impermeant fluorescent dye (New England Biolabs) according to the manufacturer's suggestions.

RNA isolation and quantitative pcr

RNA was isolated using the RNAeasy mini kit (Qiagen, Hilden, Germany) and cDNA was generated using RevertAid reverse transcriptase (Fermentas, St Leon-Rot, Germany) and random hexamer primers (Promega, Leiden, The Netherlands). Quantitative pcr reactions were carried out using Quantitekt primer assays and SybrGreen PCR Kit (both Qiagen). For relative expression, all data was normalized against expression of the household gene GAPDH.

DC-T cell cluster formation and analysis

DC-T cell clusters were induced as described previously.¹⁴ Briefly, DC and T cells were centrifuged together at 50xg in a 15 ml conical tube and incubated at 37°C for times indicated. Where indicated DC were pre-treated with 10 mM 3-methyladenine (Sigma Aldrich, Deisenhofen, Germany) for 2 hours at 37 °C after which cells were washed carefully. For antigen specific interactions, OVA₃₂₃₋₃₃₉ and control OVA₂₅₇₋₂₆₄ peptide (2 µg/ml, both AnaSpec Inc, San Jose, CA) were added. For Western Blot analysis cells were then lysed in lysisbuffer (Cell Signaling Technology, Beverly, MA) containing Protease Inhibitor Cocktail (MP Bio-medicals Inc., Solon, OH), and homogenized by ultrasound sonication. Samples were run on SDS-PAGE gels under reducing conditions and transferred to an Immobilon-P membrane (Millipore, Billerica, MA). Membranes were blocked by incubation in 5% blocking powder (Bio-Rad, Hercules, CA) and incubated with primary and secondary antibodies in 1% blocking powder (Biorad). Expression was detected by Lumilight Plus (Roche, Woerden, The Netherlands) and image analysis was performed using ImageJ software. For analysis of cytoskeletal polarization, clusters were plated on Poly-L-lysine (Sigma) coated slides and fixed in 4% paraformaldehyde. Polarization of the cytoskeleton was visualized using phalloidin-AlexaFluor 488 (Invitrogen) and scored on a three point scale by two independent observers.

Mixed lymphocyte reaction and Th subset induction

For mixed lymphocyte reactions, DC were co-cultured with allogeneic lymphocytes for 72 hours. For OVA specific reactions, BMDC were cocultured with isolated OTII splenocytes in the presence of OVA₃₂₃₋₃₃₉ peptide for 72 hours. Proliferation was measured by ³H-thymidine incorporation assay.

For analysis of Th subset induction, CD45RO⁺ T cells were isolated from peripheral blood using magnetic beads (Miltenyi Biotec) and co-cultured with autologous DC in the presence or absence of anti-IFN γ (10 μ g/mL, RnD Systems) for 4 days and restimulated during 4-6 hours using Phorbol Myristate Acetate and ionomycin (both Sigma) in the presence of Golgistop (BD Biosciences).

Time lapse analysis

DC were plated on glass bottom microwell dishes (MatTek Corp. Ashland, MA) and allowed to adhere for 2 hours at 37°C. When appropriate, OVA peptide was added during adhesion. Supernatant was removed and lymphocytes (human: peripheral blood lymphocytes, mouse: OT-II spleen cells) were added to the plates and allowed to settle for 1 minute. Sequential images were then taken every 20 seconds for 30 minutes using a Zeiss Axiovert 200M inverted microscope and processed using OpenLab 3.09 software (Perkin Elmer, Waltham, MA). The resulting image sequences were analysed by two independent observers.

Statistics

Statistical analyses were performed using SPSS (SPSS Inc., Chicago, IL) or GraphPad (Graphpad software Inc., La Jolla, CA). Analyses included Mann-Whitney U test (non paired data), Wilcoxon square rank test (paired data), Kruskal Wallis test followed by Dunn's multiple comparisons (multiple comparisons) and Spearman's Rho (correlations). Data was considered significant if $p < 0.05$.

