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RESEARCH PAPER

Exosomes transfer miRNAs from cell-to-cell to inhibit autophagy during infection with Crohn's disease-associated adherent-invasive *E. coli*

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ABSTRACT

Adherent-invasive *E. coli* (AIEC), which abnormally colonize the intestinal mucosa of Crohn's disease (CD) patients, are able to adhere to and invade intestinal epithelial cells (IECs), survive and replicate within macrophages and induce a pro-inflammatory response. AIEC infection of IECs induces secretion of exosomes that increase AIEC replication in exosome-receiving IECs and macrophages. Here, we investigated the mechanism underlying the increased AIEC replication in cells receiving exosomes from AIEC-infected cells. Exosomes released by uninfected human intestinal epithelial T84 cells (Exo-uninfected) or by T84 cells infected with the clinical AIEC LF82 strain (Exo-LF82), the nonpathogenic *E. coli* K12 strain (Exo-K12) or the commensal *E. coli* HS strain (Exo-HS) were purified and used to stimulate T84 cells. Stimulation of T84 cells with Exo-LF82 inhibited autophagy compared with Exo-uninfected, Exo-K12 and Exo-HS. qRT-PCR analysis revealed increased levels of miR-30c and miR-130a in Exo-LF82 compared to Exo-uninfected, Exo-K12 and Exo-HS. These miRNAs were transferred *via* exosomes to recipient cells, in which they targeted and inhibited ATG5 and ATG16L1 expression and thereby autophagy response, thus favoring AIEC intracellular replication. Inhibition of these miRNAs in exosome-donor cells infected with AIEC LF82 abolished the increase in miR-30c and miR-130a levels in the released Exo-LF82 and in Exo-LF82-receiving cells, thus suppressing the inhibitory effect of Exo-LF82 on ATG5 and ATG16L1 expression and on autophagy-mediated AIEC clearance in Exo-LF82-receiving cells. Our study shows that upon AIEC infection, IECs secrete exosomes that can transfer specific miRNAs to recipient IECs, inhibiting autophagy-mediated clearance of intracellular AIEC.

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KEYWORDS
Crohn's disease; AIEC; exosomes; miRNA; autophagy

Introduction

Crohn's disease (CD) is an inflammatory bowel disease (IBD) with a multifactorial etiology, involving a complex interaction between environmental, genetic and microbial factors.[1,2] An intestinal dysbiosis has been reported in CD patients, characterized by a decrease in the number of beneficial bacteria such as members of the *Firmicutes* phylum and an increase in potentially harmful bacteria such as those from the *Enterobacteriaceae* family. Our group and others have reported a high prevalence of pathogenic adherent-invasive *Escherichia coli* (AIEC) strains in the ileal mucosa of CD patients.[3–6] The CD-associated AIEC strains have been shown to adhere to and to invade intestinal epithelial cells (IECs),[7–9] to survive and replicate within macrophages[10,11] with an increase in the macrophages from CD patients compared to those from healthy controls,[12] to promote pro-inflammatory cytokine production, and to colonize the gut and induce intestinal inflammation in genetically susceptible mouse models.[13,14]

Autophagy, a lysosomal degradation process, has emerged as a key player in the maintenance of intestinal homeostasis and gut ecology, the protection against microbes and the control of appropriate intestinal immune responses.[15] Efforts have been made to reveal the mechanisms by which dysfunctional autophagy predisposes to CD development.[16] Among those, a defect in autophagy has been shown to result in impaired clearance of pathogenic bacteria including CD-associated AIEC, which is associated with aberrant immune responses.[8,9,11,13,17-19] Reversely, we reported that AIEC can limit their elimination by the autophagy machinery of the host by increasing the level of microRNAs (miRNA, miR) 30c and 130a to suppress the

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expression of ATG (Autophagy-related) 5 and ATG16L1, respectively, two key players of autophagy induction.[8] This consequently favors the intestinal colonization of AIEC and increases AIEC-induced inflammation *in vitro* and *in vivo*.[8] Given that miR-30c and miR-130a are upregulated in CD,[8] dysfunctional autophagy might not be only caused by CD-associated risk variants in autophagy-related genes, but also by the epigenetic factors that negatively regulate expression of autophagy-related genes.

Recently, our group highlighted a previously uncovered function of exosomes, small extracellular vesicles with a diameter of 30 to 100 nm,[20] in cell-to-cell communication during AIEC infection.[21] Exosomes are released from a broad range of cell types and are found in most bodily fluids.[20] Exosomes play a role in cell-to-cell communication by transferring lipids, proteins and genetic material such as mRNAs and miRNAs from a donor cell to a recipient cell.[20] Moreover, the exosomal content is functional when being transferred to recipient cells, in which the exosomal mRNAs are translated into proteins[22,23] and exosomal miRNAs are able to silence target genes.[24–26] Thus, exosomes have been implicated in many physiopathological functions, such as signaling, immunity and infection.[20] In the context of AIEC infection, we reported that AIEC infection promotes the secretion of exosomes by infected cells, which are in turn uptaken by naïve cells, leading to increased pro-inflammatory response and impaired clearance of intracellular AIEC.[21] The mechanisms underlying the effects of exosomes in recipient cells, however, remain unknown.

Since a functional autophagy is required to restrain the intracellular replication of AIEC,[19] we hypothesized that the increased AIEC replication in the cells that receive exosomes released from AIEC-infected IECs is due to impaired autophagy. Thus, in the current study, we aimed at investigating whether miR-30c and miR-130a, which were previously shown to be upregulated in AIEC-infected cells,[8] are packaged in exosomes and transferred to recipient cells to inhibit ATG5 and ATG16L1 expression, limiting autophagy-mediated clearance of intracellular AIEC.

Results

Exosomes released from AIEC LF82-infected IECs inhibit autophagy in recipient IECs

We previously reported that exosomes secreted by AIEC-infected human intestinal epithelial T84 cells increase intracellular replication of AIEC and inflammation in exosome-receiving cells.[21] Since a functional autophagy is required to restrain the intracellular replication of AIEC,[19] we hypothesized that exosomes released from AIEC-infected cells may inhibit autophagy in recipient cells, thus leading to abnormal AIEC replication. To confirm this, we purified exosomes secreted by uninfected T84 cells (designated as Exo-uninfected) or by T84 cells infected with the AIEC LF82 reference strain (designated as Exo-LF82) or the non-pathogenic *E. coli* K12 strain (designated as Exo-K12) or the commensal *E. coli* HS strain (designated as Exo-HS) for 12 h as previously described.[21] Western blot analysis showed that the purified exosomes contained the exosomal marker CD63 and CD9 but did not carry the negative marker GRP94, which was detected only in T84 cell lysate (Figure 1a). T84 cells were then stimulated with the purified exosomes, and western blot analysis for the shift of LC3-I (microtubule-associated protein 1 light chain 3; the free cytosolic form) to LC3-II (the form conjugated to phagophore and autophagosome), an indication of autophagy induction,[27] was performed. Upon stimulation with Exo-LF82, T84 cells exhibited decreased LC3-II level, suggesting impaired autophagy, compared with cells receiving Exo-uninfected, Exo-K12 or Exo-HS (Figure 1b,c). Inhibition of autophagy by Exo-LF82 was confirmed by the concomitant increase in p62/SQSTM1 (sequestosome 1), a receptor protein degraded by functional autophagy[27] (Figure 1b,c). Furthermore, Exo-LF82 also inhibited LC3-II level and p62 degradation compared to Exo-uninfected, Exo-K12 or Exo-HS in T84 cells infected with the AIEC LF82 strain (Figure 1d,e). These results suggest that exosomes derived from AIEC-infected cells may inhibit autophagy in exosome-receiving cells at basal level or following AIEC infection.

