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RESEARCH PAPER



Impaired reprogramming of the autophagy flux in maturing dendritic cells from crohn disease patients with core autophagy gene-related polymorphisms

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ABSTRACT

Crohn disease (CD) is an inflammatory bowel disease whose pathogenesis involves inappropriate immune responses toward gut microbiota on genetically predisposed backgrounds. Notably, CD is associated with single-nucleotide polymorphisms affecting several genes involved in macroautophagy/autophagy, the catabolic process that ensures the degradation and recycling of cytosolic components and microorganisms. In a clinical translation perspective, monitoring the autophagic activity of CD patients will require some knowledge on the intrinsic functional status of autophagy. Here, we focused on monocyte-derived dendritic cells (DCs) to characterize the intrinsic quantitative features of the autophagy flux. Starting with DCs from healthy donors, we documented a reprogramming of the steady state flux during the transition from the immature to mature status: both the autophagosome pool size and the flux were diminished at the mature stage while the autophagosome turnover remained stable. At the cohort level, DCs from CD patients were comparable to control in term of autophagy flux reprogramming capacity. However, the homozygous presence of *ATG16L1* rs2241880 A>G (T300A) and *ULK1* rs12303764 (G/T) polymorphisms abolished the capacity of CD patient DCs to reprogram their autophagy flux during maturation. This effect was not seen in the case of CD patients heterozygous for these polymorphisms, revealing a gene dose dependency effect. In contrast, the *NOD2* rs2066844 c.2104C>T (R702W) polymorphism did not alter the flux reprogramming capacity of DCs. The data, opening new clinical translation perspectives, indicate that polymorphisms affecting autophagy-related genes can differentially influence the capacity of DCs to reprogram their steady state autophagy flux when exposed to proinflammatory challenges.

Abbreviation: BAFA1: bafilomycin A1, CD: Crohn disease; DC: dendritic cells; HD: healthy donor; iDCs: immature DCs; IL: interleukin; J: autophagosome flux; LPS: lipopolysaccharide; MHC: major histocompatibility complex; nA: autophagosome pool size; SNPs: single-nucleotide polymorphisms; PCA: principal component analysis; TLR: toll like receptor; τ : transition time; TNF: tumor necrosis factor.

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Autophagy flux; Crohn disease; dendritic cells; single nucleotide polymorphism; inflammation


Introduction

Crohn disease (CD) is an immune-mediated chronic intestinal inflammatory disease. Its prevalence in Europe and North America is the highest reported prevalence worldwide with 322 per 100 000 patients in Germany and 319 per 100 000 patients in Canada with an increased incidence especially in developed industrialized countries [1,2]. CD usually occurs between the second to fourth decade of life and substantially affects the daily life of patients who have an increased mortality rate [3,4]. The precise etiology and pathogenesis of CD remain to be elucidated. CD manifests in genetically susceptible hosts due to an overactive host immune response to microbial factors [5,6], including strong modifications of intestinal microbiota composition, *i.e.* dysbiosis, that play a critical role by driving aberrant inflammatory innate and adaptive immune responses [7].

A meta-analysis of all genome-wide association studies/GWAS revealed a total of 71 regions of susceptibility in CD and those CD-associated risk alleles are mostly linked to bacterial sensing and innate and adaptive immunity [8,9]. The most common CD-associated single-nucleotide polymorphisms (SNPs) concern the cytosolic bacterial receptor NOD2 and the macroautophagy pathway, with variants affecting *ATG16L1*, *ULK1* or *IRGM* genes [10–12]. Macroautophagy (hereafter referred to autophagy) is a highly regulated catabolic process, which is essential for the maintenance of cellular homeostasis. The autophagy process constantly degrades obsolete components of the cell to avoid deleterious consequences of their accumulation [13]. The dynamic process of autophagy is called the autophagy flux, which is orchestrated through three tightly regulated successive steps, the initiation, the elongation and the maturation. The regulation of the initiation

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phase involves two main complexes, the ULK1 and the PIK3C3/VSP34-BECN1 complexes, leading to the generation of a phosphatidylinositol-3-phosphate-enriched phagophore, which expands and forms the double-membrane vesicle named autophagosome upon closure. The proteolytically processed form of MAP1LC3/LC3 (called LC3-I), is a soluble protein that is subjected to conjugation with phosphatidylethanolamine for insertion into the phagophore (the phagophore-anchored lipidated form of LC3-I is called LC3-II), and, together with the ATG12-ATG5-ATG16L1 complex, regulates the expansion of phagophore extremities [14,15]. Additionally, LC3-II factors are the anchoring point for soluble autophagy receptors that selectively target cytosolic cargoes (such as protein aggregates) to the nascent autophagosome in which they are ultimately sequestered. The final autophagic disposal of cargoes results from the maturation of cargo-containing autophagosomes, which involves their fusion with lysosomes [16].

Besides its crucial role in the maintenance of cellular homeostasis, autophagy has emerged as a cell-autonomous defense mechanism, as it is involved in the degradation of intracellular pathogens, a phenomenon referred to as xenophagy [17]. For instance, the sensing of bacterial compounds by the intracellular receptor NOD2 promotes antibacterial autophagy via ATG16L1 recruitment but fails to do so when NOD2 carries mutations associated with augmented susceptibility to CD [18,19]. Autophagy also modulates immunity at a larger level since it regulates inflammatory responses by degrading factors involved in inflammation-associated signaling [20] and contribute to shape the repertoire of antigenic peptides presented by major histocompatibility complex (MHC) molecules to T cells [21]. All these functions are key to the biology of dendritic cells (DCs), which play a central role in the maintenance of intestinal immune homeostasis [22–24]. Consistent with this, autophagy has been reported to regulate essential biological activities associated with immune functions of DCs [21,25,26]. DCs are innate immune cells, which can either induce immune response or immune tolerance, according to environmental contexts [27] and whose dysfunction can be associated with several inflammatory diseases, including CD [7,28]. Within tissues, DCs are found with low surface level of MHC class II and costimulatory molecules, a state called immature to reflect the fact that as such, they poorly stimulate naïve T cells. In contrast, upon pathogen detection or during inflammation, DCs upregulate the expression of both type of molecules, produce cytokines and acquire a high potency to activate naïve T cells in secondary lymphoid organs [29], a state referred to as mature [30].

Although genome-wide association studies have highlighted a link between autophagy and CD, our understanding of the impact of autophagy-related SNPs on the autophagy process in primary cells of CD patients, remains fragmentary. Previous studies using animal models of colitis revealed that alterations associated with autophagy-related SNPs, such as *ATG16L1* SNPs, in intestinal cells, macrophages but also in DCs, result in the ineffective elimination of pathogens and/or the persistent activation of the inflammatory response [18,31,32]. Although these studies have been useful in highlighting the important role of autophagy proteins, the impact

of autophagy-related SNPs on qualitative properties of autophagy process remains to be deeply investigated, particularly in DCs from CD patients. Autophagy is a dynamic process, and its activity cannot be properly examined by looking only at gene or protein expression levels. A pertinent analysis of the autophagy flux has to consider the whole dynamic process, from the biogenesis of autophagosomes to their maturation, in order to provide a relevant information on the functional status of autophagy [33–36]. Here, we chose to use monocyte-derived DCs to evaluate the mechanistic impact of some autophagy gene-related SNPs on the autophagy flux of primary DCs from CD patients. This approach provides suitable amounts of cells for autophagy flux analysis and also, allows for the comparative analysis of both mature and immature DCs from CD patients. Although these cells are certainly distinct from major DC subsets present in tissues, they may not be completely irrelevant to physiological and pathological settings as the differentiation of monocytes into DC-like cells has been observed *in vivo* at steady-state and found to be increased in mucosal or inflamed tissues both in mice and human [37,38]. In addition, transcriptomic analysis revealed great similarities between monocyte-derived DCs found *in vivo* and DCs derived from blood monocytes *in vitro* [39,40]. While calibrating the quantitative analysis of the autophagy flux with monocyte-derived DCs from healthy donors, we found that the immature to mature transition of DCs is characterized by marked changes in the autophagy flux parameters. Remarkably, monocyte-derived DCs from CD patients homozygous for two core autophagy gene-related SNPs were unable to adapt their autophagy flux during the transition toward the mature status, a defect that was not found in patients heterozygous for, or devoid of, such SNPs.

Results

Characterization of the autophagy flux signature in human primary dendritic cells

As DCs susceptible to be isolated from intestinal biopsies from CD patients would be (I) not easily accessible, (II) rare, (III) fragilized by the purification procedure, (IV) likely affected by the severe inflammatory context of the diseased tissue and (V) possibly impacted by the treatments applied to patients, we chose to use monocyte-derived DCs. While the autophagy process is highly dynamic [35], most studies on autophagy in DCs looked at the consequences of inhibition or exacerbation on cell activities at a given time point. To precisely characterize the autophagy flux of primary human DCs, we took advantage of a quantitative approach derived from metabolic control analysis by Loos et al. [34]. To do so, immature DCs (iDCs) were differentiated from peripheral blood monocytes of healthy donors and the dynamic of the conversion of soluble LC3-I into lipidated, phagophore-anchored LC3-II, which informs on the dynamic of autophagosome formation [33], was examined by western blot. The LC3-II:LC3-I protein level ratio in iDCs was analyzed prior to and after treatment with bafilomycin A₁ (BAFA₁), a potent inhibitor of the fusion of autophagosomes with lysosomes [33] and therefore, of the degradation of autophagosome-anchored LC3-II. Monitoring the variation of LC3

lipidation imposed by BAFA₁ allowed for an estimation of the autophagosome pool size (nA), a quantification of the autophagosome flux (J) and the derivation of the transition time (τ), which corresponds to the time needed to reconstitute the autophagosome pool after bringing it to zero (i.e. the autophagosome pool turnover) (Figure S1A). All three parameters were thus determined in human primary iDCs derived from 16 healthy donors (HD). We found that primary iDCs exhibited an autophagosome pool size of 0.97 ± 0.63 (median \pm SD), a flux of $10.7 \cdot 10^{-3} \pm 5.6 \cdot 10^{-3}$ and a transition time of 104 ± 30 (Figure 1A,B). These results indicated that monitoring the level of LC3 lipidation over time before and after addition of BAFA₁ does allow for a quantitative analysis of the autophagy flux of primary human iDCs derived from peripheral blood monocytes.