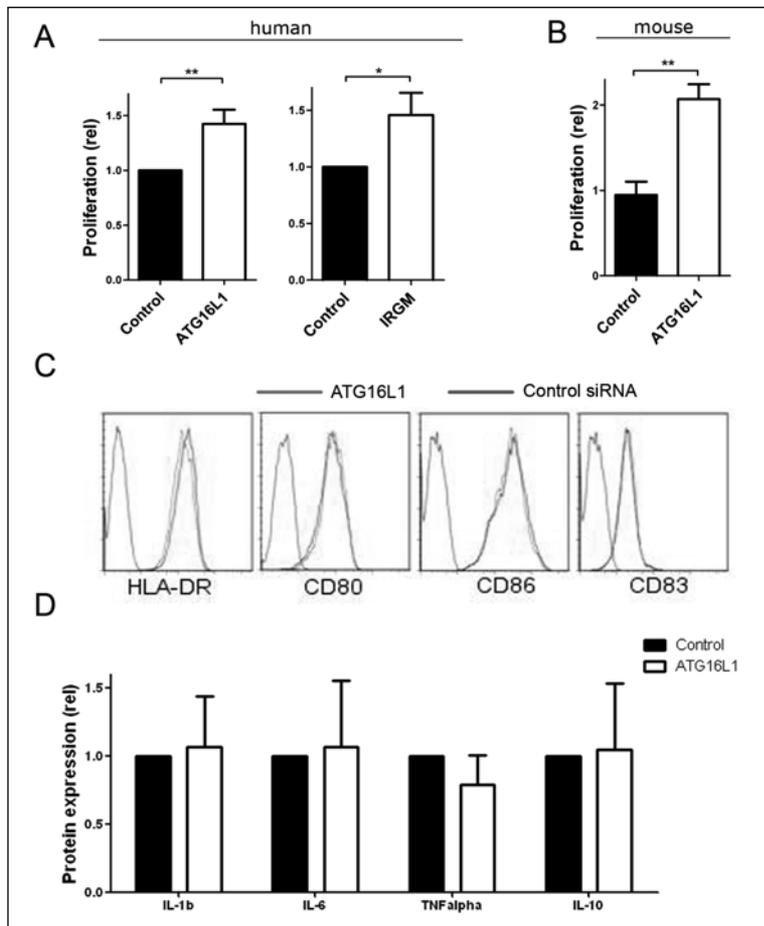
Results

Decreased DC autophagy results in increased T cell stimulatory capacity

Expression of ATG16L1 was decreased in human DC using siRNA technology and confirmed by quantitative PCR and immunoblotting for the autophagy target p62 (Supplementary Figure 1A/B). Although autophagy has been described as a cell death pathway, under our experimental (nutrient sufficient) conditions cell viability was not altered in ATG16L1^{low} DC (Supplementary Figure 1C).

ATG16L1^{low} DC induced significantly more proliferation in allogeneic lymphocytes than control DC (Figure 1A). To verify that the increased stimulatory capacity of ATG16L1^{low} cells was due to diminished autophagy rather than an unknown function of ATG16L1, results were confirmed by inhibition of a second autophagy related protein, IRGM (Figure 1A). Additionally, autophagy knockdown in murine BMDC resulted in increased proliferation of ovalbumin-specific T cells in the presence of their cognate antigen (Figure 1B), suggesting the mechanism also operates in antigen specific responses and is conserved across species. Expression of activation markers including HLA-DR, CD80, CD86 and CD83 was not altered after knockdown, neither before nor after co-culture with lymphocytes (Figure 1C and data

Figure 1 Decreased DC autophagy results in increased T cell proliferation without phenotypic maturation.



(a) Inhibition of autophagy genes ATG16L1 or IRGM in DC results in increased allogeneic T cell stimulation in an MLR culture. Cells were co-cultured for 72-96 hours, data normalized against proliferation in the control sample of the same donor, $n=10$ and $n=9$ respectively. (b) Decreased autophagy in BMDC pulsed with OVA results in increased OTII proliferation. Cells were co-cultured for 96-120 hours, data normalized against control sample, mean and s.e.m. of three independent experiments shown. (c) Inhibition of ATG16L1 expression does not alter DC maturation. DC were treated with ATG16L1 (green line) or control (red line) siRNA for 48 hours and analyzed by flow cytometry, blue lines indicate control staining. Data is representative of four independent experiments. (d) Inhibition of ATG16L1 expression does not alter DC cytokine secretion. Cytokine secretion of DC co-cultured with allogeneic lymphocytes (ratio 1:5) was determined in supernatants by cytometric bead array. Data normalized against control of the individual donors. Mean and s.e.m. of three individual experiments shown. (a,b) * indicates $p < 0.05$, ** $p < 0.01$

not shown). Furthermore, decreased expression of ATG16L1 did not affect the secretion of proinflammatory cytokines IL-1 β , IL-6 and TNF- α , nor of the regulatory cytokine IL-10 (Figure 1D), suggesting that the immunogenic phenotype was not due to increased DC maturation.

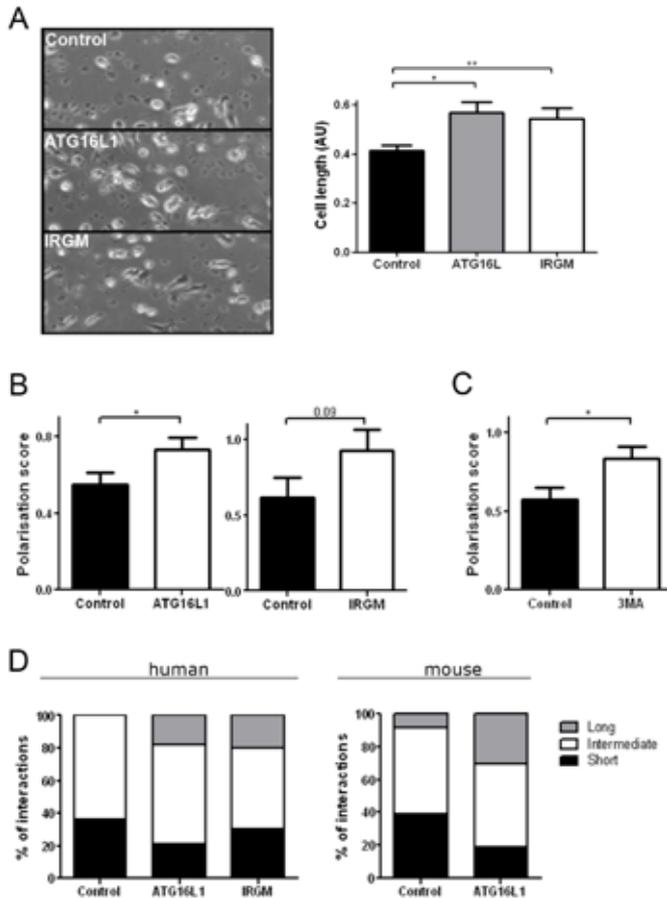
Decreased autophagy increases interaction strength in DC – T cell interactions

Interestingly, knockdown of ATG16L1 and IRGM gene expression resulted in a change in the gross morphology of DC, in that cells showed a more elongated phenotype (Figure 2A). This suggests that autophagy affects the cytoskeletal organization of DC. As polarization of the actin cytoskeleton is a key feature of DC-T cell interactions, we reasoned that such cytoskeletal effects may also affect DC mediated immune responses. Upon antigen recognition, DC and T cells form an immunological synapse, a highly organized molecular structure at the site of contact between the two cells.¹³ One characteristic of the immunological synapse is the polarization of the actin skeleton towards the site of interaction. The relevance of this polarization is shown by the fact that inhibition of cytoskeletal rearrangements results in decreased T cell activation.¹⁸ We hypothesized that autophagy knockdown would alter polarization of the DC actin skeleton. Therefore, allogeneic DC-T cell interactions were induced *in vitro* and the level of polarization was measured. Significantly stronger actin polarization was seen in DC in which autophagy was inhibited either by ATG16L1 or IRGM knock-down (Figure 2B). Again, this finding was consistent in OVA-specific murine BMDC-T cell interactions (Supplementary fig. 2A).