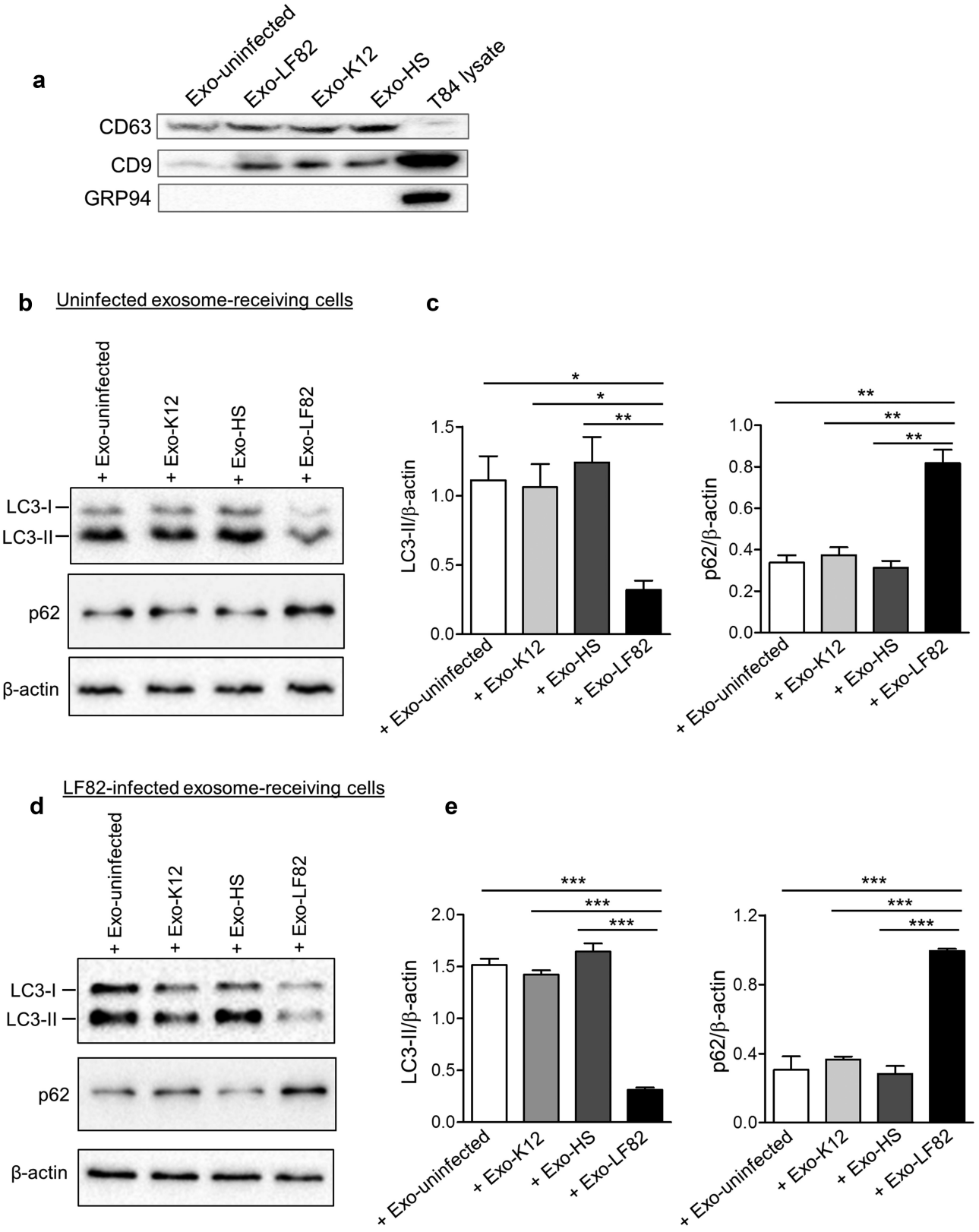

Figure 1. AIEC LF82-infected IECs release exosomes that can inhibit autophagy in recipient cells. Exosomes were purified from the culture supernatant of uninfected T84 cells (Exo-uninfected) or T84 cells infected with the AIEC LF82 strain (Exo-LF82), the *E. coli* K12 MG1655 strain (Exo-K12) or the commensal *E. coli* HS strain (Exo-HS) for 12 h. (a) Western blot analysis of the exosomal markers CD63 and CD9, and the negative marker GRP94 using 30 µg of exosomal protein lysate or T84 protein lysate. (b-e) T84 cells were stimulated for 8 h with Exo-uninfected, Exo-LF82, Exo-K12 or Exo-HS (80 µg of exosome/mL of culture medium) and then uninfected (b, c) or infected with LF82 for 4 h (d, e). Representative western blot analysis (b, d) and quantification of LC3-II/β-actin band intensity (c, e) are shown. Data are representative of three independent experiments and are presented as means ± SEM. Statistical analysis was performed using the one-way ANOVA test followed by a Bonferroni posttest. *$P < 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$.

MiR-30c and miR-130a levels are increased in the exosomes released from AIEC LF82-infected IECs and in exosome-receiving IECs

Our previous study demonstrated that upon AIEC infection, the levels of miR-30c and miR-130a are increased in host IECs, thus inhibiting expression of ATG5 and ATG16L1, respectively, and subsequently impairing autophagy.[8] As previously observed, qRT-PCR analysis showed increased levels of miR-30c and miR-130a in T84 cells in response to AIEC LF82 infection compared to uninfected IECs or IECs infected with the K12 MG1655 or *E. coli* HS strains (Figure 2a). We hypothesized that in response to AIEC infection, miR-30c and miR-130a were packaged in exosomes and transferred to recipient cells, in which they inhibit ATG5 and ATG16L1 expression and suppress autophagy. To confirm this, exosomes secreted from uninfected T84 cells or T84 cells infected with the AIEC LF82, or K12 MG1655 or *E. coli* HS strain were purified, and the levels of miR-30c and miR-130a in these exosomes were analyzed by qRT-PCR. As shown in Figure 2b, the levels of miR-30c and miR-130a were significantly increased in Exo-LF82 compared to Exo-uninfected, Exo-K12 or Exo-HS.

Furthermore, T84 cells stimulated with Exo-LF82 exhibited increased miR-30c and miR-130a levels compared with those receiving Exo-uninfected, Exo-K12 or Exo-HS (Figure 2c). This suggests that these miRNAs were transferred from exosomes to recipient cells.

MiR-30 c and miR-130a are efficiently transferred via exosomes from donor cells to recipient cells, where they target and inhibit ATG5 and ATG16L1 expression

To demonstrate that miR-30c and miR-130a are directly transferred from cell-to-cell *via* exosomes, we transfected T84 cells with Alexa Fluor 555-labeled-miR-30c or Alexa Fluor 555-labeled-miR -130a, then extracted the released exosomes. Naïve T84 cells were then incubated with the purified exosomes. Confocal microscopic analysis clearly showed the localization of Alexa Fluor 555-labeled miRNAs inside exosome-receiving cells (Figure 3a,b), indicating that these miRNAs are successfully transferred from donor cells to recipient cells *via* exosomes.

We next examined whether miR-30c and miR-130a transferred from exosomes can regulate ATG5 and ATG16L1 expression in recipient cells. Naïve T84 cells were transfected with a construct in which the 3′-untranslated region (UTR) of *ATG5* or *ATG16L1* mRNA was cloned downstream a luciferase-coding sequence in the pMIR-REPORT^{TM} luciferase vector (designated as *ATG5*-3′-UTR-luc or *ATG16L1*-3′-UTR-luc construct, respectively). Transfected cells were then stimulated with Exo-uninfected, Exo-K12, Exo-HS or Exo-LF82. Exo-LF82 significantly reduced luciferase activity in recipient cells transfected with *ATG5*-3′-UTR-luc or *ATG16L1*-3′-UTR-luc construct compared to Exo-uninfected, Exo-K12 or Exo-HS, indicating that the exosomal miR-30c and miR-130a bind to the 3′-UTRs of *ATG5* and *ATG16L1* mRNAs, respectively (Figure 4a). However, in cells transfected with the empty luciferase reporter, luciferase activity was not reduced upon stimulation with Exo-LF82 compared to Exo-uninfected, Exo-K12 or Exo-HS, indicating the specificity of binding between the exosomal miR-30c or miR-130a and the 3′-UTR of *ATG5* or *ATG16L1* mRNA, respectively (Figure 4a). Importantly, T84 cells receiving Exo-LF82 exhibited significantly reduced levels of *ATG5* and *ATG16L1* mRNAs compared to those receiving Exo-uninfected, Exo-K12 or Exo-HS, as analyzed by qRT-PCR (Figure 4b). Furthermore, western blot analysis revealed a significant reduction of ATG5 and ATG16L1 protein expression in cells receiving Exo-LF82 compared to that in cells receiving Exo-uninfected, Exo-K12 or Exo-HS (Figure 4c).

Together, these results show that upon AIEC infection, host cells can release exosomes that transfer miR-30c and miR-130a to recipient cells to inhibit expression of their target genes *ATG5* and *ATG16L1* at both mRNA and protein levels, by directly targeting the 3′-UTRs of *ATG5* and *ATG16L1* mRNAs.