Reprogramming of the autophagy flux in primary dendritic cells undergoing transition from their immature to mature status

To determine whether the autophagy flux signature is modulated during the conversion of iDCs into mature DCs (mDCs), we determined the flux parameters of cells having

confronted maturation-inducing signals. To this end, iDCs were exposed to either the TLR4 (toll like receptor 4) ligand lipopolysaccharide (LPS), the TLR7/8 ligand R848, both to mimic infectious pathogen sensing, or to TNF/TNF α (tumor necrosis factor) to mimic an inflammatory microenvironment. In all maturation-inducing conditions used, iDCs efficiently differentiated into mDCs as determined by a strong increase in the cell surface expression of MHC class II molecules (HLA-DR) and co-stimulatory molecules (CD80, CD86) (Figure S1B). In addition, iDCs exposed to TLR agonists acquired the ability to secrete the pro-inflammatory cytokines IL6 (interleukin 6) and IL8 (Figure S1, C and D). Autophagy flux parameters were then determined in DCs induced to undergo maturation (LPS: 15 donors, R848: 9 donors, TNF: 10 donors) and compared to their immature counterparts derived from the same donors (Figure S2A,B). Strikingly, regardless of the maturation condition used, both the autophagosome pool size and the flux were significantly diminished in mDCs relative to their immature counterparts (Figure 2A-C, left and middle panels). In contrast, the transition time remained remarkably stable (Figure 2A-C right panels). Thus, although DC maturation was associated with

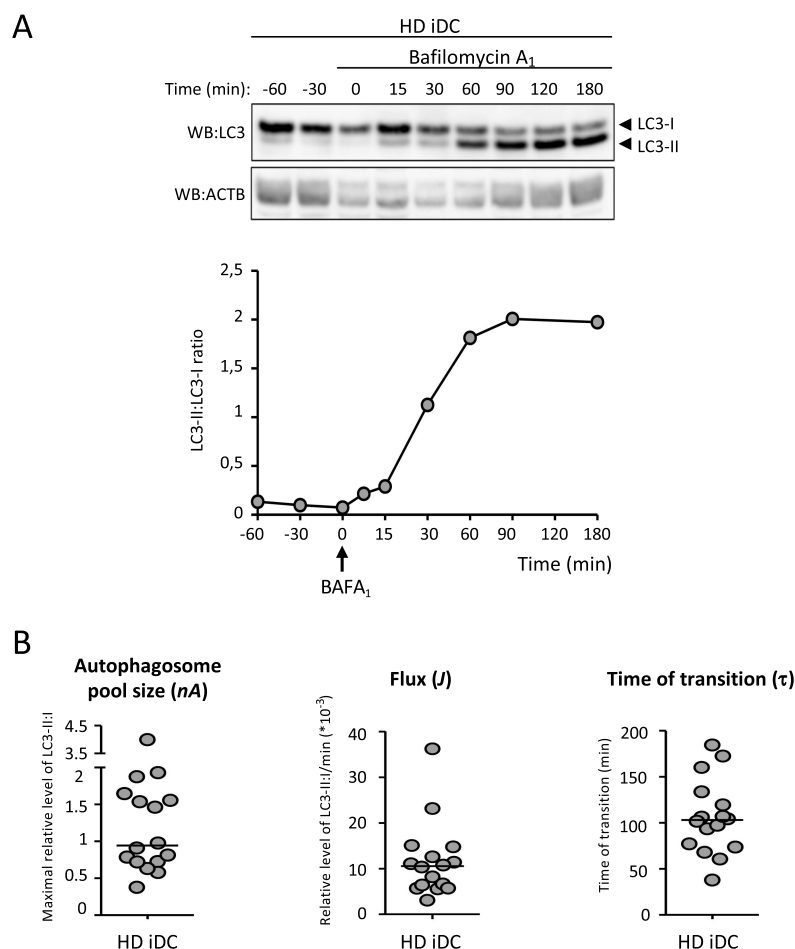


Figure 1. Quantitative autophagy flux analysis in human primary dendritic cells. Immature dendritic cells differentiated from healthy donor blood monocytes (HD iDC) were harvested at the indicated time points for analyzing both LC3 and ACTB/ACTIN expression by western blot, prior or after cell incubation with bafilomycin A₁ (BAFA₁). (A) a representative western blot obtained from iDCs of one donor treated with BAFA₁ is shown, along with the graph corresponding to the quantification of the LC3-II:LC3-I ratio determined for each time point. (B) the autophagosome pool size (maximal LC3-II:LC3-I ratio), the flux (slope of the curve) and the time of transition (turnover to replenish the pool of autophagosome) parameters were mathematically extracted from individual curves, as explained in Methods and Figure S1A, and were determined from iDCs of 16 donors treated with BAFA₁. for each parameter, the median is indicated with a bar.

a marked negative modulation of both the autophagosome pool and the autophagy flux, the time necessary for the renewal of the autophagosome pool remained constant. Down modulation of both nA and J values during LPS-induced DC maturation could also be documented when the quantitative analysis of the autophagy flux was monitored by a separate approach, namely the quantification of LC3-positive punctiform structures (LC3⁺ dots) after LC3 immunostaining and confocal microscopy analysis (Figure S2, C-E).

For an integrated view of the iDC/mDC analysis, flow cytometry data on surface expression of the maturation markers and autophagy flux parameters were globally examined by using a principal component analysis (PCA) for each maturation condition used. As shown in Figure 2D, the PCA consistently revealed two distinct clusters corresponding respectively to iDCs (blue dots), with low surface marker expressions along with relatively high autophagosome pool size and flux, and mDCs (red dots), with augmented surface marker expressions, and lowered autophagosome pool size and flux. The PCA also underlined the stability of the transition time. These clustered distributions were also seen when the PCA did not distinguish between maturation conditions (Figure S2F left panel). Finally, if the PCA focuses solely on data from mDCs, no significant cluster was observed, with the main parameter of segregation between the three maturation contexts being the expression level of maturation markers (Figure S2F right panel). The results indicate that, upon induction by both microbial components and inflammatory stimuli, DC maturation is associated with a reprogramming of autophagy parameters, with a reduced autophagosome pool and a lowered flux in mDCs relative to iDCs. Interestingly, the transition time remained unchanged in mDCs, suggesting that the biosynthetic rate of autophagosome formation of DCs is kept under tight regulation regardless of the maturation status of the cells. Finally, the results indicate that the early increase in DC autophagic activity that can be induced by TLR engagement [41,42], gets modulated upon stabilization of the maturation status. In relation with the pathophysiological context of CD, we also examined the autophagy flux of DCs from a healthy donor when exposed to a particular pathotype of *Escherichia* that is highly prevalent in ileal lesions of CD patients, adherent-invasive *Escherichia coli* (AIEC) [43]. Live or heat-killed AIEC-LF82 strain induced a strong maturation of DCs as identified by the increased surface expression of HLA-DR, CD80 and CD86 markers (data not shown). Importantly, as with LPS, live or heat-killed AIEC-LF82-induced DC maturation was characterized by a down modulation of the autophagic flux (Figure 2E,F). Hence, the autophagic activity of DCs is also negatively reprogrammed when they confront a pathobiont bacteria that is highly prevalent in the diseased ileum of CD patients.

Synchronized reciprocal reprogramming of autophagy flux and maturation markers expression during dendritic cell maturation

The process of DC maturation is associated with important modulations of transcriptional and biological activities to which autophagy is thought to contribute [44]. To identify the relationship between autophagy flux reprogramming and acquisition of maturation features, we monitored the autophagy flux parameters

of DCs progressing toward a full maturation status. We found that, in iDCs exposed to LPS, the evolution of autophagy flux parameters was progressive and well correlated with the kinetic of the phenotypic changes that characterize maturation: both the estimated autophagosome pool size and the flux progressively decreased as the expression level of induced costimulatory markers progressively augmented between 12 h and 48 h (Figure 3A and Figure S3A-B). As previously observed, the transition time did not substantially vary during maturation. Thus, during the immature to mature transition, the negative regulation of autophagy flux parameters and the positive regulation of maturation markers surface expression were progressive and synchronized. To assess whether an active autophagic flux is required for the complete acquisition of phenotypic maturation markers, iDCs were treated independently with two inhibitors of autophagy prior to cell culture with the maturation agent LPS, either BAFA₁, that interferes with autophagosome maturation [33] or spautin-1, that inhibits initiation of autophagosome formation by promoting the degradation of the PIK3C3/VSP34-BECN1 complex [45]. Both inhibitors efficiently altered the autophagy process, as assessed by quantification of the LC3-II:LC3-I protein level ratio (data not shown). Although, in most cases, the data did not reach statistical significance, we noticed a trend toward a negative regulation of the expression of the CD86, CD83 and ICAM1/CD54 markers when either autophagosome recycling or autophagy initiation were pharmacologically compromised (Figure 3B,C and Figure S3, C and D). Of note, the perturbed HLA-DR expression seen in the presence of BAFA₁ was expected as MHC class II molecules are known to traffic through lysosomal compartments for peptide loading. Finally, we looked at the impact of stimulated autophagy on the phenotypic maturation of DCs by treating iDCs with rapamycin, an inducer of autophagy that alleviates the negative regulation of autophagy by the serine/threonine protein kinase TORC1 [33], before LPS treatment. We found that rapamycin-mediated autophagy activation had no effect on the surface expression of maturation markers (Figure 3D and Figure S3E). Thus, the manipulation of autophagy by pharmacological means did not substantially influence the phenotypic changes associated with monocyte-derived DC maturation.