We further confirmed the role of the autophagy pathway as a whole using the autophagy inhibitor 3-methyladenine (3-MA). Since it has been shown previously that T cells require some level of autophagy to prevent activation induced cell death,¹⁹ it was not possible to add 3-MA directly to the cultures. Instead, DC were pre-incubated with 3-MA for two hours. Cells were then washed carefully, and used in subsequent polarization experiments. Indeed, pharmacological inhibition of autophagy also resulted in increased cytoskeletal polarization (Figure 2C), suggesting a role for the pathway as a whole.

The outcome of immune responses is not only determined by the polarization and recruitment of molecules, but also by the duration of the interaction.^{20, 21} As has been described previously, inhibition of proper immunological synapse formation by the myosin II inhibitor blebbistatin resulted in decreased duration of DC-T cell interactions and decreased T cell activation (data not shown). To measure the effect of decreased autophagy on the duration of DC-T cell interactions, we performed time lapse experiments. Both ATG16L1^{low} and IRGM^{low} DC engaged in more prolonged interactions with allogeneic T cells (Figure 2D, left). In accordance with a conserved role for autophagy in immune regulation, similar results were obtained for the interaction between murine BMDC loaded with cognate antigen and OTII splenocytes (Figure 2D, right). Together this data suggests that in DC, autophagy is a regulatory mechanism, controlling the strength and duration of DC-T cell interactions and thereby the ensuing immune response.

Figure 2 Decreased autophagy leads to increased cytoskeletal rearrangements in DC and prolonged DC-T cell interactions.

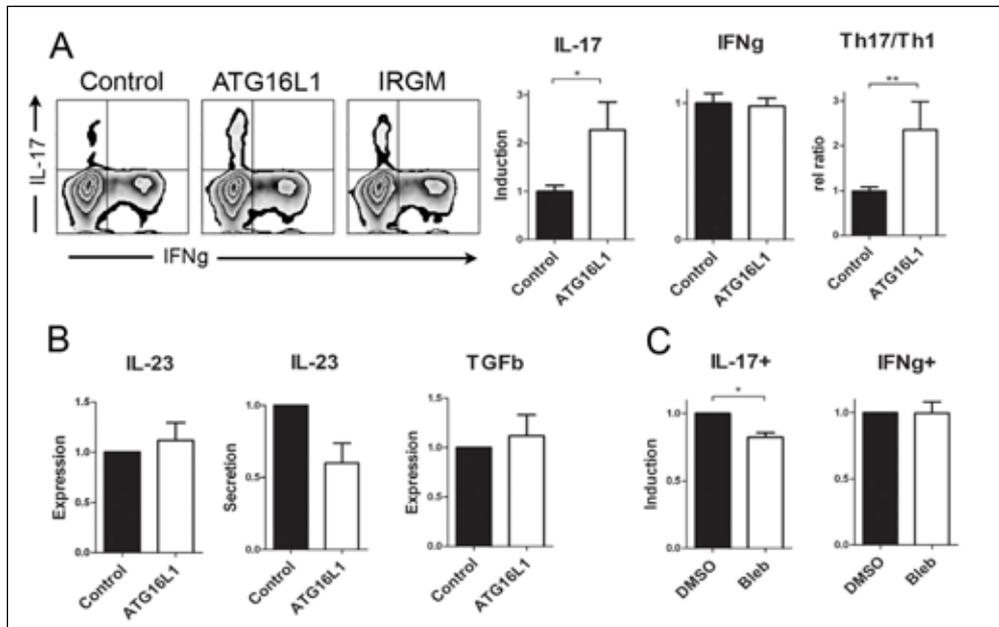


(a) DC generated from siRNA treated monocytes show an elongated phenotype. Representative light microscopy image of each condition (magnification 200x, left). Cell length was measured by image analysis in arbitrary units (right). Mean and s.e.m. of >50 cells per condition is shown. (b,c) DC-T cell clusters were generated by centrifugation at 50g and maintained for 30 min. Actin skeleton was visualized by phalloidin staining and scored for actin polarization on a three point scale (0-2). Bars represent mean and s.e.m., representative figure of three independent experiments. (c) DC were pre-treated with 3MA for 2 hours and washed carefully prior to clustering with T cells. (d) Time lapse analysis of DC-T cell interactions. Allogeneic T cells were added to adherent siRNA treated human DC (ratio 5:1, left panel) and OTII cells were added to adherent siRNA treated BMDC in the presence of cognate antigen (OVA₃₂₃₋₃₃₉ peptide, 1 μ g/ml, ratio 1:5, right panel). All samples were analyzed for 30 minutes by time lapse microscopy and total duration of individual interactions were calculated in duplicate samples. Interactions were categorized as short (< 3 minutes, black), intermediate (3-15 minutes, white) or long >15 minutes, grey). Results are representative of five independent experiments. * indicates $p < 0.05$, ** $p < 0.01$

Decreased DC autophagy results in Th17 polarization

One important function of DC in the regulation of immune responses is the skewing of T cells. Although the sharp distinction between Th1, Th2 and Th17 cells has proven less obvious in humans than in rodents, human T cell subsets also produce varying degrees of the effector cytokines IFN γ , IL-4 and IL-17. The stability of DC-T cell interactions has been shown to influence the Th1/Th2 balance,²² but data regarding the effect on Th17 has not been available thus far. Given the potential role of the Th17 axis in inflammatory bowel disease,^{23, 24} we tested the effect of decreased autophagy on the polarization of Th17 cells. To this end, DC were co-cultured with CD45RO+ T cells without the addition of exogenous cytokines. Both ATG16L1^{low} and IRGM^{low} DC induced significantly more IL-17 producing cells in an array of donors (Figure 3A). In contrast, the level of IFN γ producing cells was not altered (Figure 3A).