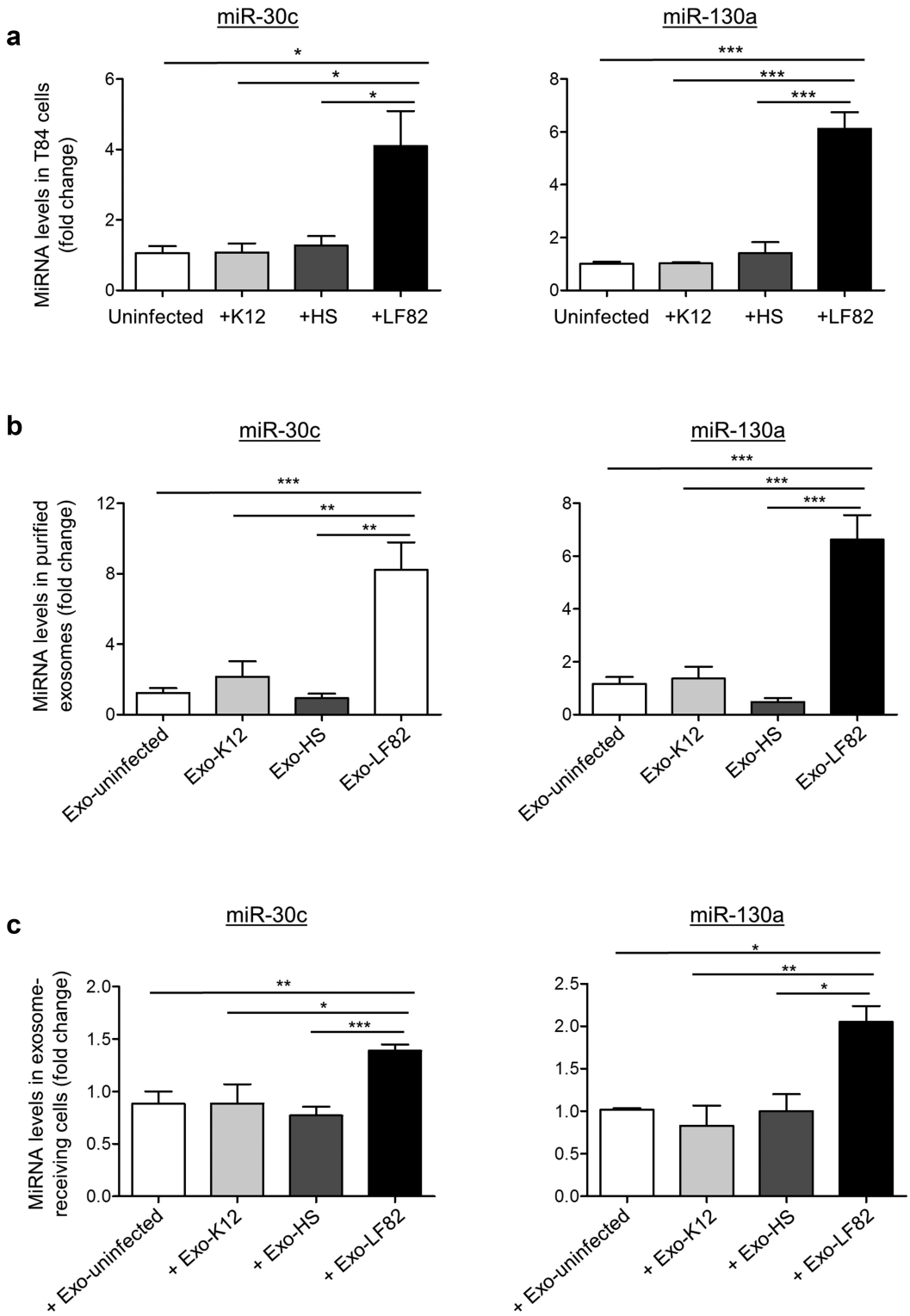

Figure 2. MiR-30c and miR-130a levels are increased in the exosomes released from AIEC LF82-infected IECs and in exosome-receiving IECs. (a, b) Exosomes were purified from uninfected T84 cells (Exo-uninfected) or T84 cells infected with the AIEC LF82 strain (Exo-LF82), the *E. coli* K12 MG1655 strain (Exo-K12) or the *E. coli* HS strain (Exo-HS) for 12 h. The levels of miR-30c and miR-130a in exosome-donor cells (a) and in the purified exosomes (b) were analyzed by qRT-PCR. (c) T84 cells were stimulated with the purified exosomes (80 µg of exosome/mL of culture medium), and the levels of miR-30c and miR-130a in these cells were analyzed by qRT-PCR. Data are representative of three independent experiments and are presented as means ± SEM. Statistical analysis was performed using the one-way ANOVA test followed by a Bonferroni posttest. *$P < 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$.