The chronic inflammatory status associated with Crohn Disease does not induce persistent changes in the capacity of monocyte-derived dendritic cells to reprogram their autophagy flux during maturation

Because various inflammatory contexts, such as pathogen infection or vaccination, are capable of persistently modifying metabolic and epigenetic features of bone marrow progenitors of innate immune cells [46], we wondered whether the chronic supraphysiological inflammation that prevail in CD patients could lead to persistent changes able to affect the functioning of the autophagy machinery in DCs. To address this issue, we quantitatively analyzed the autophagy flux of iDCs, and their LPS-induced mDCs counterparts, derived from peripheral blood monocytes of CD patients. Twenty-six CD patients with or without SNPs affecting autophagy-related genes were included in our study with their main demographic and disease-related characteristics reported in

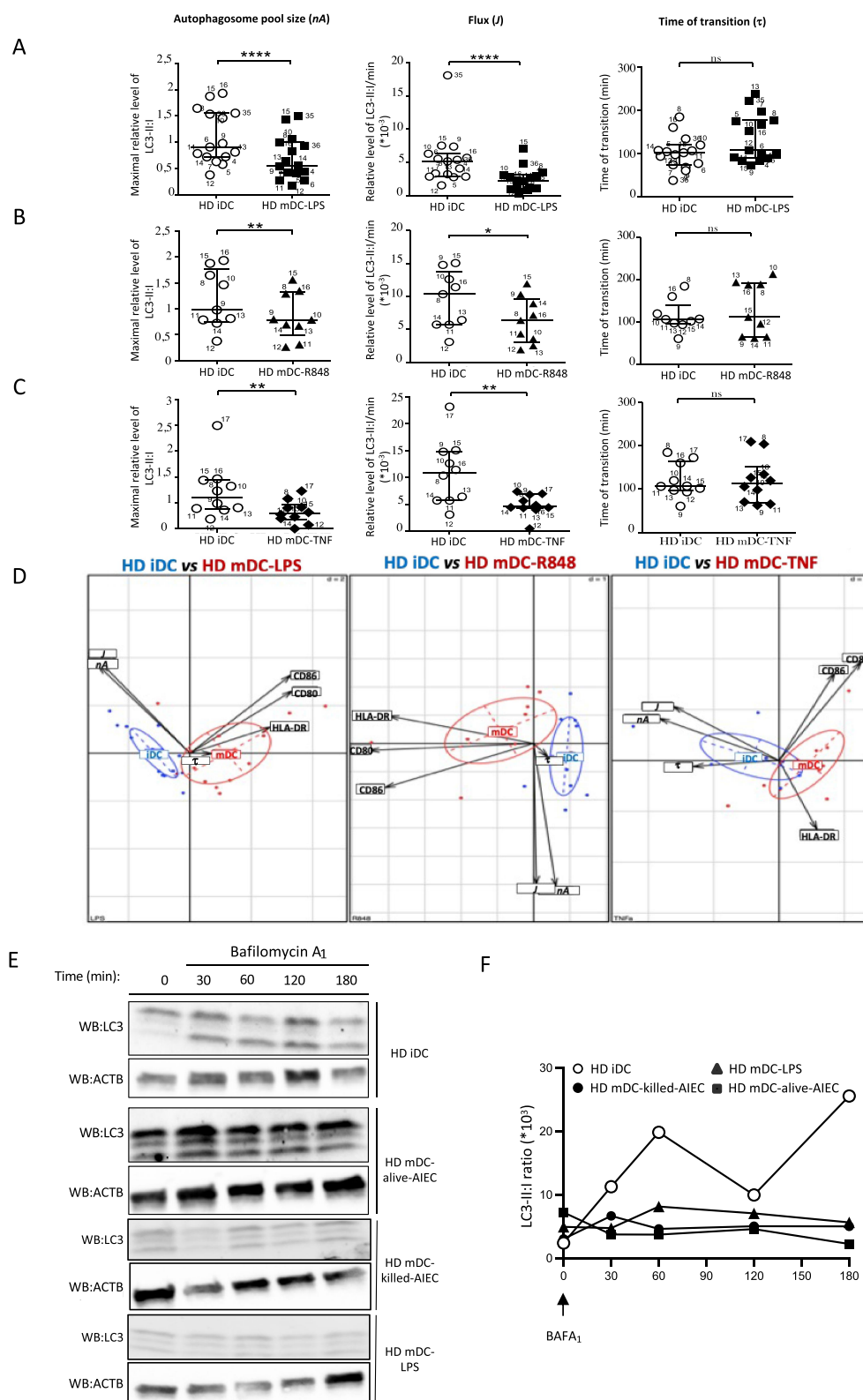


Figure 2. Modulation of the autophagy flux in mature dendritic cells. (A–C) Immature dendritic cells differentiated from healthy donor blood monocytes (HD iDC) were eventually matured (HD mDC) by a 48-h culture with LPS (A, $n = 15$), R848 (B, $n = 9$) or TNF (C, $n = 10$). The indicated autophagy flux parameters were mathematically extracted from graphs determined following the quantification of the LC3-II:LC3-I ratio obtained from iDC or mDC harvested at different time points, prior or after cell incubation with BAFA₁ (see Figure S2A/B for representative western blots and graphs). iDC that were matured in mDC from individual donors are numerated. ns: not significant, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. (D) Principal component analysis (PCA) including FACS-determined median of fluorescence of cell surface expression of HLA-DR and CD80, CD86 as determined from iDC and mDC-LPS, iDC and mDC-R848, or iDC and mDC-TNF and autophagy flux parameters: the autophagosome pool size, the flux and the time of transition. (E–F) Immature dendritic cells differentiated from healthy donor blood monocytes (HD iDC) were eventually matured by a 48-h culture with LPS, live or heat-killed AIEC-LF82 (HD mDC-AIEC) ($n = 1$). (E) Western blot analysis of LC3 obtained for iDC and mDC matured with AIEC-LF82, prior and after treatment with BAFA₁. (F) Curve of LC3-II:LC3-I ratio determined over time after BAFA₁ treatment.

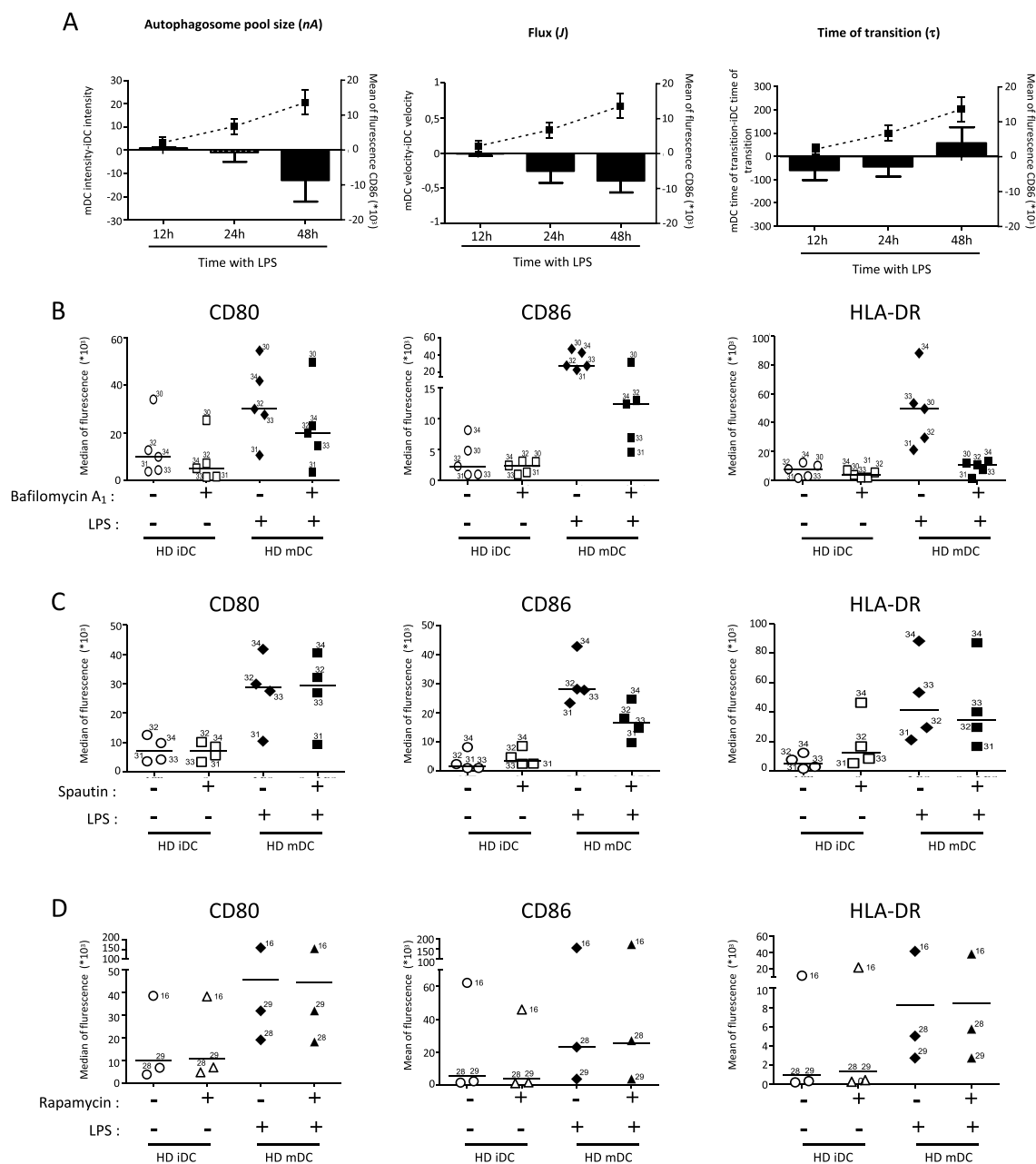


Figure 3. Modulation of autophagy flux and dendritic cell maturation. (A) Immature dendritic cells differentiated from healthy donor blood monocytes (HD iDC) were matured (HD mDC) with LPS and harvested at the indicated time points for analyzing the autophagic flux by quantification of the LC3-II:LC3-I ratio after cell incubation with BAF_{A1}. For each parameter determined, results were expressed as LPS-treated DC (mDC) minus iDC. The dotted line represents median of fluorescence of the maturation marker CD86 at the corresponding time points. For clarity only CD86 values are shown but consistent curves were obtained for CD80 and HLA-DR (not shown). (B–D) iDC differentiated from healthy donor blood monocytes were pre-treated or not with (B) BAF_{A1}, (C) spautin or (D) rapamycin and matured or not for 48 h with LPS. Maturation markers expression (HLA-DR, CD80, CD86) were analyzed by flow cytometry and results were expressed as median of fluorescence. For each parameter, the median is indicated with a bar.