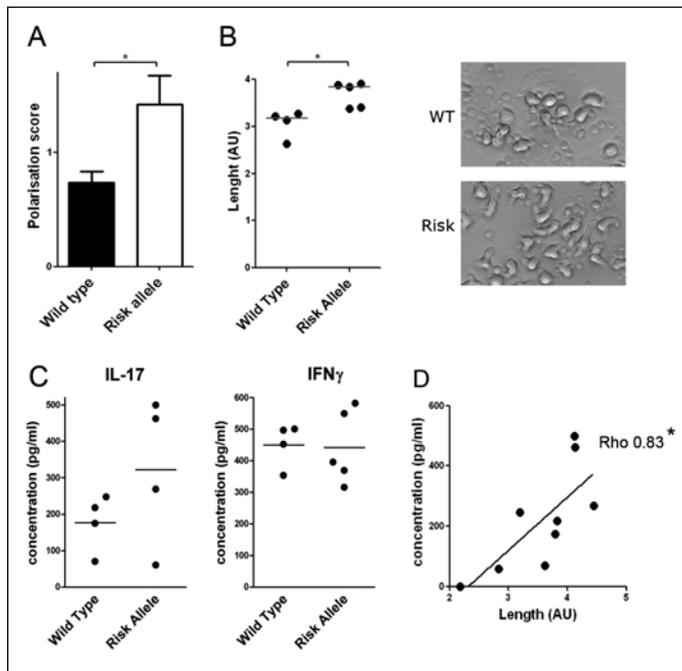
Figure 3 Immunological synapse hyperstability results in increased Th17 polarization.



(a,b) Isolated CD45RO+ T cells were co-cultured with autologous siRNA treated DC for 4 days, restimulated by PMA/ionomycin and analyzed by flow cytometry (a), quantitative pcr or ELISA (b). (a) Left panel shows representative data, bars represent mean (normalized against control sample in each donor) and s.e.m. of duplicate experiments in five different donors. (b) Bars represent mean relative mRNA expression and s.e.m. (left and right panel, n=3 and n=4 respectively) normalized against GAPDH and CD11c expression in each individual donor. Middle panel represents mean protein level and s.e.m. of four individual donors. (c) Isolated CD45RO+ T cells were co-cultured with autologous siRNA treated DC for 4 days in the presence of blebbistatin (5 μ M) or vehicle control (DMSO), restimulated by PMA/ionomycin and analyzed by flow cytometry. Data are normalized against vehicle control for each donor and represent mean and s.e.m. (n=5). * indicates $p < 0.05$, ** $p < 0.01$

Various cytokines have been implicated in the development of Th17 cells *in vitro*, including IL-23, IL-6 and TGF- β . However, IL-23 mRNA levels were unaltered and protein levels were decreased rather than increased in ATG16L1 siRNA treated DC (Figure 3B). Furthermore, DC production of TGF β 1 and IL-6 was also comparable in autophagy^{low} and control cells (Figure 3B and Figure 1D), suggesting that increased secretion of Th17 inducing cytokines is not the mechanism underlying the T cell skewing observed. To further analyze the role of synaptic stability in the activation of Th17 responses, we used the myosin II inhibitor blebbistatin. Complete myosin inhibition would quench all cellular interactions and be toxic in long term cultures, such as those required for induction of Th17 responses. Therefore, we used a relatively low dose in order to destabilize but not completely abolish immunological synapse formation. Interestingly, we found that in the presence of blebbistatin polarization towards the Th17 phenotype was decreased, whereas the induction of Th1 was not significantly affected (Figure 3C). This results in a decreased Th17/Th1 balance, suggesting that

Figure 4 ATG16L1 Crohn's disease susceptibility allele induces DC hyperpolarisation and tilted Th17/Th1 balance.



(a) Monocyte-T cell clusters were induced by centrifugation at 50g, actin was stained using phalloidin and actin polarization was scored on a three point scale (0-2, n=9). (b) Monocytes isolated from peripheral blood were cultured in the presence of GM-CSF and IL-4 for six days, two images were taken from each well at random locations and processed by image analysis (n=9), right panel shows representative images from 2 donors. (c) DC were co-cultured with allogeneic T-cells for 96 hours. Supernatant was then collected for cytokine measurements by ELISA (d) Cell length as measured in section (a) was correlated to IL-17 concentration in supernatants using Spearman's Rho. * indicates $p < 0.05$

the stability of the immunological synapse itself may be involved in determining the polarization of the immune response.