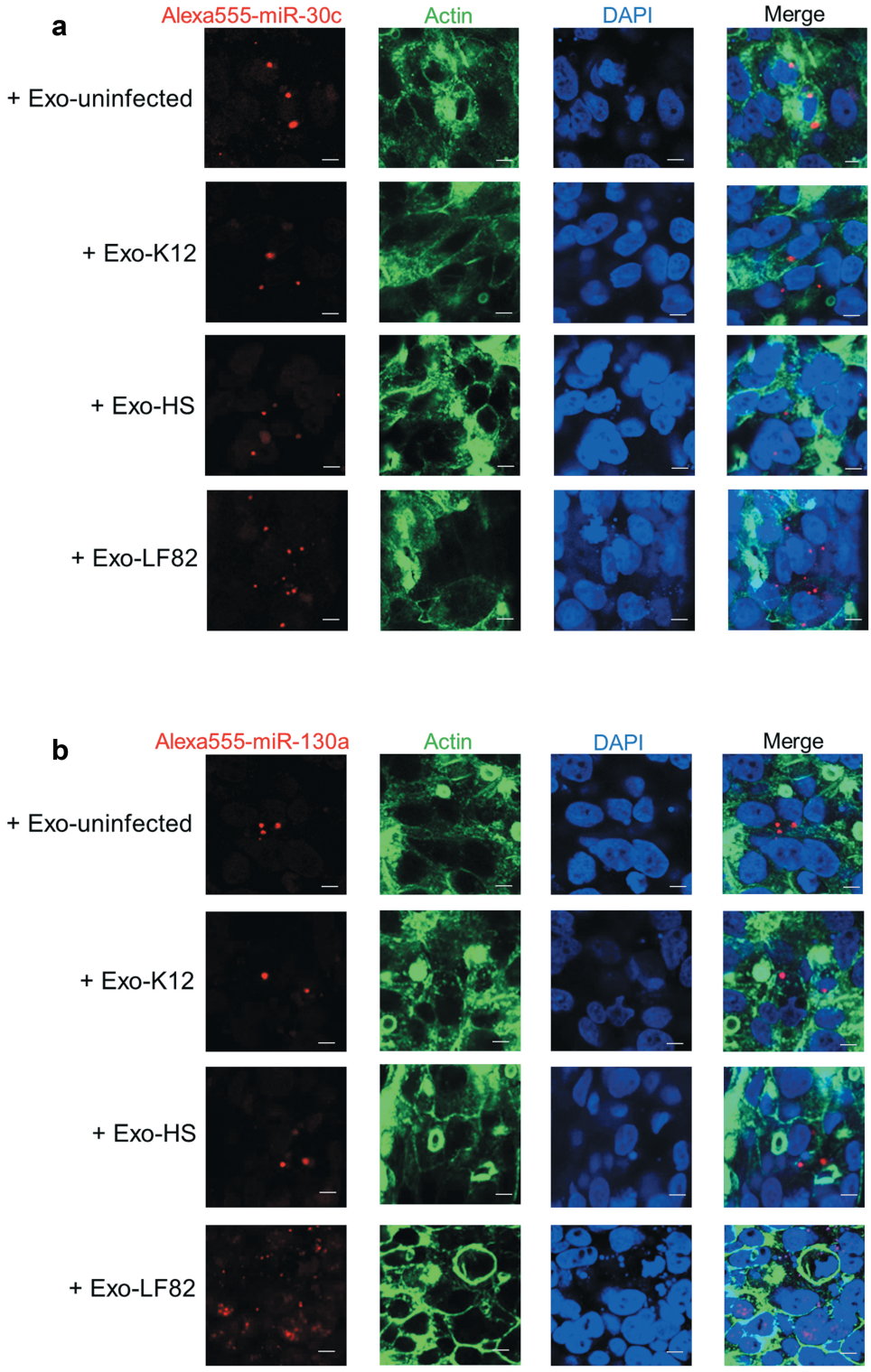

Figure 3. MiR-30c and miR-130a are efficiently transferred *via* exosomes from donor to recipient cells. T84 cells were transfected with 50 nM of Alexa Fluor 555-labeled-miR-30c (a) or Alexa Fluor 555-labeled-miR-130a (b) for 24 h, and uninfected or infected with the AIEC LF82, the *E. coli* K12 or the *E. coli* HS strain with a multiplicity of infection (MOI) of 10 for 12 h. Exosomes released from these cells were extracted and designated as Exo-uninfected, Exo-LF82, Exo-K12 and Exo-HS, respectively. Naïve T84 cells were incubated with the purified exosomes (80 µg of exosome/mL of culture medium) for 8 h. Confocal microscopic analysis of T84 cells that received exosomes from cells transfected with miR-30c (a) or miR-130a (b) labeled with Alexa Fluor 555 (red). Actin cytoskeleton was stained with FITC-phalloidin (green). Nuclei were stained with DAPI (blue). Scale bars = 5 µm.

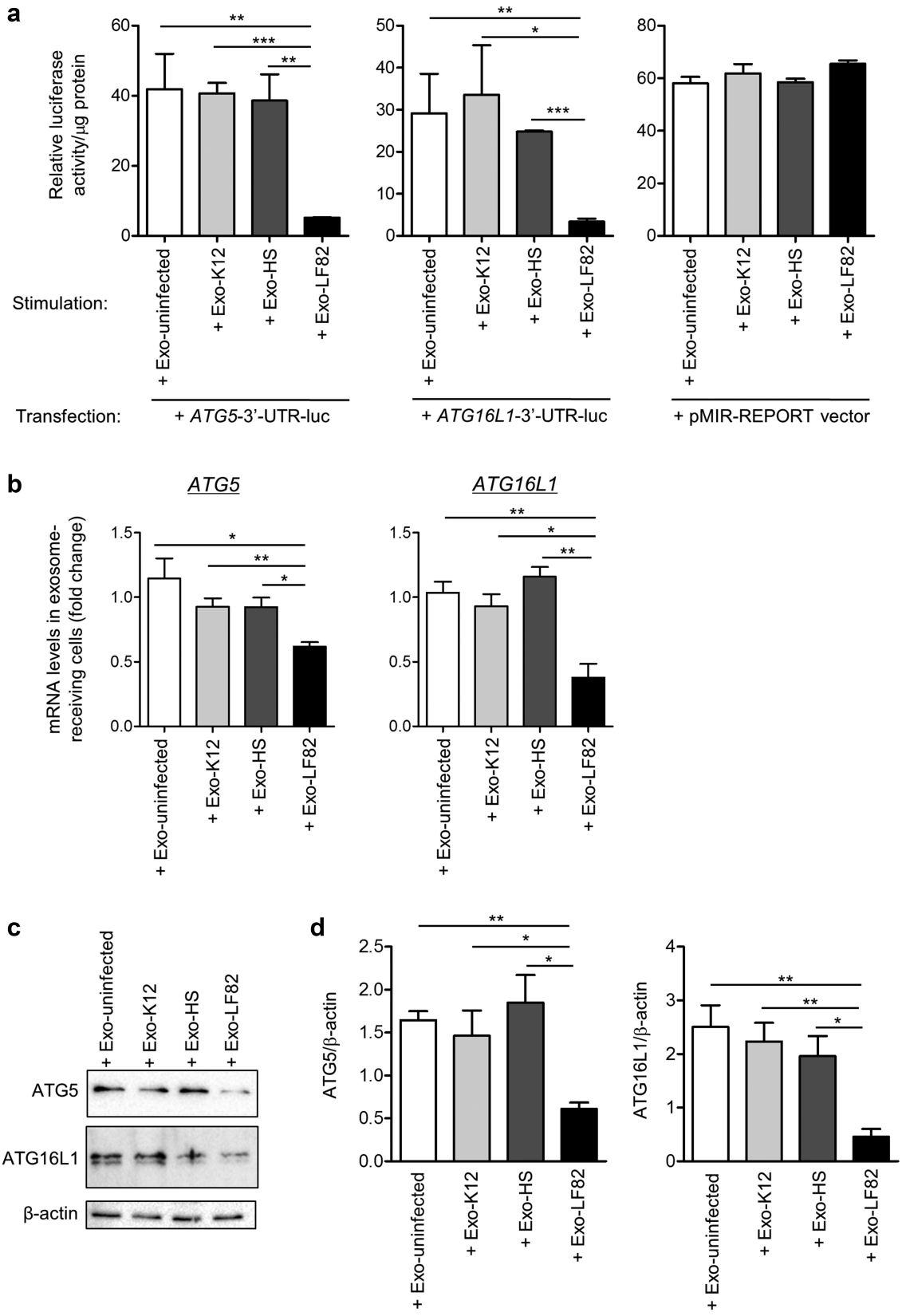

Figure 4. MiR-30c and miR-130a transferred from exosomes inhibit ATG5 and ATG16L1 expression in exosome-receiving IECs. Exosomes were purified from uninfected T84 cells (Exo-uninfected) or T84 cells infected with the AIEC LF82 (Exo-LF82), the *E. coli* K12 MG1655 (Exo-K12) or the HS strain (Exo-HS) for 12 h. (a) T84 cells were transfected with *ATG5*-3′-UTR-luc or *ATG16L1*-3′-UTR-luc construct, in which the 3′-UTR of *ATG5* or *ATG16L1* mRNA was cloned downstream a luciferase-coding sequence in the pMIR-REPORT luciferase vector, or with the empty vector. Sixteen hours after transfection, the cells were incubated with Exo-uninfected, Exo-K12,

Inhibition of miR-30 c and miR-130a in AIEC LF82-infected cells abolishes the increase in miR-30c and miR-130a levels in the released exosomes and in exosome-receiving cells

To further demonstrate that the inhibitory effect of Exo-LF82 on autophagy in exosome-receiving cells is dependent on miR-30c and miR-130a, we inhibited the levels of these miRNAs using their antisenses, designated as anti-miR-30c and anti-miR -130a, in T84 donor cells uninfected or infected with LF82 or *E. coli* HS strain, and examined the effect of exosomes released by these cells on autophagy in recipient cells. As shown in Figure 5a, in T84 cells transfected with the anti-miR negative control (anti-miR-NC), LF82 infection led to an increase in miR-30c and miR-130a levels compared to *E. coli* HS infection or uninfected condition. However, in cells transfected with anti-miR-30c and anti-miR-130a, this increase was abolished (Figure 5a).

Furthermore, naïve T84 cells were transfected with *ATG5*-3′-UTR-luc or *ATG16L1*-3′-UTR-luc construct and then incubated with Exo-uninfected, Exo-HS or Exo-LF82 released from cells transfected with anti-miR-NC or a combination of anti-miR-30c and anti-miR -130a. In cells receiving the exosomes from anti-miR-NC-transfected cells, reduced luciferase activity was observed upon incubation with Exo-LF82 compared to Exo-uninfected or Exo-HS (Figure 5b). This result, which was in agreement with the data in Figure 4a, indicates that increased levels of miR-30c and miR-130a in Exo-LF82 derived from anti-miR-NC-transfected cells were transferred to exosome-receiving cells, where they bound to the target 3′-UTRs of *ATG5* and *ATG16L1* mRNAs. However, in cells receiving the exosomes secreted by cells transfected with anti-miR-30c and anti-miR -130a, no significant difference in luciferase activity upon incubation with Exo-uninfected, Exo-HS or Exo-LF82 was observed (Figure 5b). This suggests that the inhibition of miR-30c and miR-130a in exosome-donor cells abolishes the increase in the levels of these miRNAs in cells receiving Exo-LF82 *versus* Exo-uninfected or Exo-HS. As expected, miR-30c and miR-130a were detected at very low levels in Exo-uninfected, Exo-HS and Exo-LF82 derived from cells transfected with anti-miR-30c and anti-miR-130a with no significant difference (data not shown). Importantly, Exo-LF82 derived from cells transfected with anti-miR-NC can increase miR-30c and miR-130a levels in recipient cells compared to Exo-uninfected or Exo-HS, and this effect was not observed for Exo-LF82 derived from cells transfected with anti-miR-30c and anti-miR-130a (Figure 5c).