Table 1. When we looked at the patient cohort at large, we found that like DCs derived from healthy donors, mDCs from CD patients revealed lower values for both the autophagosome pool size and autophagy flux relative to iDCs. Moreover, as observed for healthy donors DCs, both iDCs and mDCs from CD patients displayed comparable transition time (Figure 4A). Hence, in the CD patient cohort considered globally, DC maturation was normally associated with a reprogramming of autophagy flux parameters. This observation suggests that, if occurring in the chronic inflammatory context that characterizes CD, inflammation-triggered

metabolic and epigenetic changes susceptible to persistently modify innate immune cell progenitors do not affect the capacity of blood monocyte-derived DCs to reprogram their autophagy flux.

Altered capacity of autophagy flux reprogramming in dendritic cells from Crohn Disease patients homozygous for polymorphisms that affect core autophagy genes

Polymorphisms affecting genes encoding autophagy-related proteins are frequently found among CD patients. However, the

Table 1. Clinical characteristics of Crohn disease patients.

	CD patients <i>ATG16L1</i> ^{T300A}	CD patients <i>ULK1</i> rs12303764 (G/T)	CD patients <i>IRGM</i> rs10065172 313C>T	CD patients <i>NOD2</i> ^{R702W}	CD patients "WT" <i>ATG16L1</i> - <i>NOD2</i> - <i>ULK1</i> - <i>IRGM</i>
Number of patients (%)	8 (30%)	3 (12%)	2 (8%)	7 (27%)	6 (23%)
Age at inclusion (years), mean ± SD	38,1 ± 13	31 ± 14	29 ± 9	36 ± 11	39 ± 16
Female gender, n (%)	4 (50%)	0 (0%)	1 (50%)	0 (0%)	3 (50%)
Active smokers, n (%)	2 (25%)	1 (33%)	1 (50%)	3 (43%)	1 (17%)
Duration of CD (years), mean ± SD	10 ± 12	14 ± 14	2 ± 0	16 ± 10	11 ± 9
Prior bowel resection, n (%)	1 (12,5%)	3 (100%)	0 (0%)	5 (71%)	1 (17%)
Montreal Classification					
CD location					
L1, n (%)	5 (62,5%)	0 (0%)	1 (50%)	0 (0%)	1 (17%)
L2, n (%)	3 (37,5%)	1 (33%)	0 (0%)	2 (27%)	0 (0%)
L3, n (%)	0 (0%)	2 (66%)	1 (50%)	5 (71%)	5 (83%)
CD behavior					
B1, n (%)	2 (40%)	1 (33%)	2 (100%)	2 (28,5%)	4 (67%)
B2, n (%)	3 (60%)	0 (0%)	0 (0%)	3 (43%)	2 (33%)
B3, n (%)	0 (0%)	2 (66%)	0 (0%)	2 (28,5%)	0 (0%)
Perianal lesions, n (%)	5 (62,5%)	2 (66%)	0 (0%)	4 (57%)	3 (50%)
Prior medications					
Thiopurines, n (%)	4 (50%)	2 (66%)	0 (0%)	7 (100%)	2 (33%)
Methotrexate, n (%)	2 (25%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Anti-TNF agents					
Infliximab, n (%)	1 (12,5%)	1 (33%)	0%	4 (57%)	1 (17%)
Adalimumab, n (%)	4 (50%)	3 (100%)	2 (100%)	3 (43%)	3 (50%)
Golimumab, n (%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Ustekinumab, n (%)	0 (0%)	1 (33%)	0 (0%)	0 (0%)	1 (17%)
Vedolizumab, n (%)	1 (12,5%)	1 (33%)	0 (0%)	0 (20%)	1 (17%)
At least two biologics, n (%)	1 (12,5%)	1 (33%)	0 (0%)	2 (28%)	1 (17%)
Disease's activity at inclusion					
Clinical activity					
HBI, mean ± SD	6 ± 5,3	4 ± 4	5,5 ± 2,1	8 ± 2,1	0,7 ± 1,6
HBI >4, n (%)	3 (37%)	1 (33%)	1 (50%)	2 (28,5%)	1 (17%)
Biological activity					
CRP, mean ± SD	1,7 ± 16	1,7 ± 16	1,7 ± 16	1,7 ± 16	1,7 ± 16
CRP >5 mg/L, n (%)	2 (28,5%)	1 (33%)	1 (50%)	2 (25%)	1 (17%)
Concomitant use of medication					
Steroids	0 (0%)	1 (33%)	0 (0%)	0 (0%)	0 (0%)
Immunosuppressors					
No one	5 (62,5%)	3 (100%)	2 (100%)	7 (100%)	2 (33%)
Thiopurines	3 (37,5%)	0 (0%)	0 (0%)	0 (0%)	4 (66%)
Methotrexate	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Anti-TNF agents					
Infliximab, n (%)	5 (62,5%)	1 (33%)	0 (0%)	3 (43%)	4 (66%)
Adalimumab, n (%)	2 (25%)	0 (0%)	2 (100%)	0 (0%)	1 (17%)
Golimumab, n (%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Ustekinumab, n (%)	0 (0%)	0 (0%)	0 (0%)	1 (14%)	0 (0%)
Vedolizumab, n (%)	1 (12,5%)	0 (0%)	0 (0%)	1 (14%)	0 (0%)

exact impact of such gene alteration on autophagy activity, including in DCs, remains poorly characterized. To examine the status of the autophagy flux in relation with autophagy gene polymorphisms that predispose to CD, we selected patients with SNPs most frequently associated with increased risk of disease and monitored LC3 lipidation in the corresponding blood monocyte-derived DCs. We first focused on patients ($n = 8$) bearing the *ATG16L1* rs2241880 A>G SNP that translates into a threonine to alanine substitution (T300A) in the region of the C-terminal WD-repeat domain leading to altered selective autophagy [19,47], secretion of antimicrobial peptides by Paneth cells and inflammasome-dependent cytokine production [32,48,49] due to sensitivity to caspase-mediated degradation [50]. We found that the homozygous presence of this variant totally abolished the capacity of DCs to reprogram their autophagy flux during LPS-induced maturation: neither the autophagosome pool size nor the flux were modified during the conversion to the mature status of DCs after LPS treatment

(Figure 4B,C, Figure S4, A and B and Figure S5). Not surprisingly in this context, the transition time of iDCs and mDCs displayed similar values (Figure 4C). In contrast, DCs from CD patients heterozygous for the *ATG16L1* rs2241880 A>G T300A variant ($n = 8$) were not defective in their capacity to reprogram their autophagic activity during the maturation process (Figure 4D).

We found comparable results when we examined the autophagy flux of DCs from CD patients homozygous for a polymorphism affecting another core autophagy gene: *ULK1*, encoding a kinase whose activation regulates autophagy initiation. Although available in a more limited number ($n = 4$), patients homozygous for the *ULK1* rs12303764 (G/T) SNP, that corresponds to a G-T substitution in the intron with unclear consequence [12], revealed a trend toward a lack of autophagy flux reprogramming during DC maturation (Figure 5A). However, in the case of patients with monoallelic expression of this SNP ($n = 6$), we found that

All combined genotype CD patients

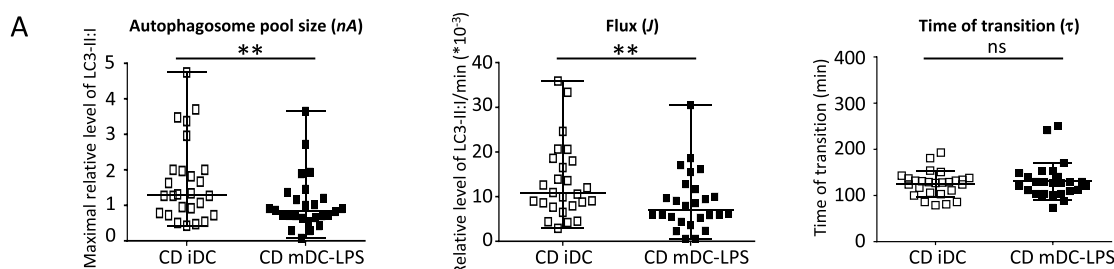
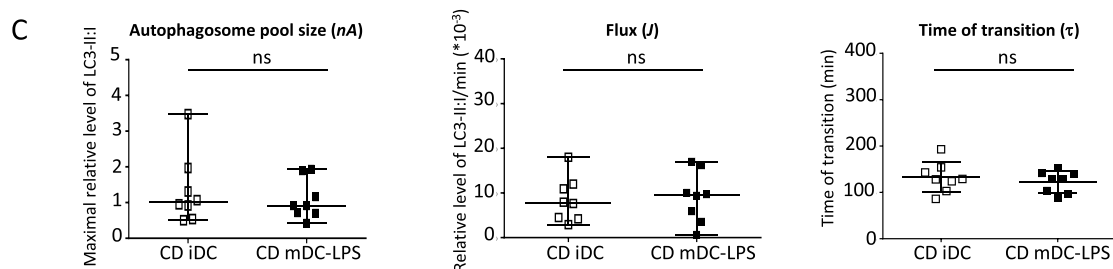
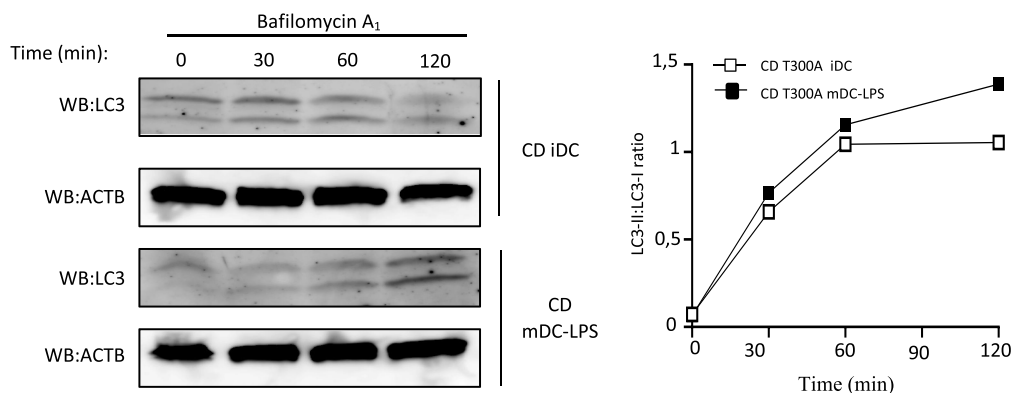
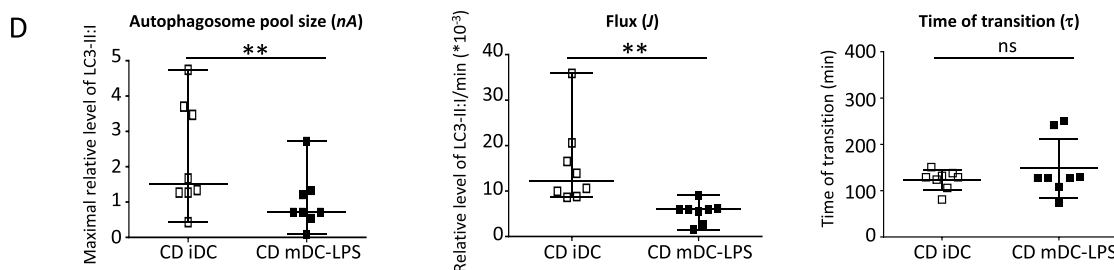
**B** Homozygous *ATG16L1*^{T300A} CD patientsHeterozygous *ATG16L1*^{T300A/G} CD patients