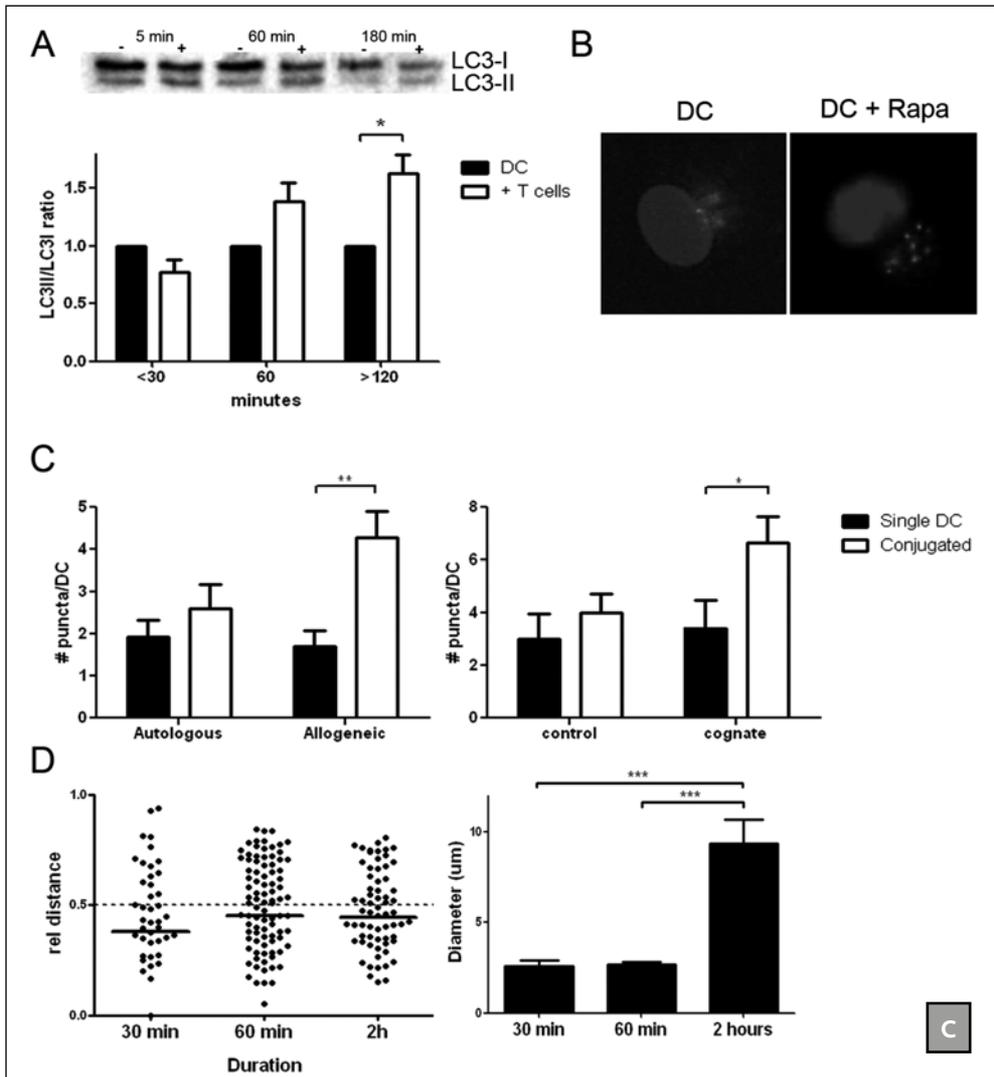
ATG16L1 risk allele carrier DC display hyperpolarization and increased Th17 induction

To confirm the physiological relevance of autophagy-based immune regulation, we recruited Crohn's disease patients homozygously carrying either the wild type or the T300A risk allele for ATG16L1.¹ Since protein interactions between ATG16L1 and NOD2 have been described previously, we excluded carriers of the NOD2 3020insC risk allele to avoid confounding by this polymorphism. In accordance with our previous data, monocytes obtained from ATG16L1 risk allele carriers exhibited significant hyperpolarization of the actin cytoskeleton, indicating more stable synapses (Figure 4A). Furthermore, DC generated from patients carrying the risk allele were significantly longer than DC from patients expressing the wild type allele, similar to the data obtained using gene knock-down (Figure 4A). Furthermore, co-culture of CD45RO+ T cells and T300A expressing DC resulted in a trend towards increased levels of IL-17 but not IFN γ secretion compared to wild type DC (Figure 4B). Strikingly, the level of IL-17 secretion was strongly correlated to DC length, but not to activation marker expression (Figure 4C and data not shown), further supporting the link between the cytoskeletal aberrancies and the increased presence of Th17 cells.

Immunological synapse formation induces autophagy in DC

To further decipher the mechanism by which autophagy regulates stability of the immunological synapse, we evaluated whether autophagy is upregulated by synapse formation. During autophagy, the autophagosomal protein LC3 is converted from 18 kD (LC3 I) to 16 kD (LC3 II), leading to an increased LC3 II/ LC3 I ratio.²⁵ This ratio can therefore be used as a measure of the level of autophagy. GFP-LC3 transfected DC were incubated in the presence or absence of allogeneic T cells, and the LC3 II/I ratio was determined by immunoblotting against GFP. Indeed, DC co-incubated with allogeneic T cells for more than 30 minutes showed autophagic processing compared to DC alone (Figure 5A).