Together, these data show that inhibition of miR-30c and miR-130a in AIEC LF82-infected donor cells abolishes the increase in miR-30c and miR-130a levels in the released exosomes and in exosome-receiving cells.

Inhibition of miR-30c and miR-130a in AIEC-infected cells abolishes the inhibitory effect of exosomes released from these cells on autophagy-mediated AIEC clearance in recipient cells

Exo-LF82 released from anti-miR-NC-transfected cells, compared to Exo-uninfected and Exo-HS, triggered the decreased expression of ATG5 and ATG16L1 at both mRNA and protein levels (Figure 6a–c) and inhibited autophagy response, as shown by decreased LC3-II level (Figure 6b,c) in recipient cells. This subsequently led to increased LF82 intracellular replication, presented as percentage of LF82 colony-forming units (CFU) determined at 24 h *versus* 4 h post-infection, defined as 100% (Figure 6d). The CFU of LF82 determined at 4 h post-infection in the cells that received Exo-LF82, Exo-uninfected and Exo-HS were not different (data not shown). Transfection of donor cells with anti-miR-30c and anti-miR-130a abolished the inhibitory effects of Exo-LF82 on ATG5 and ATG16L1 expression (Figure 6a–c) and on autophagy response (Figure 6b,c) in recipient cells. This resulted in an

Exo-HS or Exo-LF82 (80 µg of exosome/mL of culture medium) for 8 h. Luciferase activity was measured and normalized to the protein concentration of cell lysate. (b-d) T84 cells were stimulated with exosomes as in afor 8 h, and ATG5 and ATG16L1 expression at mRNA and protein levels were analyzed by qRT-PCR (b) and western blot (c), respectively. (d) Quantification of band intensity in c. Data are representative of three independent experiments and are presented as means ± SEM. Statistical analysis was performed using the one-way ANOVA test followed by a Bonferroni posttest. *$P < 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$.

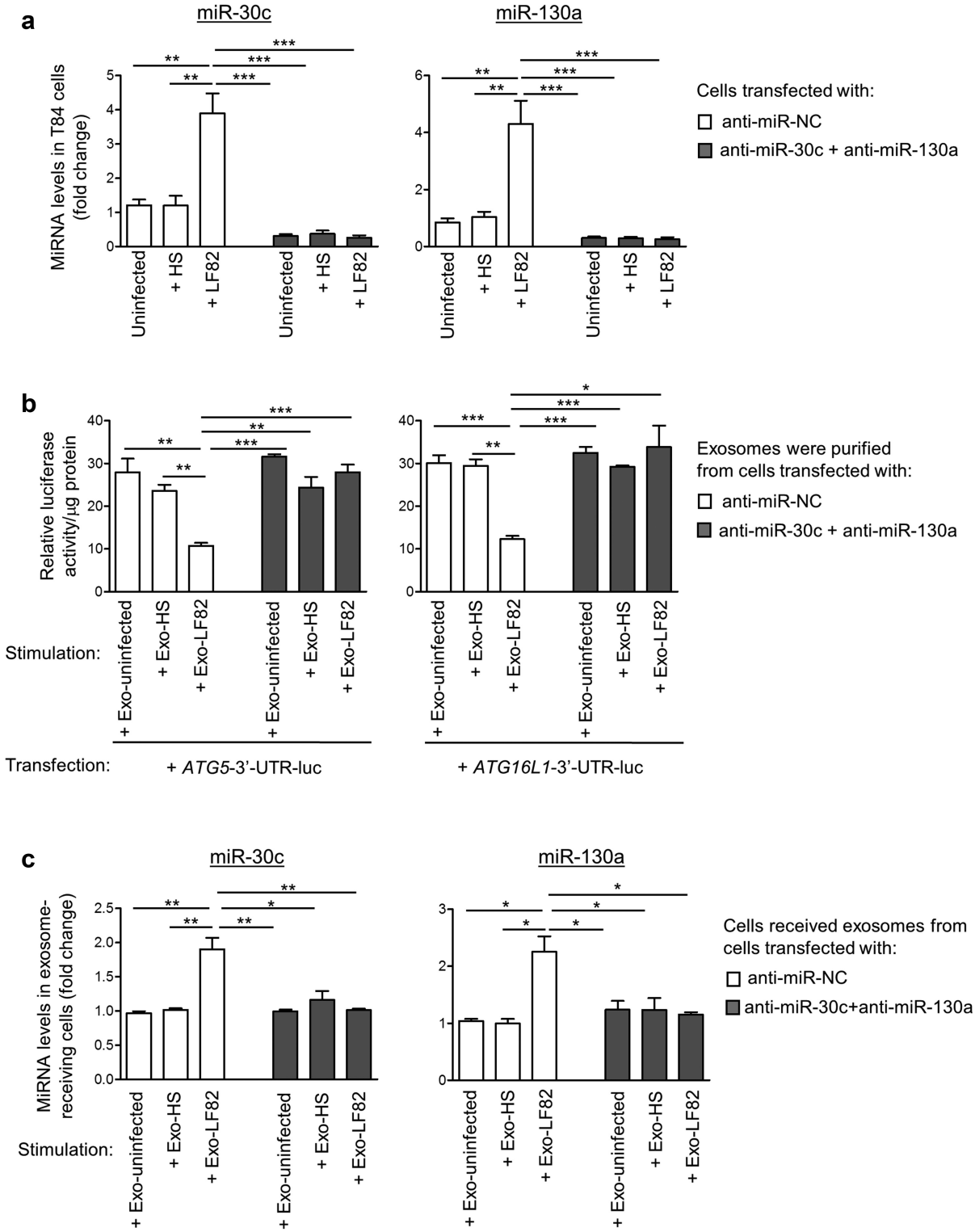

Figure 5. Inhibition of miR-30c and miR-130a in AIEC-infected T84 cells abolishes the increase in miR-30c and miR-130a levels in exosomes and in exosome-receiving cells. (a) T84 cells were transfected with vehicle or with 50 nM of anti-miR-negative control (anti-miR-NC) or with a combination of anti-miR-30c and anti-miR-130a. One day post-transfection, the cells were uninfected or infected with the AIEC LF82 or the commensal *E. coli* HS strain for 12 h. The levels of miR-30c and miR-130a were analyzed by qRT-PCR. (b) Naïve T84 cells were transfected with the *ATG5*-3′-UTR-luc or *ATG16L1*-3′-UTR-luc construct, in which the 3′-UTR of *ATG5* or *ATG16L1* mRNA was cloned downstream a luciferase-coding sequence in the pMIR-REPORT luciferase vector. Sixteen hours after transfection, the cells were stimulated with the exosomes purified from the culture supernatant of the cells in a(designated as Exo-uninfected, Exo-LF82 and Exo-HS) for 8 h (80 µg of exosome/mL of culture medium). Luciferase activity was measured at 1 day post-transfection and was normalized to the protein concentration of cell lysate. (c) Naïve T84 cells were stimulated with the purified exosomes as in b for 8 h, and miR-30c and miR-130a levels in the exosome-receiving cells were analyzed by qRT-PCR. Data are representative of three independent experiments and are presented as means ± SEM. Statistical analysis was performed using the one-way ANOVA test followed by a Bonferroni posttest. *$P < 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$.