Figure 4. Autophagy flux analysis in *ATG16L1*^{T300A} dendritic cells from Crohn disease patients. iDCs differentiated from CD patient blood monocytes (CD iDC) were matured (CD mDC) by a 48-h culture with LPS and harvested at the indicated time points for analyzing autophagic flux by western blotting LC3 and ACTB/ACTIN, prior or after cell incubation with BAFA₁. (A) Autophagy flux parameters of the autophagosome pool size, the flux and the time of transition obtained from CD patients ($n = 26$); (B) a representative western blot obtained from CD iDCs and CD mDCs derived from blood monocytes of one CD patient expressing homozygous *ATG16L1*^{T300A} variant after treatment with BAFA₁, which is accompanied with the graph corresponding to the quantification of the LC3-II:LC3-I ratio determined for each time point are shown. (C) Autophagy flux parameters of the autophagosome pool size, the flux and the time of transition obtained from CD patients with homozygous expression of *ATG16L1*^{T300A} variant ($n = 8$) or (D) heterozygous expression of *ATG16L1*^{T300A/G} variant ($n = 8$). For each parameter, the median is indicated with a bar. ns: not significant ** $p < 0.01$.

the capacity of DCs to significantly reprogram their autophagy flux during maturation was clearly intact (Figure 5C). From a mechanistic point of view, we tried to assess whether the sole presence of the T300A substitution could be intrinsically responsible for the lowered autophagic potential seen in CD patients homozygous for the *ATG16L1* rs2241880 A>G SNP. To this aim, we used HeLa epithelial cells engineered to lack *ATG16L1* expression (*ATG16L1* KO) and ectopically complemented them with plasmids coding for either control *ATG16L1* (*ATG16L1* WT) or CD-associated *ATG16L1*^{T300A} prior to analyzing the autophagy flux (Figure 6A). *ATG16L1* KO HeLa cells displayed no autophagic flux activity as quantified up to 3-h of BAFA₁ treatment (Figure 6B,C). Interestingly, while both *ATG16L1* WT and *ATG16L1*^{T300A} proteins were expressed at similar levels (Figure 6A), the monitoring of the LC3 status through LC3-II:LC3-I ratio western blot analysis and quantification of LC3-positive punctiform structures (LC3⁺ dots) over time after BAFA₁ treatment revealed a compromised capacity of *ATG16L1*^{T300A}-expressing cells to mount a sizable autophagic activity relative to cells expressing *ATG16L1* WT (Figure 6B, Figure S6A and B). Accordingly, *nA* and *J* values were lowered in *ATG16L1*^{T300A}-expressing cells (Figure 6C, Figure S6C). Thus, presence of the *ATG16L1*^{T300A} variant is sufficient to compromise the autophagic potential of model cells and is likely to participate in the altered capacity of DCs from *ATG16L1* rs2241880 A>G homozygous patients to reprogram their autophagic activity during maturation. Overall, these observations indicate that CD-associated SNPs that affect two major core-autophagy genes preamp primary DCs from CD patients from reprogramming their autophagy flux during the immature to mature transition. They also reveal that this effect is gene dose dependent.

Unaltered autophagy flux reprogramming in dendritic cells from Crohn disease patients with homozygous expression of polymorphism affecting the non-core autophagy gene *NOD2*

We also examined the autophagy flux of DCs from CD patients (*n* = 9) bearing the rs2066844 variant of the innate receptor gene *NOD2* where a C>T nucleotide change in exon 4 leads to an arginine to tryptophan substitution (R702W) in the leucine rich repeat domain and which is among the most prevalent variants associated to CD susceptibility (*NOD2* rs2066844 c.2104 C>T (R702W)) [51]. Unlike *ATG16L1* rs2241880 A>G (T300A) and *ULK1* rs12303764 (G/T), the homozygous *NOD2*^{R702W} status did not affect the capacity of DCs from CD patients to reprogram their autophagy flux during maturation (Figure 5D). Within the cohort, we also noticed patients homozygous for a SNP that affects the human GTPase [11] *IRGM*. The *IRGM* rs10065172 c.313C>T consists in an exonic synonymous C>T nucleotide change in marked linkage disequilibrium with a deletion polymorphism of 20kb upstream of the *IRGM* gene. This SNP renders *IRGM* resistant to microRNA-mediated down regulation leading to dysfunctional anti-bacterial autophagy in CD [52]. However, we get access to too few patients (*n* = 2) to

make a conclusion on a possible effect of this polymorphism on the autophagy flux status during DC maturation (Figure 5B). Thus, these results indicate that not all genes whose polymorphism is markedly associated with augmented susceptibility to CD preamp DCs to reprogram their autophagy flux during the maturation process. Notably, DCs from CD patients homozygous for *NOD2* rs2066844 c.2104 C>T R702W normally reprogrammed their autophagy flux during maturation.

The SNP-associated defect in flux reprogramming does not impact the phenotypic maturation of dendritic cells from Crohn disease patients

To determine whether the altered capacity to reprogram their autophagy flux impacted the potential of patient iDCs with polymorphism to adopt a mature DC phenotype, we examined the surface expression level of MHC class II (HLA-DR), costimulatory (CD80 and CD86) and activation (CD83) molecules after LPS-induced maturation. We observed that cells from patients homozygous for the polymorphisms that confer alteration in flux reprogramming (*ATG16L1* rs2241880 A>G (T300A) and *ULK1* rs12303764 (G/T)) behaved similarly to cells homozygous for *NOD2* rs2066844 c.2104 C>T (R702W) and *IRGM* rs10065172 c.313C>T, which do not show flux reprogramming alteration: all cells were able to upregulate the surface expression level of HLA-DR, CD80, CD86 and CD83 to the same extend (Figure 7A-D). This result was somehow in line with our observation that in healthy donor cells, the pharmacological neutralization of autophagy did not substantially alter the capacity of dendritic cells to acquire a mature phenotype (Figure 3B,C). Finally, the acquisition of a mature DC phenotype was comparable for cells homozygous or heterozygous for the above-mentioned polymorphisms (not shown). Thus, under the experimental conditions used, alteration in the autophagy flux reprogramming capacity did not significantly impact acquisition of the mature phenotype by DCs exposed to a microbial component.

Discussion

Besides its crucial role in the maintenance of cellular homeostasis, autophagy has emerged as a central element of the immune response due to its involvement in the regulation of inflammatory responses, its ability to degrade intracellular pathogens and its contribution to the repertoire of antigenic peptides for MHC molecule presentation to T cells [20]. All these immune functions are key activities of DCs, and dysfunctions of these cells is a hallmark of CD. Nevertheless, although CD is associated with several genetic risk factors that affect genes known to regulate the autophagy process at steady state (e.g. *ATG16L1* or *ULK1*) or upon intracellular bacterial infections (e.g. *NOD2*), to which extend the autophagic activity is indeed impacted by certain SNPs remains poorly characterized.