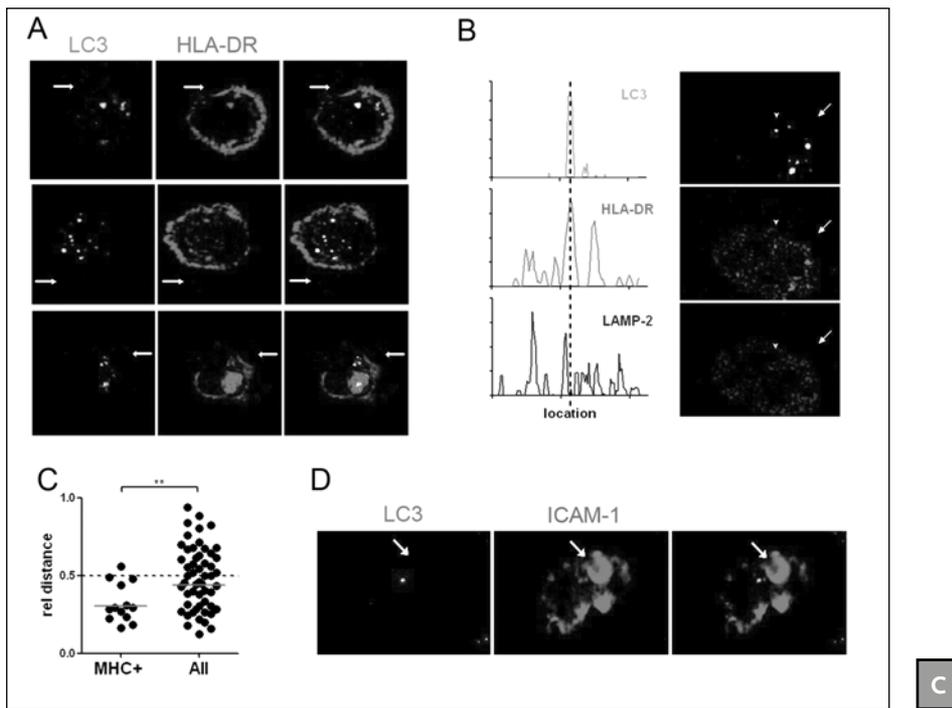
This finding was further confirmed by quantification of GFP-LC3 puncta (which are formed upon induction of autophagy, Figure 5B) in single and clustered DC in the same image field. The number of LC3 puncta was increased significantly in clustered DC compared to single DC, suggesting the induction of autophagy (Figure 5C). To verify that autophagy is induced by the actual formation of the immunological synapse rather than the mere proximity of a lymphocyte, we compared allogeneic and autologous DC-T cell clusters. In allogeneic clusters, a real synapse is formed, including polarization of the actin cytoskeleton, while in an autologous setting, clusters are formed, but these do not lead to the formation of a full synapse.¹⁴ Again, in the allogeneic setting, a significantly higher level of autophagy was present in clustered cells compared to single DC. However, in autologous interactions the number of puncta in clustered DC was not increased compared to the number of LC3 puncta in single DC, indicating that under these conditions, autophagy was not induced (Figure 5C). Similarly, a significant increase in autophagy occurred in BMDC-OTII splenocyte clusters when DC presented the cognate OVA₃₂₃₋₃₃₉ peptide resulting in a full synapse. However, when DC were loaded with the control OVA₂₅₇₋₂₆₄ peptide (Figure 5c) autophagy remained unaltered, emphasizing the fact that it is not the mere clustering of DC and T cells that leads to autophagy, but the actual cognate interaction.

Figure 5 Immunological synapse formation induces autophagy in DC.

(a) GFP-LC3 transfected DC were incubated in the absence (black bars) or presence (white bars) of allogeneic lymphocytes (times indicated above lanes), samples were then blotted for GFP and LC3II/LC3I ratio was calculated by image analysis. Western blot representative of three independent experiments. Data normalized against control samples, bars represent mean and s.e.m. of duplicate experiments. (b) GFP-LC3 transfected DC were treated with rapamycin (right, 20 μ g/ml) and analyzed by immunofluorescence. (c) Clusters were induced between GFP-LC3 transfected DC and autologous or allogeneic T cells (left), or between BMDC and OTII cells in the presence of control or cognate antigen (right). Number of LC3 puncta/DC were then scored in unconjugated DC (black bars) and DC conjugated to T cells (white bars) in the same image fields. Data representative of three individual experiments, bars represent mean and s.e.m. (d) Clusters were induced between GFP-LC3 transfected DC and allogeneic T cells and maintained for indicated periods. Cells were then imaged by confocal microscopy and relative distance was calculated as (distance of LC3+ spot to contact site / maximum width of the cell at site of synapse). Spot size was measured by image analysis. * indicates $p < 0.05$, ** $p < 0.01$

To further establish whether this autophagy induction was related to stability of the immunological synapse, we measured the relative distance between the autophagosome and the synapse (that is, the distance between the LC3⁺ spot and the site of cell-cell contact relative to the maximum width of the cell). If the autophagosomes are distributed randomly throughout the cell, this relative distance would have an average of 0.5. However, when measuring the relative distance over a large number of clusters, the average distance is significantly below 0.5, suggesting a preferential localization towards the immunological synapse (Figure 5D).

Figure 6 Autophagosomes are involved in degradation of synaptic components.



(a) Clusters of GFP-LC3 transfected DC and allogeneic T cells were induced for 60 minutes and stained for expression of HLA-DR (red). Subcellular localization of positive staining was determined by confocal imaging. Three individual clusters are shown, arrows indicate position of T cell in cluster. (b) Position of LC3⁺/MHC⁺ and LC3⁺/MHC⁻ spots were calculated as relative to maximum width of the cell. Data representative of three independent experiments in four donors. (c) Clusters of GFP-LC3 transfected DC and allogeneic T cells were induced for 60 minutes and stained for expression of HLA-DR (red) and LAMP2 (blue). Arrows indicate location of T cell in cluster. Arrowheads indicate line of analysis shown in line graphs. Graphs show relative signal intensity along the same line for all three stainings. Data representative of three individual experiments in different donors. (d) GFP-LC3 and SNAP-ICAM transfected DC were surface stained for ICAM (red). ICAM localized around phagocytic cups (indicated by arrows) and co-localized with LC3. Picture representative of two independent experiments. (a,c,d) All images magnification 630x and taken at a single Z-stack level in each cell