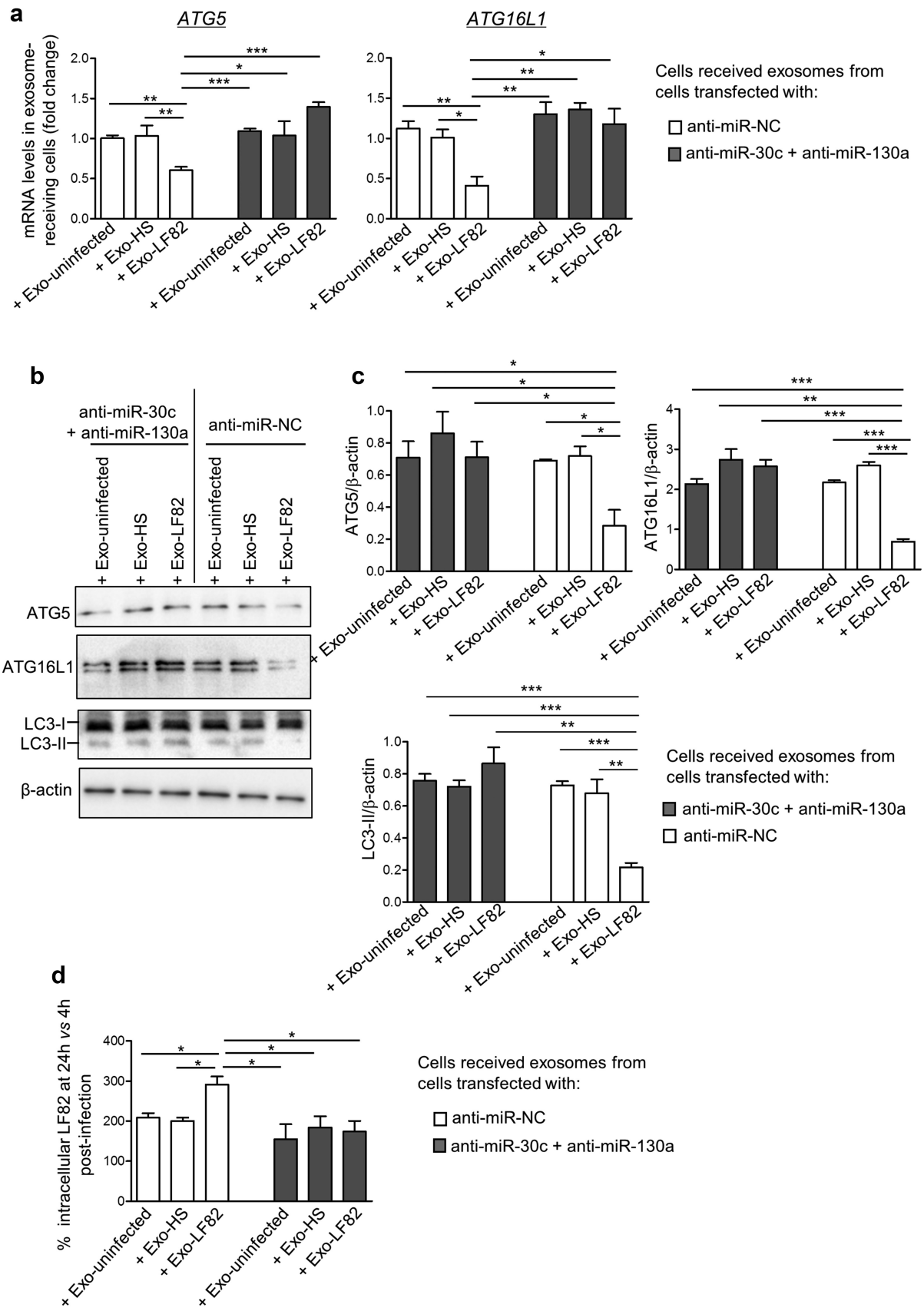

Figure 6. Inhibition of miR-30c and miR-130a in AIEC-infected T84 cells abolishes the inhibitory effect of exosomes on autophagy-mediated AIEC clearance in recipient cells. T84 cells were transfected with vehicle or with 50 nM of anti-miR-negative control (anti-miR-NC) or a combination of anti-miR-30c and anti-miR-130a. One day post-transfection, the cells were uninfected or infected with the AIEC LF82 or the commensal *E. coli* HS strain for 12 h, and exosomes were purified from the culture supernatant (designated as

efficient clearance of intracellular LF82 in cells receiving Exo-LF82 compared to Exo-uninfected or Exo-HS since similar levels of LF82 intracellular replication in these cells were observed (Figure 6d).

Together, these data show that during AIEC infection, the inhibitory effect of exosomes on autophagy in recipient cells is mediated by miR-30c and miR-130a, which are transferred from donor cells to recipient cells *via* exosomes.

Discussion

In recent years, significant progresses have been made in elucidating the mechanistic network underlying the role of exosomes in antibacterial defense, immunity and pathogenesis of several human diseases.[20] In the current study, we showed that the exosomal shuttle can transfer specific miRNAs from cells to cells during CD-associated AIEC infection, which leads to impaired autophagy-mediated clearance of intracellular AIEC.

Exosomes have been emerged as an important intermediator participating in cellular crosstalk during pathogen infection by delivering their content composed of proteins, lipids, and RNAs. It has been shown that exosomes can directly transfer pathogen-related molecules from cells to cells, thereby impacting the infection progress.[28] For example, cells intoxicated with the lethal toxin virulence factor, a toxin secreted by *Bacillus anthracis*, secrete exosomes transporting this toxin to naïve cells.[29] Similarly, the cytotoxin-associated gene A (CagA), a major virulence factor secreted by *Helicobacter pylori*, was found in exosomes secreted by gastric epithelial cells inducibly expressing the *cagA* gene as well as in exosomes derived from the serum of patients carrying *cagA*-positive *Helicobacter pylori*.[30] Furthermore, exosomes isolated from the serum of patients exhibiting a chronic gastritis and have *H. pylori* infection can induce expression of the soluble IL-6 receptor, which increases IL-1α expression, in gastric epithelial cells.[31] It has been reported that *Mycobacterium tuberculosis*-infected macrophages release extracellular vesicles including exosomes that can deliver pathogen-associated molecular patterns (PAMPs), such as mycobacterial proteins, lipids, and nucleic acids, to naïve macrophages, activating or inhibiting immune responses.[23,32-35] Moreover, exosomes from *Mycobacterium tuberculosis*-infected macrophages or from the serum of *Mycobacterium tuberculosis*-infected mice can activate endothelial cells, suggesting a role for these exosomes in promoting leukocyte adhesion and cell migration as well as inflammation upon *Mycobacterium tuberculosis* infection.[35] However, the exosomal components responsible for these effects remain to be identified. Regarding the exosomal miRNAs, increasing evidence has highlighted the effect of pathogen infection on the miRNA composition of host cell-derived exosomes, which may have an impact on exosome-receiving cells and host immune responses, thus inhibiting the infection or, on the contrary, promoting the immune escape of the pathogen.[28] In the context of viral infection, exosomes secreted by epithelial cells infected with Newcastle disease virus, a zoonotic virus affecting all species of birds, exhibit increased levels of miR-1273f, miR-1184 and miR-198.[24] These exosomal miRNAs inhibit interferon pathway in exosome-receiving cells, thus promoting viral infection.[24] Similarly, infection of human HT-29 colon cancer cells with enterovirus 71 induces the secretion of exosomes that can transfer miR-146a to recipient cells, in which this miRNA inhibits expression of IRAK1 (interleukin-1 receptor-associated kinase 1), TRAF6 (tumor necrosis factor receptor-associated factor 6) and STAT1 (signal transducer and activator of transcription 1), thus suppressing IFN response and facilitating enterovirus 71 infection.[36] Conversely, exosomes derived from bronchoalveolar lavage fluid of mice infected with influenza virus display an increased level of miR-483-3p, which exacerbates pro-inflammatory and anti-viral responses in

Exo-uninfected, Exo-LF82 and Exo-HS, respectively). Naïve T84 cells were stimulated with the purified exosomes for 8 h (80 µg exosome/mL of culture medium). (a) *ATG5* and *ATG16L1* mRNA expression levels in exosome-receiving cells were analyzed by qRT-PCR. Western blot analysis of ATG5, ATG16L1 and LC3 expression (b) and band intensity quantification (c). (d) Exosome-receiving cells were infected with the AIEC LF82 strain, and the number of intracellular LF82 bacteria was determined using gentamicin protection assay. The results are presented as percentage of CFU of LF82 determined at 24 h *versus* 4 h post-infection, defined as 100%. Data are representative of three independent experiments and are presented as means ± SEM. Statistical analysis was performed using the one-way ANOVA test followed by a Bonferroni posttest. *$P < 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$.