Most studies looking at autophagy either focused on the level of expression of genes/proteins or focused on punctual impact on the autophagy flux. However, the analysis of the

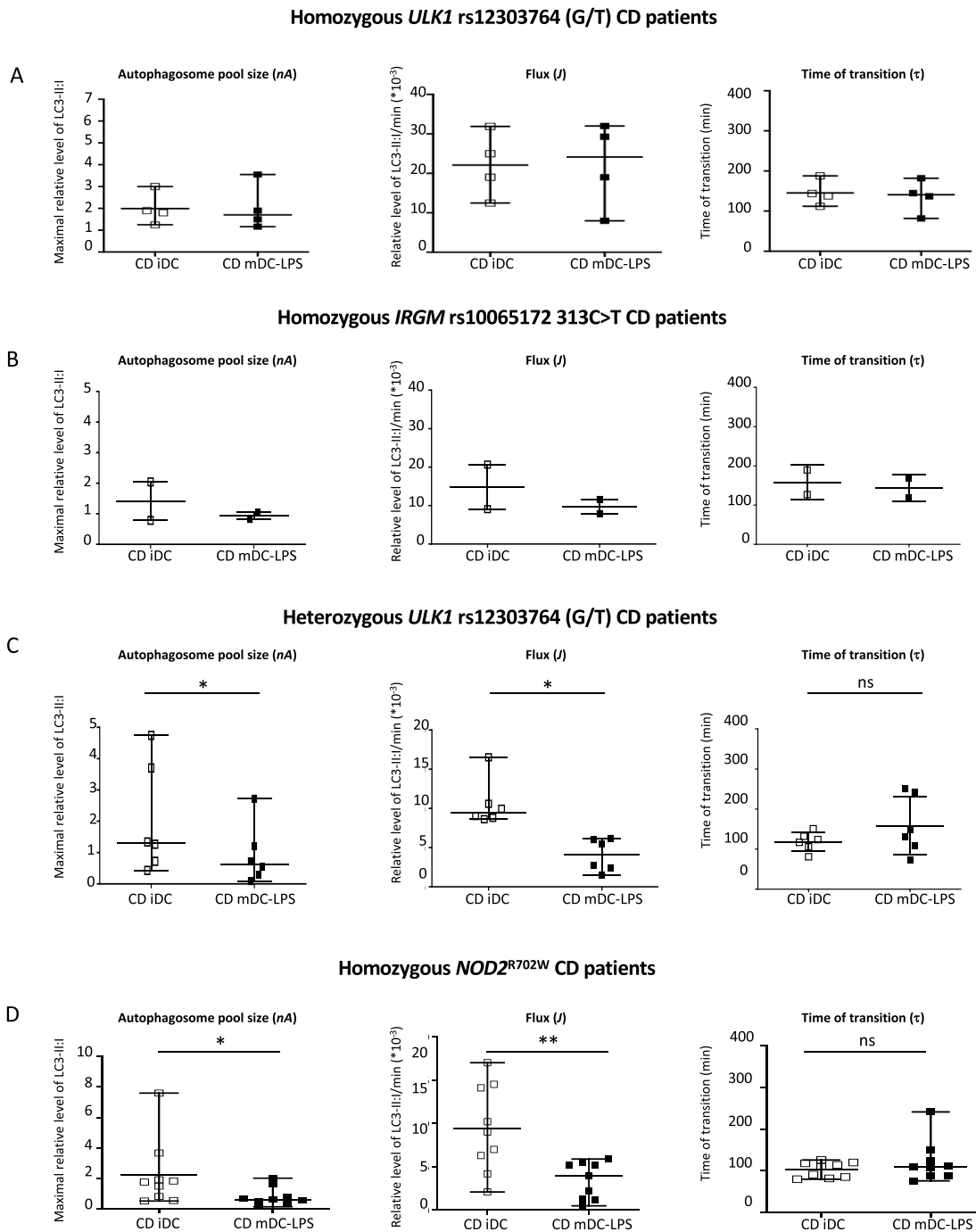


Figure 5. Autophagy flux analysis in *ULK1* rs12303764 (G/T), *IRGM* rs10065172 313C>T, *NOD2*^{R702W} dendritic cells from Crohn disease patients. (A-D) iDCs differentiated from Crohn disease patients blood monocytes with homozygous expression of *ULK1* rs12303764 (G/T) variant (A, $n = 4$), homozygous expression of *IRGM* rs10065172 313C>T variant (B, $n = 2$), heterozygous *ULK1* rs12303764 (G/T) variant (C, $n = 6$), homozygous expression of *NOD2*^{R702W} variant (D, $n = 9$) were matured (CD mDC) or not (CD iDC) by a 48-h culture with LPS. Autophagy flux parameters were extracted from quantification of the LC3-II:LC3-I ratio obtained from CD iDC and CD mDC harvested at different time points, prior or after cell incubation with BAFA₁. For each parameter, the median is indicated with a bar. ns: not significant * $p < 0.05$, ** $p < 0.01$.

complete dynamic properties of the autophagy flux should be considered if we aim at understanding specific alteration of autophagic activity and the associated physiological perturbations.

Here, we demonstrate that monocyte-derived DCs from healthy donors progressively reprogram the features of their autophagy flux upon maturation, what is impaired in DCs from CD patients homozygous for SNPs affecting core

autophagy genes, i.e. genes required for the orchestration of autophagy. Such a reprogramming is characterized by a decrease of both, the autophagosome pool size and the flux. As iDCs are highly endocytic, the elevated autophagy flux that characterize them could maximize the contribution of autophagy to the degradation of extracellular environmental antigens, including intestinal microorganisms, and the generation of the peptide repertoire for presentation the

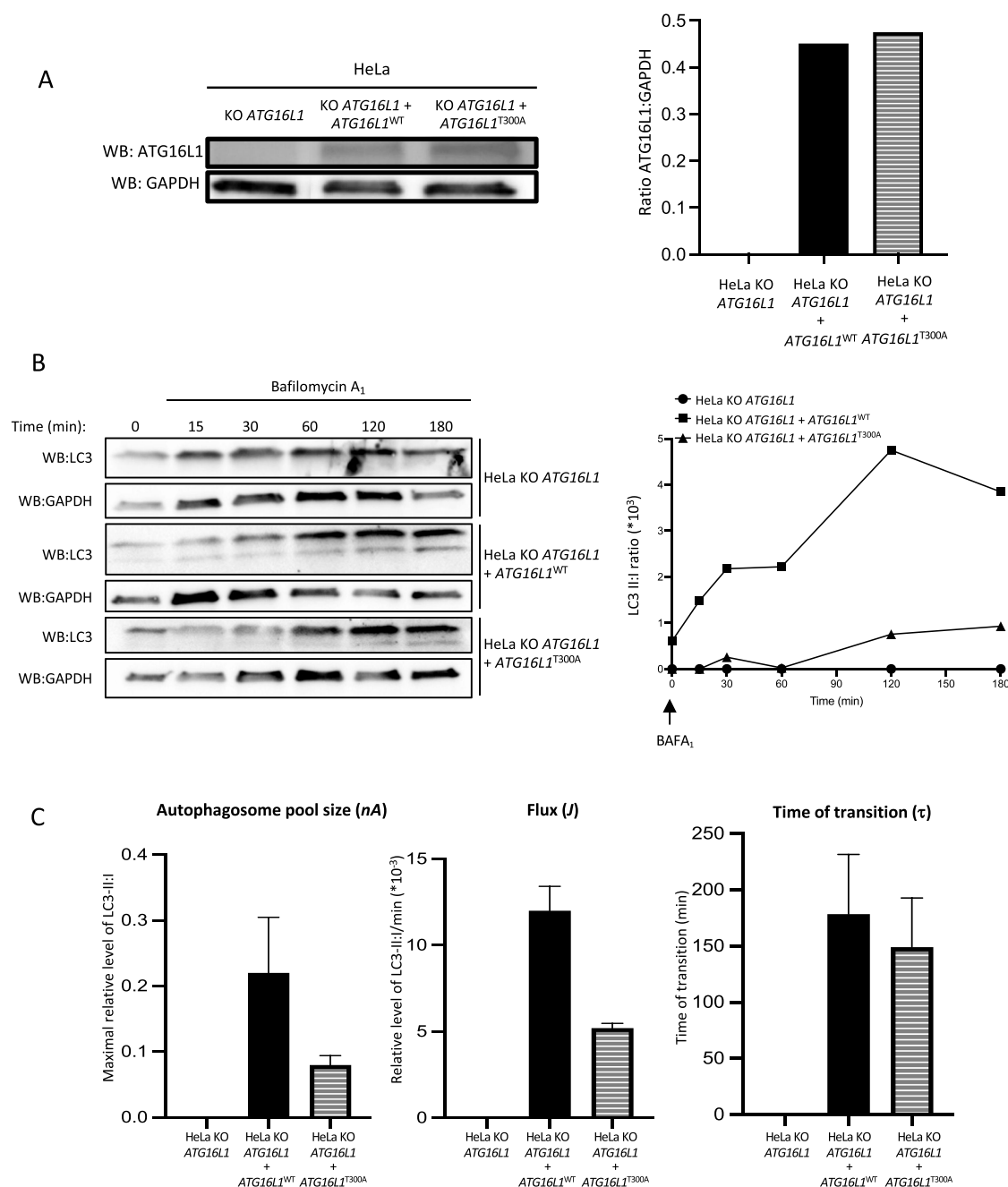


Figure 6. Impact of *ATG16L1*^{T300A} expression on the autophagy flux profile of model cells. (A-C) HeLa epithelial cells lacking *ATG16L1* (KO *ATG16L1*) were transfected with plasmids coding for either *ATG16L1*^{WT} or *ATG16L1*^{T300A} during 48 h. (A) a representative western blot showing the similar efficiency of plasmid transfections as assessed by the quantification of the *ATG16L1*:GAPDH protein detection ratio. (B) a representative set of western blot obtained after BAFA₁ treatment of parental and transfected HeLa cells accompanied by the graph corresponding to the quantification of the LC3-II:LC3-I ratio determined for each time point. (C) Autophagy flux parameters were extracted from the kinetic of the quantified LC3-II:LC3-I ratio of parental and transfected cells. A-B show one experimental result representative of 3 independent experiments. C is the mean of 3 independent experiments.