Autophagosomes close to the immunological synapse contain synaptic components

When we analyzed the formation of autophagosomes during formation of the immunological synapse, we not only observed a preferential localization towards the contact site, but also an increase in the size of autophagosomes between 30 minutes and 2 hours of DC-T cell interactions (Figure 5D). Given that autophagy is mainly involved in the degradation of cellular components, we hypothesized that these growing structures are sites of degradation of synaptic components, thereby destabilizing the synapse. To test this, clusters of GFP-LC3 transfected DC and allogeneic T lymphocytes were induced as described before, and localization of the central immunological synapse component MHC II was determined by confocal imaging. In line with our hypothesis, clustered DC showed clear colocalization of autophagosomes and MHC II (Figure 6a). Furthermore, distance measurements revealed that while autophagosomes in general are localized somewhat preferentially near the synapse, LC3+ spots containing MHC II molecules are localized significantly closer to the synapse (Figure 6B). To exclude the possibility that the MHC II/LC3 colocalization was caused by fusion of autophagosomes and late endosomes/lysosomes, ⁶ samples were also stained for the endo/lysosomal marker LAMP2. Although the majority of spots stained positive for all three markers, puncta positive for LC3 and MHCII but negative for LAMP2 were also observed, showing these spots are in fact autophagosomes containing non-lysosomal derived MHCII (Figure 6C). To further confirm this data, colocalization of the immunological synapse component ICAM-1 was determined using SNAP-tag technology. After expression in the cell of interest, the SNAP-tag can be labeled fluorescently using a cell non-permeable dye, resulting in the selective staining of proteins present on the outside of the cell membrane. We created an SNAP-ICAM-1 fusion construct, and labeled membrane ICAM-1 during formation of the immunological synapse. Similar to MHC II, ICAM-1 colocalized with LC3 puncta close to the synapse (Figure 6D). Since ICAM-1 staining was only possible at the cell surface these ICAM-1 molecules have to be membrane derived. The presence of immunological synapse derived molecules in autophagosomes close to the synapse strongly suggests that these autophagosomes play an active role in degradation and thereby regulation of the synapse.

Discussion

We demonstrate that cognate interactions between DC and T cells induce autophagy which in turn negatively regulates the interaction. Decreased autophagy in DC resulted in increased formation of the immunological synapse, prolonged DC-T cell interactions and increased T cell activation and Th17 polarization.

Autophagy was first described over three decades ago, but was initially only considered a cell survival mechanism. The identification of several autophagy genes as susceptibility genes for Crohn's disease has led to renewed attention for the field, in particular its role in the immune system. Autophagy contributes to innate immunity through degradation of intracellular pathogens, including *Mycoplasma Tuberculosis*, *Listeria Monocytogenes* and *Salmonella Enterica*.^{26, 27, 28} Diminished autophagy may therefore result in defective innate immunity, excessive microbial expansion and unbalanced adaptive immune activation. This mechanism, known as the theory of innate immunodeficiency, has been proposed to be

responsible for the elevated immune activation seen in Crohn's disease patients carrying these SNP. However, although impaired clearance of intracellular pathogens may contribute to the excessive immune responses seen in Crohn's disease, our study shows that a deficient autophagy can also directly hyperactivate T cell responses.

Previous studies into formation of the immunological synapse mainly focused on the T cell. The 'other side' of the synapse (ie, the DC) has received relatively little attention, leading to a void in the knowledge on this topic. However, inhibition of actin polarization specifically in DC hampers the formation of a proper synapse and significantly decreases T cell activation and proliferation.¹⁴ Additionally, a recent study showed recruitment of the signaling molecule phosphatidylinositol-4,5-bisphosphate to the synapse as well as local activation of Akt1 in DC suggesting active signaling in DC.²⁹ Our study further confirms the active participation of DC in immunological synapse formation, as autophagy knockdown specifically in DC results in altered synapses. This effect appears to be part of a negative feedback mechanism, as autophagic activity increases upon formation of a mature immunological synapse. Dendritic cells have a basal level of constitutive autophagy, which is increased upon synapse induction. Most likely only part of these autophagosomes are involved in regulation of the immunological synapse, whereas others play a role in cellular homeostasis. This may explain why localization of autophagosomes is somewhat polarized to the synapse, but still displays a relative big spread. Synaptic components such as MHCII and ICAM-1 are engulfed by autophagosomes, targeting them for degradation. Although no data is available on the relationship between the number of molecules present and the strength of the immunological synapse, it is not hard to imagine that a decrease in the number of interacting molecules leads to a relative destabilization of the synapse. Previously, it has been shown that membrane expression of MHCII is regulated through ubiquitination of the molecule and that ubiquitination can target proteins to the autophagosomes.³⁰⁻³² Therefore, ubiquitination of synaptic components may be the mechanism behind the trafficking observed. Interestingly, ubiquitination-related polymorphisms have recently been associated with the development of Crohn's disease.^{33, 34} Whether these polymorphisms have similar consequences for the regulation of cell-cell interactions as autophagy related defects would be an interesting topic of investigation.

Interestingly, a recent study shows that the protein encoded by another Crohn's disease susceptibility gene, NOD2, is necessary for proper localization of ATG16L1 at the cell membrane.³⁵ The susceptibility variant of NOD2 lacks this function, resulting in abnormal cytoplasmic ATG16L1 distribution. Given the important role of the membrane in cell-cell interactions, it is tempting to speculate that membrane localization of ATG16L1 is also necessary for proper regulation of DC-T cell interactions. Indeed, in our experiments, decreased expression of NOD2 resulted in a DC hyperpolarization similar to that observed in autophagy^{low} DC (Supplementary Figure 2B). This suggests that NOD2 and ATG16L1 risk alleles can independently lead to decreased pathogen degradation as well as increased adaptive immune activation, thus explaining why the risks carried by these alleles appear to be independent rather than cumulative.