recipient lung epithelial cells *in vitro*.[37] In the context of bacterial infection, it was shown that upon lipopolysaccharide (LPS) exposure, miR-155 and miR-146a from murine bone marrow-derived dendritic cells (BMDC) are released into exosomes and are efficiently transferred to recipient BMDC.[25] Upon being uptaken, these miRNAs mediate target gene repression to modulate inflammatory gene expression and reprogram cellular response to endotoxins.[25] Similarly, exosomes derived from the serum of a sepsis-related lung injury mouse model established by intraperitoneal injection of LPS are selectively enriched in miR-155.[38] *In vitro*, these exosomes stimulate NF-κB activation, induce production of TNF-α and IL-6, and favor macrophage proliferation. *In vivo*, the intravenous injection of these exosomes to naïve mice increased the number of pro-inflammatory M1 macrophages in the lungs and induce lung inflammation.[38] MiR-155 level was also found increased in exosomes derived from *Helicobacter pylori*-infected macrophages, and this exosomal miRNA regulates inflammatory responses in exosome-receiving macrophages to limit *H. pylori* replication and prevent the gastritis caused by *H. pylori* infection.[39] Likewise, upon infection of macrophages with *Mycobacterium bovis* Bacillus Calmette-Guerin (BCG), the miRNA content of macrophage-derived exosomes is altered.[40] *In silico* analysis suggested that the differentially expressed exosomal miRNAs may subvert host metabolic pathway to enable BCG survival within infected macrophages.[40]

Evidence has also suggested that extracellular vesicles, which include exosomes, seem to play a role in IBD. The extracellular vesicles purified from the intestinal lumen of IBD patients convey a higher amount of IL-6, IL-8, IL-10 and TNF-α compared to those from healthy subjects, and the levels of these pro-inflammatory mediators were positively correlated with CD severity score.[41] The extracellular vesicles isolated from the lumen of IBD patients also activate IECs *in vitro*, leading to increased IL-8 secretion.[41] Furthermore, these extracellular vesicles, as well as extracellular vesicle-treated IECs, induce the migration of a greater number of macrophages than untreated IECs.[41] A higher amount of human proteins associated with oxidative antimicrobial activity was also shown in extracellular vesicles (including exosomes, microvesicles and bacterial outer membrane vesicles) collected from the interface between intestinal mucosa and intestinal lumen of IBD patients compared to those collected from control subjects, and was correlated with an alteration of microbial functions in IBD patients.[42]

In the context of CD-associated AIEC infection, we recently reported that exosomes are new mediators of host-AIEC interaction with their capacity to trigger a pro-inflammatory response and promote AIEC intracellular replication in exosome-receiving cells.[21] However, the mechanisms behind the functional effects of exosomes on recipient cells remain unknown. Previous works of our group have shown that autophagy is a key mechanism of host defense to limit the intracellular replication of AIEC,[9,11,19] and that the CD-associated risk variants in the autophagy-related genes *ATG16L1* and *IRGM* lead to defective autophagy and impaired elimination of intracellular AIEC.[17,19] Conversely, AIEC is able to counteract autophagy defense by increasing the level of miR-30c and miR-130a to suppress the expression of the autophagy-related proteins ATG5 and ATG16L1 in host cells, thus favoring AIEC colonization and promoting AIEC-induced inflammation.[8] In the present study, we identified the mechanism by which exosomes derived from AIEC-infected cells modulate AIEC intracellular replication in recipient cells. We showed that exosomes secreted by AIEC LF82-infected human intestinal epithelial T84 cells exhibited an increased level of miR-30c and miR-130a compared with exosomes secreted by uninfected cells or cells infected with nonpathogenic *E. coli* strains. These miRNAs were efficiently transferred *via* exosomes from donor to recipient T84 cells, in which they directly targeted the 3′-UTRs of *ATG5* and *ATG16L1* mRNAs, thus inhibiting ATG5 and ATG16L1 expression at both mRNA and protein levels. This consequently resulted in impaired autophagy-mediated clearance of AIEC. Along with other studies, our results showed that bacterial infection-induced miRNA transfer within exosomes plays a role in cell-to-cell communication *via* modulating innate immune responses, thus favoring pathogen survival.

To further confirm that the observed effects are specifically dependent of miR-30c and miR-130a, we inhibited the levels of these miRNAs in exosome-donor cells and the functional consequences of this inhibition were examined. We showed that inhibition of miR-30c and miR-130a in exosome-

donor cells before AIEC LF82 infection abolished the increase in their levels in the secreted exosomes and in exosome-receiving cells. Consequently, the inhibitory effect of the exosomes secreted from LF82-infected cells on autophagy in exosome-receiving cells was not observed; thus, autophagy was sustained and LF82 intracellular replication was well controlled. Collectively, these results indicated that the exosomal miR-30 c and miR-130a, increased in response to AIEC infection, are sufficient to impair autophagy in exosome-receiving cells, thus leading to increased AIEC intracellular replication. Moreover, these results highlighted for the first time a role for exosomal miRNAs in modulating autophagy in exosome-receiving cells, and reinforce the importance of autophagy, miRNAs and exosomes in CD pathogenesis.

In conclusion, together with our previous study showing that AIEC can subvert host autophagy response by upregulating the levels of miR-30c and miR-130a to replicate inside host cells, we propose that AIEC dysregulate host miRNAs to inhibit autophagy, and this can be amplified from cell to cell *via* the exosomal shuttle, thus favoring AIEC colonization (Figure 7). This importantly suggests that the loss of tightly regulated autophagy, caused by miRNA dysregulation, in handling AIEC, which might contribute to the pathogenesis of CD, could happen at local and distant sites in a host. This should be kept in mind while developing future personalized strategies to treat CD patients with abnormal AIEC colonization.

Materials and methods

Bacterial strains

The AIEC LF82 reference strain isolated from a chronic ileal lesion of a CD patient,[7] the nonpathogenic *E. coli* K-12 MG1655 strain and the commensal *E. coli* O9:H4 strain (*E. coli* HS)[43] were used. Bacteria were grown in Luria-Bertani (LB) broth (Pronadisa) overnight at 37°C without shaking.

Cell culture

Human intestinal epithelial T84 cells (ATCC CCL-248™) were maintained in an atmosphere containing 5% CO_2 at 37°C in a Dulbecco's Modified Eagle Medium/Nutrient F-12 HAM culture medium (Gibco) supplemented with 10% fetal bovine serum (FBS, Dutscher), 1% L-glutamine (Gibco), 1% minimal essential medium vitamins 100X free from L-Glutamine (Dutscher), and 1% antibiotic and antimycotic solution (10,000 U of penicillin, 10 mg of streptomycin, and 0.025 mg of amphotericin B per mL; GE Healthcare HyClone).

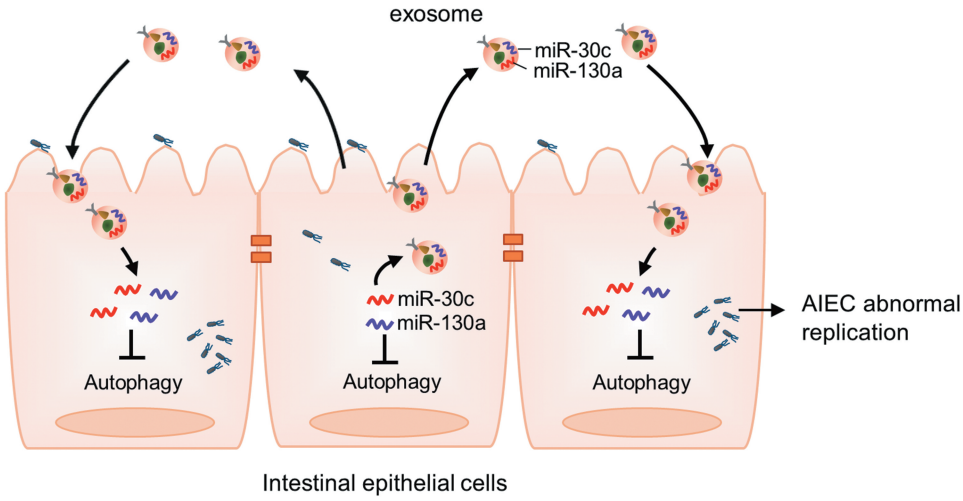

Figure 7. Model for the role of exosomes in transferring miR-30c and miR-130a from cell-to-cell to inhibit autophagy during infection with CD-associated AIEC. AIEC upregulates miR-30c and miR-130a in host cells to inhibit autophagy and this can be amplified from cells to cells *via* the exosomal shuttle, which transfers these miRNAs to adjacent cells. This consequently leads to impaired autophagy-mediated AIEC clearance, favoring AIEC colonization.