MHC class II pathway. In contrast, mDCs that are specialized in antigen presentation to naïve T cells, display a reduced capacity to internalize microorganisms [30,53–55]. Our results may suggest a coordinated adjustment of the autophagy flux and the endocytic activity during maturation. How this coordination precisely takes place remains to be understood. As endosome recycling has been proposed to contribute to the autophagy process [56], a lowered endocytic activity in mDCs could have a direct consequence on the magnitude of the autophagy flux. Modulation of the

autophagy flux might also be associated with a modified autophagosomal proteome targeted for degradation, in terms of quantity and/or quality, modifying the nature of the peptide repertoire displayed by mDCs, what could impact adaptive immune responses. Interestingly, despite the marked changes in the autophagosome pool size and flux of iDCs, the time of transition of was virtually unchanged after maturation, possibly suggesting that it may represent a stable cell-type characteristic. This stability may indeed fit with the notion that autophagy is influential for the biology of

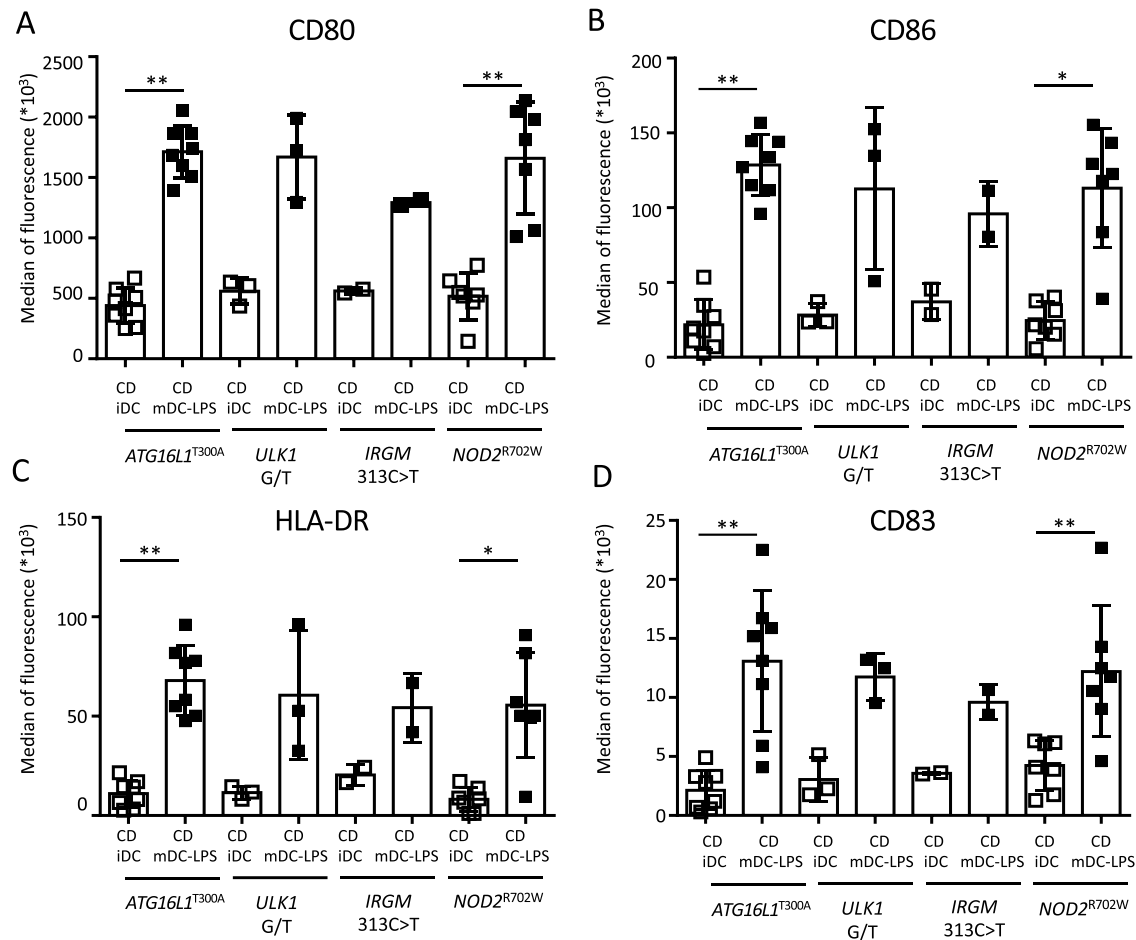


Figure 7. Adaptation of autophagy flux during dendritic cell maturation process. (A-D) Immature dendritic cells differentiated from CD patients' blood monocytes (CD iDC) expression the indicated CD-associated SNP were matured (CD mDC) by a 48-h culture with LPS and maturation markers expression (CD80 [A], CD86 [B], HLA-DR [C] and CD83 [D]) were analyzed by flow cytometry. Results were expressed as median of fluorescence. * $p < 0.05$, ** $p < 0.01$.

DCs [42,57,58]. Whether autophagy flux features influence either the immunogenic or tolerogenic potential of DCs in physiological conditions is unknown.

Looking at the autophagy flux in DCs from CD patients, we found that flux reprogramming was taking place normally when observed at the level of the whole cohort but was strongly compromised in maturing DCs from patients homozygous for polymorphisms affecting two major core autophagy factors: *ATG16L1* rs2241880 A>G (T300A) and *ULK1* rs12303764 (G/T). *ATG16L1* associates with the covalent ATG5–ATG12 complex to coordinates the elongation of the phagophore up to the formation of a sealed autophagosome [14,15]. *ATG16L1* also interacts with CLTC (clathrin heavy chain) and is involved in the formation of *ATG16L1*-positive early autophagosome precursors [59]. The *ATG16L1*^{T300A} polymorphism renders the protein highly susceptible to a proteolytic cleavage mediated by activated caspase 3/7 [32,50] in stressed cells, what can be further increased upon *ATG16L1*^{T300A} phosphorylation by *ULK1* under infectious conditions [60]. *ULK1* is indeed a kinase whose activation regulates the induction of autophagy. Although identified has a risk allele for CD, the functional consequence of expressing the *ULK1* rs12303764 (G/T) variant is unknown [12]. We report here that the homozygous expression of this variant, as that of the *ATG16L1*^{T300A} variant,

prevents the reprogramming of the autophagy flux in mDC, indicating a direct impact on the functionality of the autophagy machinery. In such patients, the absence of autophagy flux reprogramming during DC maturation could impair their immune functions and actively contribute to the pathogenesis of CD through a dysfunctional autophagic control of inflammatory responses and a deregulated autophagic response to invasive bacteria. Indeed, a decrease in antibacterial defense in murine cells with autophagy-related SNPs has been reported [18,31,32]. An intrinsic defective capacity to adjust the dynamic of this flux during DC maturation could possibly affect antigen degradation and/or the nature of epitopes presented to T lymphocytes.

Regarding the *IRGM* rs10065172 c.313C>T SNP, we identified too few homozygous patients to determine whether its homozygous presence impacted the ability of maturing DC to reprogram their autophagic flux yet, such an effect cannot be excluded. *IRGM* is a human GTPase whose function in autophagy is associated with innate immunity regulation and contribute to autophagy activation in infectious and inflammatory contexts. Interestingly, by interacting with *ULK1*, and with *ATG16L1*, *IRGM* was found to stabilize autophagy-regulating complex and to regulate autophagy initiation in xenophagic settings [61].

A recent study reported that the monoallelic expression of rare variants affecting the *ATG4A* and *MAP1LC3B2* autophagy genes, alter the autophagic response to herpes simplex virus (HSV)-2 infection and is associated with susceptibility to recurrent lymphocytic meningitis, indicating that haploinsufficiency can translate into pathological manifestations [62]. In contrast, we found here that the monoallelic expression of *ATG16L1* rs2241880 A>G (T300A) and *ULK1* rs12303764 (G/T) variants were not affecting the functionality and plasticity of the autophagy flux, at least in monocyte-derived DCs. In the same line, only the homozygous expression of the *ATG16L1*^{T300A} variant in human DCs was found to be associated with marked cytoskeletal defects limiting the formation of membrane protrusion and cell migration [63,64] within particular, a dysfunctional autophagic regulation of the small Rho GTPase Rac1. The reasons for which such observations differ from data obtained in *Atg16L1*^{T300A} knock-in mice, where both homozygous and heterozygous macrophages showed autophagy alterations [65] remain to be elucidated.

In contrast to *ATG16L1* rs2241880 A>G (T300A) and *ULK1* rs12303764 (G/T), the expression of the *NOD2*^{R702W} variant, which is among the most frequent risk variant associated with CD, is not associated with an intrinsic default of autophagy [19]. *NOD2* is an innate receptor which can induce autophagy upon bacterial detection by recruiting *ATG16L1* to the entry site and by interacting with *IRGM* and *ULK1* [61]. Possibly, a defect of autophagy in *NOD2*^{R702W} expressing cells could be limited to few autophagosomes, those induced downstream of *NOD2* engagement, while innate receptors other than *NOD2* induce an efficient autophagy [55].

Autophagy has been found dysfunctional in multiple human disorders [66–69] and therapeutic interventions aiming at modulating autophagy to improve the management of such disorders are being considered for more than a decade [70–74]. At the moment, our knowledge on the exact dynamic of autophagy in disease settings remains very fragmentary and means to accurately study the status of the autophagy flux directly in tissues represent major challenges. Despite difficulties, strategies and considerations to gain insights into these issues are nevertheless emerging [35,36]. Currently, the ex-vivo analysis of the autophagy flux performed very rapidly on whole blood samples might represent a suitable option to define the autophagy status during acute phase of disease or to monitor the effects of treatments on the autophagic activity [75]. On the other hand, means to document the intrinsic features of autophagic activity in the absence of inflammatory/pharmacological influences prior to precisely configuring autophagy targeted therapies are greatly needed. Here, we describe an approach to assess the intrinsic functional status of the autophagy flux in patients by examining the dynamic of its reprogramming in easily accessible cells: blood monocyte-derived DCs. In the case of healthy individuals, such cells normally recalibrate two parameters of the flux while they progress toward the mature status which is the status that render them capable of instructing subsets adaptive immune cells. Interestingly, this flux reprogramming capacity appears not to be compromised by chronic supraphysiological levels of inflammation such as the ones that characterizes CD. We

propose that this approach can be part of the strategies used to precisely document the features of the autophagy flux functioning in patients in general, and to detect constitutive alterations of this functioning in inflammatory/infectious contexts in particular.

Materials and methods

Ethics statement

Experiments were performed at Biological Safety Level 2 in accordance with the regulations set forth by the national French committee of genetic. The clinical study including CD patients has been approved by an ethics committee in accordance with French regulations (Comite de Protection des Personnes, Ile-de-France VII, CPP number #2019-A0149-57) and sponsored by Hospices Civils de Lyon (#69HCL180-0027).