Several studies have highlighted the importance of autophagy in the antigen presentation by MHC class II molecules. When influenza matrix antigen was targeted to the autophagosome, increased presentation and T cell activation was observed.⁶ More recently, *Atg5^{lox}CD-11c^{cre}* mice lacking autophagy specifically in DC show decreased MHC II antigen presenta-

tion due to decreased autophagosomal-lysosomal fusion.³⁶ Consequently, these DC were less capable of inducing T cell activation and mice showed impaired immune responses. The different results in this study compared to ours may be explained by the fact that the previous study used a complete knockout model, whereas in our model DC display decreased autophagy levels, but not a complete deficiency. Differential levels of autophagy may be required for regulation of antigen processing versus regulation of immunological synapse stability. Whereas the complete knockout is a relevant model to study if a specific gene plays any role in a given process, our model may be more reflective of the effect of hypomorphic alleles as seen in Crohn's disease patients. Indeed, several of the effects seen in our autophagy^{low} DC are mirrored by results in DC generated from Crohn's disease patients carrying the ATG16L1 risk allele, confirming the physiological relevance of our findings.

The role of the immunological synapse in the polarization of T helper responses has been investigated previously in the context of Th1/Th2 skewing. For example, synapses resulting in Th1 polarization contain a clear ICAM-1 ring structure and exclusion of CD45 from the central synapse, while synapses resulting in Th2 skewing contain more phosphotyrosines.³⁷ To the best of our knowledge, no data are available on the role of the synapse in the induction or activation of Th17 cells. In our experiments, secretion of the Th17 inducing factors IL-23, IL-6 and TGF- β ³⁸ was not increased in autophagy^{low} DC, indicating that the Th17 polarization observed is not due to altered cytokine secretion. Although we can not formally exclude that the increase in Th17 is the result of alterations in DC other than increased immunological synapse formation, we have not observed any such alterations. Furthermore, destabilization of the synapse using blebbistatin resulted in a decreased Th17 skewing, further supporting our hypothesis that hyperstability of the immunological synapse positively correlates with the induction of Th17 responses.

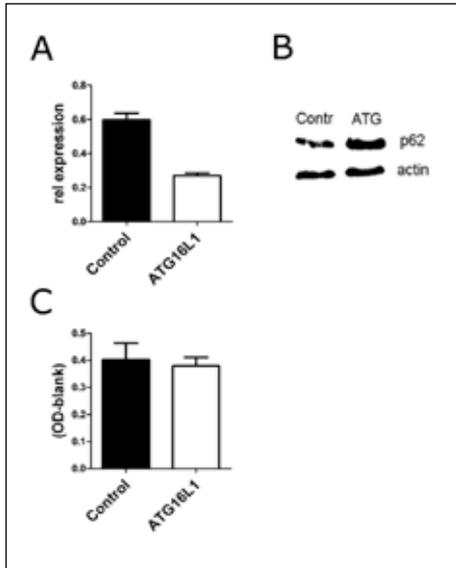
Our results demonstrate a novel role for autophagy in the regulation of immunological synapse stability and subsequent strength and polarization of the adaptive immune response. The clinical implications of these findings are shown by the fact that Crohn's disease patients carrying polymorphisms in ATG16L1 display similar alterations in their DC, including hyperpolarization and a tendency towards increased induction of IL-17 producing T cells. Interestingly, azathioprine, a frequently used therapy for Crohn's disease, has been shown to destabilize DC-T cell interactions through inhibition of the Rac pathway.³⁹ Our data suggest that this may in fact be a therapeutic mechanism for this drug in Crohn's disease. Additionally, other medications interfering with immunological synapse formation may be new candidates for Crohn's disease treatment.

In summary, this study shows that upon formation of the immunological synapse, autophagy is induced, which then acts as a negative feedback regulator of DC-T cell interactions by destabilizing the synapse. In Crohn's disease patients carrying the ATG16L1 risk allele, autophagic activity is decreased, leading to hyperstable DC-T cell interactions and increased Th17 activation.

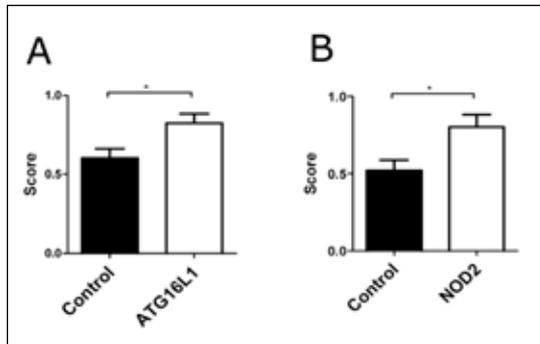
Competing interests: none

Supplementary figures

Supplementary figure 1.



(a) DC were treated with specific or control siRNA for 48 hours and analyzed by quantitative PCR. All data was normalized against GAPDH. Bars indicate mean expression and s.e.m., representative of >10 experiments. (b) siRNA treated DC were stimulated with rapamycin for 12 hours (20 $\mu\text{g}/\text{ml}$) and immunoblotted for expression of p62. Data representative of three donors. (c) siRNA treated DC were cultured for three days in RPMI culture medium containing 10% FCS and tested for viability by MTS assay. Bars represent mean and s.e.m. of three individual donors

Supplementary figure 2.

Clusters were formed between T cells and DC (a) pre-treated with 3MA (1 mM, 2 hours) or (b) transfected using NOD2 siRNA. Data representative of three individual experiments. Actin skeleton was visualized by phalloidin staining and scored for actin polarization on a three point scale (0-2). Bars represent mean and s.e.m. of >100 cells/experiment, ** p<0.01

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