Bacterial infection of human T84 cells and determination of intracellular bacterial number using gentamicin protection assay

For exosome extraction, T84 cells were seeded on 150 cm^2 cell culture flask (Falcon) and infected with a multiplicity of infection (MOI) of 10. T84 cells were maintained in contact with bacteria for 3 h in culture medium without antibiotics (infection medium). Then, cells were washed with phosphate-buffered saline (PBS; Gibco) and incubated for 9 h with culture medium containing 10% exosome-depleted FBS (System Biosciences, Mountain View, CA) instead of regular FBS to avoid coextraction of bovine exosomes and 100 µg/mL of gentamicin (Euromedex) to remove extracellular bacteria.

For infection of exosome-receiving cells, T84 cells were seeded on 12-well or 24-well plates (Falcon), stimulated with exosomes (80 µg of exosomes per 1 ml of culture medium) for 8 h and infected with a MOI of 10. Cells were maintained in contact with bacteria for 3 h in infection medium and were washed with PBS. Cells were then incubated with infection medium containing 100 µg/mL of gentamicin for the indicated time.

Invasion assay was performed as described previously.[8] Briefly, cells were lysed using 1% Triton X-100 (Euromedex) in deionized water. Serial dilutions of samples were made and plated onto LB agar plate, and the number of colony-forming units (CFU) was counted to determine the number of intracellular bacteria.

Isolation of exosomes from cell culture supernatant

Twelve hours after infection, the supernatant of infected cells was collected, and exosomes were extracted using the ExoQuick™ Exosome Precipitation Solution (System Biosciences) following the manufacturer's protocol. Briefly, supernatant was centrifuged at 3,000 *g* for 15 min to eliminate cell debris. Then, supernatant was passed through a Mustang E membrane 0.2-µm pore size filters (Pall Corporation) to remove bacterial LPS and microvesicles larger than exosomes. ExoQuick™ was added to the supernatant with a volume ratio of 1:5, the suspension was mixed by inverting the tubes and incubated at 4°C overnight. Finally, the suspension was centrifuged at 1,500 *g* for 30 min to pellet the exosomes.

Protein extraction and western blot analysis

T84 cells or purified exosomes were lysed in radioimmunoprecipitation assay (RIPA) lysis buffer containing 150 mM NaCl, 0.5% sodium deoxycholate, 50 mM Tris-HCl pH 8, 0.1% sodium dodecyl sulfate, 0.1% Nonidet *P*-40, and supplemented with protease inhibitors (Roche). Proteins were quantified using Bio-Rad DC™ protein assay kit according to the manufacturer's instructions (Bio-Rad).

Cell and exosome lysates were separated on sodium dodecyl sulfate polyacrylamide gels, transferred to nitrocellulose membranes, blocked in 5% bovine serum albumin (BSA) in PBS containing 0.1% Tween-20 and then probed with appropriate primary antibodies: anti-CD63 (EXOAB-CD63A-1, System Biosciences), anti-CD9 (EXOAB-CD9A-1, System Biosciences), anti-GRP94 (sc-11402, Santa Cruz), anti-ATG16L1 (#8089, Cell Signaling Technology), anti-ATG5 (#12994, Cell Signaling Technology), anti-LC3 (L7543, Sigma), anti-p62 /SQSTM1 (sc-28359, Santa Cruz) and anti-β-actin (#4970, Cell Signaling Technology). After washes, membranes were incubated with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies (Cell Signaling Technology and System Biosciences), and blots were revealed using the Clarity Western enhanced chemiluminescence detection kit (Bio-Rad) and the ChemiDoc™ XRS System (BioRad).

In vitro transfection and luciferase assay

Anti-miR negative control #1 and the antisenses of miR-30c and miR-130a were purchased from Ambion. T84 cells were transfected with 50 nM of antisenses of miRNAs using Lipofectamine 2000 (Invitrogen) and Opti-MEM I reduced serum medium (Invitrogen) 24 h before being infected with AIEC LF82, *E. coli* K12 MG1655 or *E. coli* HS strain for 12 h. Exosomes were purified from the culture supernatant of these cells and designated as Exo-uninfected, Exo-LF82, Exo-K12 and Exo-HS, respectively.

The 3′-untranslated regions (UTRs) of human *ATG5* and *ATG16L1* mRNAs were cloned into the pMIR-REPORT™ Luciferase vector (Ambion) to generate *ATG5*-3′-UTR-luciferase or *ATG16L1*-3′-UTR-luciferase construct, respectively, as previously described.[8] For luciferase assay, T84 cells were seeded on 48-well plate. Twenty-four hours later, cells were transfected with 750 ng of *ATG5*-3′-UTR-luciferase or *ATG16L1*-3′-UTR-luciferase construct or of the pMIR-REPORT™ Luciferase vector, and 16 h after transfection, the cells were stimulated with 80 µg of exosomes per 1 ml of culture medium for 8 h. Firefly luciferase activity was measured using the dual-luciferase reporter assay system (Promega) and a Luminoskan Ascent luminometer (Thermo Electron Corp.). Relative luciferase activity was normalized to µg of protein, determined from cell lysate using Bio-Rad DC™ protein assay kit according to the manufacturer's instructions (BioRad).

RNA extraction, cDNA synthesis and quantitative RT-PCR (qRT-PCR) analysis

Total RNAs were extracted from purified exosomes or cultured cells using the miRNeasy mini kit (Qiagen) according to the manufacturer's instructions. For exosomal samples, 25 fmol of each spike-in controls *Caenorhabditis elegans* cel-mir-39-3p, cel-mir-54-3p and cel-mir-238-3p (mirVana™ miRNA mimic, Life Technologies) were added into the lysis solution per extraction.

Total RNAs were reversely transcribed using the miRNA 1st strand cDNA synthesis kit (Agilent) to quantify mature miRNA levels or the PrimeScript RT reagent kit (Takara) to quantify mRNA expression levels. qRT-PCR was performed using SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad) on a CFX96 Touch™ Real-Time PCR detection system (Bio-Rad) using specific primers listed in **Table S1**. For the quantification of mature miRNA levels, the universal reverse primer provided in the miRNA 1st strand cDNA synthesis kit was used with the specific forward primers indicated in **Table S1**. Spike-in controls cel-mir-39-3p, cel-mir-54-3p and cel-mir-238-3p or U6 were used as internal controls for quantification of miRNAs in exosomes or in cells, respectively. Human 18S was used as an internal control for mRNA quantification in cells. Fold-induction was calculated using the comparative threshold cycle number (Ct) method as follows: $\Delta\Delta Ct = (Ct_{\text{target mRNA/miRNA}} - Ct_{\text{internal control}})_{\text{condition of interest}} - (Ct_{\text{target mRNA/miRNA}} - Ct_{\text{internal control}})_{\text{control condition}}$, and the final data were derived from $2^{-\Delta\Delta CT}$. When spike-in controls cel-mir-39-3p, cel-mir-54-3p and cel-mir-238-3p were added into samples and used as internal controls, the average of Ct of the three miRNAs was used as $Ct_{\text{internal control}}$.

Fluorescent microscopy

T84 cells were transfected with AlexaFluor555-labeled -miR-30c or AlexaFluor555-labeled-miR-130a (Ambion) for 24 h, and uninfected or infected with the AIEC LF82 strain or the *E. coli* K12 or *E. coli* HS strain with a MOI of 10 for 12 h. The supernatants of these cells were collected and exosomes were purified. T84 cells seeded on coverslips were stimulated with the purified exosomes (80 µg exosome/mL of culture medium) for 8 h. Cells were then fixed with 4% formaldehyde, permeabilized with 0.1% Triton X-100 for 10 min, saturated with PBS containing 0.025% Triton X-100, 3% BSA and 5% FBS for 1 h. Actin cytoskeleton was stained with FITC-phalloidin (A12379, Life technologies), and nuclei were stained with 2-(4-Amidinophenyl)-6-indolecarbamidine dihydrochloride (DAPI; Sigma) for 45 min at room temperature. Coverslips were mounted in EUKITT® medium (O. Kindler). Images were taken using Zeiss LSM 800 with Airyscan confocal microscope.

Statistical analysis

Values are expressed as means ± standard error of the mean (SEM). Statistical analyses between several groups were performed using ANOVA followed by a Bonferroni posttest (Kruskal–Wallis if not parametric) with GraphPad Prism version 7 software (GraphPad Software, La Jolla, CA). A P value less than 0.05 was considered statistically significant. $^{*}P < 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.001$.

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Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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