Crohn disease patients

Crohn disease patients were prospectively recruited between June 2020 and July 2023 at the Department of Gastroenterology, Lyon-Sud University Hospital, Hospices Civils de Lyon, France. The main clinical data included disease history and severity criteria were summarized in Table 1. Blood samples were drawn in heparin tubes regarding monocyte separation for DCs generation and in PAXgene® Blood DNA Tube (Qiagen 761,115) for genetic analysis. DNA was extracted using PAXgene Blood DNA Kit (Qiagen 761,133). All CD patients included were genotyped for single nucleotide polymorphisms (SNPs) predisposing to CD: *ATG16L1* rs2241880 T300A [10], *ULK1* rs12303764 [12], *IRGM* rs10065172 313C>T [52] and *NOD2* rs2066844 R702W [76] using Ion Chef robotic platform (ThermoFisher) allowing PCR amplification and library construction by ligation. Allelic discrimination was achieved using Ion Torrent PGM sequencer (ThermoFisher).

Cells and cell culture

DCs were differentiated from human peripheral blood monocytes. Blood samples were obtained from the EFS (Etablissement Français du Sang, healthy donors) or from the hospital Lyon Sud Gastroenterology department (CD patients), to generate immature DCs (iDCs) from healthy donors (HD iDC) or from Crohn disease patients (CD iDC), respectively. Blood CD14⁺ monocytes were purified from peripheral blood mononuclear cells (PBMC) using a dedicated CD14⁺ isolation kit (Miltenyi, 130-050-201). To differentiate monocytes in iDCs, monocytes were cultured in RPMI 1640-Glutamax (Gibco 61,870) supplemented with 10% fetal bovine serum (FBS; Corning 15,393,691), 1% HEPES (Gibco 15,630,106), pH 7.4, and 1% penicillin-streptomycin (Gibco 15,070,063), 20 ng/mL CSF2/GM-CSF (Miltenyi, 130-093-866) and 50 ng/mL IL4 (Miltenyi, 130-093-922) for 7 days. The efficiency of DC differentiation was confirmed from each healthy donor or CD patient by FACS analysis based on the acquisition of the expression of CD1A and the

loss of the expression of CD14. Eventually, iDCs were incubated for the last 12 h to 48 h of culture with either 1 µg/mL of purified LPS (Invivogen, 0111:B4, tlr1-3pelps), 3 µg/mL of R848 (Invivogen, tlr1-r848), 200 ng/mL of TNF (Miltenyi, 130-094-014) or with adherent-invasive *Escherichia coli* (AIEC strain LF82, MOI = 2.5; AIEC were eventually heat-killed by 40-min incubation at 70°C, before to be cultured with DC), in order to induce mature dendritic cells (mDCs). Maturation status was characterized by FACS analysis for the intensity of cell surface expression of CD80, CD86, ICAM1/CD54, CD83 and HLA-DR.

HeLa *ATG16L1* KO cells were maintained in Dulbecco's Modified Eagle Medium (DMEM; Gibco 10,566,016) complemented with 10% FBS, 50 µg/mL of gentamicin (Gibco 15,750,037).

Antibodies and reagents

Anti-CD80 FITC (Immunotech, MAB104), anti-CD1A PE (clone HL149), anti-CD86 PE-Vio770 (FM95), anti-CD83 APC (HB15) (all from Miltenyi), HLA-DR PE-CF594 (G46-6) and anti-ICAM1/CD54 (555512) (all from BD) were used for FACS analysis (LSRII and FlowJo 7.6.5, both from Becton Dickinson). Analysis of FACS data was done on fixed DCs with IOTest3 Fixative solution (Beckman Coulter, A07800), and after exclusion of dead cells using Cell Viability Dyes (Becton Dickinson 566,332). Anti-ACTB/ACTIN (Sigma, A2066) and HRP anti-rabbit (Sigma, A0545) were used for protein detection by western blot and anti-LC3B (L7543, Sigma) was used for western blot and confocal microscopy. Pharmacological reagents used were 100 nM bafilomycin A₁ (Invivogen, tlr1-baf1), 20 µM spautin-1 (Calbiochem 567,569), and 100 nM rapamycin (Calbiochem 553,210).

Western blotting

Cells were lysed with a lysis buffer (Pierce 8,990,089,901), containing 4 mM protease cocktail inhibitor (Roche 04,693,132,001). Soluble proteins were reduced in 5 mM TCEP solution (ThermoFischer 77,720) and 1X LDS sample buffer (Invitrogen 2,045,288), and boiled 10 min. Proteins were separated on NuPAGE 4–12% Bis-Tris Gel (Invitrogen, NP0335BOX) and transferred to nitrocellulose membrane with Trans-Blot Turbo RTA Transfer Kit (Bio-Rad 1,704,271). Membranes were blocked for 1 h in 1X PBS (Gibco 10,010,023), 0.1% Tween 20 (Sigma, P9416), 5% milk (Millipore 70,166). Primary rabbit polyclonal antibodies, anti-ACTB/ACTIN and anti-LC3B and secondary HRP-linked anti-rabbit were used, and antigen-antibody complexes were visualized by enhanced chemiluminescence using western lightning Plus-ECL (PerkinElmer, NEL104001EA) with the ImageQuant LAS400 (GE Healthcare). Individual western blot detected bands for LC3-I and LC3-II were determined with ImageJ Software (NIH) to determine the LC3-II:LC3-I ratio.

Autophagy flux analysis

DCs or HeLa cells (1.10⁵ per well) were collected (time point –60 min) or seeded in 48-well plates and collected after half

an hour (time point –30 min) or one hour (time point 0 min) of culture. One hour post seeding (corresponding to the time point 0 min), cells were incubated with bafilomycin A₁ (BAFA₁) and collected at several time points up to 180 min of culture (time points 15 min to 180 min). Cells were lysed and the LC3-I and LC3-II levels of expression were analyzed by western blot, allowing for each time point the determination of the LC3-II:LC3-I level of expression ratio (reflecting the conversion of soluble LC3-I in autophagosome-anchored LC3-II) and reported on individual curves allowing for the determination of three qualitative parameters of the autophagic flux (Figure S1A): J = Flux, slope of the curve; nA = autophagosome pool size, (maximal LC3-II:LC3-I ratio after BAFA₁ treatment (plateau of the curve)); and τ = ($nA - nA_0$)/ J = time of transition or turnover, which corresponds to the time required for the renewal of the pool of autophagosomes to reach the basal level in a steady state condition.

Cytokines production analysis

IL6 and IL8 were assayed using Enzyme-Linked Immunosorbent Assay (ELISA) method (Human IL6 DuoSet Elisa and Human IL8 DuoSet Elisa; R and D system, DY206 and DY208). Briefly, supernatants of DCs were collected after 2 days of culture in complete medium with or without LPS (1 µg/ml), R848 (3 µg/ml) or TNF (200 ng/mL). All supernatants were analyzed using TECAN infinite 200Pro system (Lifesciences Tecan) and results were expressed as the mean amount of cytokine per ml (pg/ml ± S.D.).

Confocal microscopy

iDC and mDCs cells from a healthy donor were first plated on pre-treated poly-D-lysine (0.1 mg/ml) glass slices for 2 h, and treated with BAFA₁ (time points 30 min, 60 min, 120 min). After treatment with saponin (0.02%; Sigma 84,510) for 1 min, cells are fixed with 2% paraformaldehyde diluted in PBS for 15 min at room temperature. Primary antibodies were incubated overnight in staining buffer (5% goat serum (Abcam, 7481), 3% rabbit serum (Abcam 166,640), 1% BSA (Sigma, A3294), PBS). Secondary antibodies were then incubated for 75 min at room temperature in staining buffer. Nuclei were labeled with NucBlue (Invitrogen, R37606). Slices were mounted using Fluoromount (Agilent, S302380–2). All images were taken on LSM710 confocal microscope (Zeiss) with a 63X plan apochromat objective. Image analysis was performed using ImageJ software.

HeLa cell transfection and autophagy flux analysis

The day prior transfection, 3.10⁵ KO *ATG16L1* HeLa cells (provided from Pr. N. Barnich, Université Clermont Auvergne, Inserm U1071, Clermont Ferrand, France) were seeded in 6-well plates in DMEM medium (Gibco 61,965–026), supplemented with 10% FBS (Corning 15,393,691), 1% HEPES (Gibco 15,630,106) and 1% penicillin-streptomycin (Gibco 15,070,063). Cells were transfected with 1 µg of plasmid (*ATG16L1*^{WT} or *ATG16L1*^{T300A} provided from Pr. J.H. Brumell; Hospital for Sick Children,

Toronto, ON, Canada) per well using DreamFect™ Gold (OZ Biosciences, DG81000) following the manufacturer's instructions for 48 h. Then, 3.10^5 cells were seeded in 24-well plates for western blot analysis or on glass slides for confocal microscopy analysis in the same medium condition the day before treatment with BAFA₁. Cells were collected one hour before BAFA₁ treatment (corresponding to the time point 0 min), or collected at several time points up to 180 min of culture (time points 15 min to 180 min for western blot and 30 min to 120 min for confocal microscopy). For western blot analysis, HeLa cells were finally lysed and the LC3-I and LC3-II levels of expression were analyzed by western blot. For confocal microscopy, cells were treated with saponin (0.05%) for 1 min, and then fixed with acetone/methanol for 7 min for analyzing LC3-positive punctiform structures (LC3⁺ dots).

Statistics

Parameters of autophagic flux were compared on pairs of data between iDC and mDC with the Wilcoxon matched-pairs signed rank test. Subsequently, principal component analysis (PCA) was performed including as parameters the intensity of cell surface expression of CD80, CD86 and HLA-DR, the nature of the agent used for DC maturation (LPS, R848 and TNF) as well as the flux, the autophagosome pool size and the time of transition of the autophagy flux determined from each individual donor/patient, using the R package ade4. Bars represent median and ns: not significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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