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RESEARCH PAPER

Aging is associated with hypermethylation of autophagy genes in macrophages

Hany Khalil[a], Mia Tazi[b,c], Kyle Caution[b,c], Amr Ahmed[b,c], Apurva Kanneganti[b,c], Kaivon Assani[d], Benjamin Kopp[d], Clay Marsh[c], Duaa Dakhlallah[c], and Amal O. Amer[b,c]

[a]Department of Molecular Biology, Genetic Engineering and Biotechnology Research Institute, University of Sadat City, Sadat City, Egypt; [b]Department of Microbial Infection and Immunity, Center for Microbial Interface Biology, The Ohio State University, Columbus, OH; [c]The Davis Heart and Lung Research Institute, The Ohio State University, Columbus, OH; [d]The Research Institute at Nationwide Children's Hospital, The Ohio State University, Columbus, OH

ABSTRACT

Autophagy is a biological process characterized by self-digestion and involves induction of autophagosome formation, leading to degradation of autophagic cargo. Aging is associated with the reduction of autophagy activity leading to neurodegenerative disorders, chronic inflammation, and susceptibility to infection; however, the underlying mechanism is unclear. DNA methylation by DNA methyltransferases reduces the expression of corresponding genes. Since macrophages are major players in inflammation and defense against infection we determined the differences in methylation of autophagy genes in macrophages derived from young and aged mice. We found that promoter regions of *Atg5* and *LC3B* are hypermethylated in macrophages from aged mice and this is accompanied by low gene expression. Treatment of aged mice and their derived macrophages with methyltransferase inhibitor (2)-epigallocatechin-3-gallate (EGCG) or specific DNA methyltransferase 2 (DNMT2) siRNA restored the expression of Atg5 and LC3 *in vivo* and *in vitro*. Our study builds a foundation for the development of novel therapeutics aimed to improve autophagy in the elderly population and suggests a role for DNMT2 in DNA methylation activities.

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Aging; autophagy; DNA methylation; EGCG; macrophages

Introduction

Autophagy is a fundamental, conserved physiological process by which cells target long-lived cytosolic proteins and organelles for lysosomal degradation. Typically, formation of autophagosomes is initiated upon starvation or stress. The pre-autophagosomal membrane acquires autophagy proteins BECN1/Atg6, Atg12, Atg5 and Atg16L. Then, Atg8/LC3 is recruited to the membrane and processed to yield LC3-II. Therefore, the transformation of LC3 to LC3-II denotes autophagy stimulation and autophagosome formation. The accumulation of LC3-II on forming autophagosomes can be detected by confocal microscopy as puncta and by Western blot as a lower LC3 band. Subsequently, the formed autophagosome fuses with the lysosome, where the contents are degraded. The lack of a single Atg is sufficient to abort the completion of autophagosomes. Autophagy cooperates with apoptosis, inflammatory cytokines, and the adaptive immune system to orchestrate the cellular homeostasis and the appropriate immune response against endogenous or exogenous danger signals.[1] Dysfunctional aged macrophages are a major contributor to the aging process and, in particular, to the low-grade pro-inflammatory phenotype that is associated with aging and deterioration of the innate and adaptive immune responses accompanying the aging process.[2,3] Autophagy controls inflammation through interactions with innate immune pathways by removing endogenous inflammasome components and affects the secretion of immune mediators.[1,4] The decline in autophagy activity with aging has been implicated in a variety of age-related disease conditions such as Alzheimer's, Parkinson's, Huntington's diseases, cancer, and susceptibility to specific infections. However, the mechanism for reduced autophagy with aging remains obscure.[5-9]

Epigenetic modifications, such as DNA methylation, are described as changes occurring on a chromosome without altering the DNA sequence. DNA methylation mainly occurs on cytosine at position 5 in a CpG dinucleotide and is mediated by DNA methyltransferases (DNMTs). Five forms of methyltransferases exist: DNMT1, DNMT2, DNMT3a, DNMT3b, and DNMT3L. Methylation of CpG islands within a promoter region suppresses the expression of corresponding genes due to poor recognition by transcription factors.[10,11] Consequently, the DNA methylation pattern modifies corresponding protein expression profile that is ultimately associated with various human disorders, such as Alzheimer's and cancer.[12]

Here, to determine the mechanism for low autophagy activity in innate immune cells derived from aged mice, we investigated the expression of autophagy genes and their methylation status. Our data indicate that the low expression of autophagy molecules in aged macrophages is due to high levels of DNA methyltransferase 2 (DNMT2), which is associated with high levels of methylated autophagy molecules Atg5 and LC3B. Interestingly, treatment with the methylation inhibitor (2)-epigallocatechin-3-gallate (EGCG), a plant flavonoid with anti-inflammatory and anti-oxidant

CONTACT Amal O. Amer amal.amer@osumc.edu

effects, leads to re-expression of autophagy genes *in vitro* and *in vivo*. This effect could be due to inhibition of DNMT2 methylation activity. Therefore, our data provides a novel mechanism for the low autophagy activity associated with aging. The identification of a role for DNA methyl inhibitors in the improvement of autophagy in senescence provides new therapeutic options for age-associated disorders with underlying autophagy defects.

Results

Resting macrophages from aged mice exhibit characteristics of aging cells and low expression of autophagy proteins

The strong implication of autophagy in age-related diseases and evidence that it declines with age prompted us to seek the mechanism of deteriorating autophagy activity with aging. Macrophages from 62-64 weeks aged mice were derived from femurs, as previously described,[13-15] and compared to their young 8 weeks aged counterparts. We examined the expression of several autophagy molecules, such as Atg5, LC3A, and LC3B, in macrophages. Quantitative reverse transcription PCR (qRT-PCR) showed that Atg5 and LC3B mRNAs are significantly reduced in macrophages from aged mice ($P = 0.002$ and $P = 0.02$, respectively) (Fig. 1A); however, the expression of LC3A showed normal levels ($P = 0.13$). The expression of corresponding proteins was also reduced, as demonstrated by Western blot with specific antibodies to Atg5/Atg12 and LC3 (Fig. 1B). We next conducted a macrophage functional assay to examine the ability of macrophages from aged mice to form autophagosomes during resting conditions. Macrophages from young and aged mice were fixed and LC3 was detected with the corresponding antibody and secondary fluorescent antibody. Upon starvation, macrophages from young mice demonstrated a moderate number of LC3-labeled autophagosomes (puncta), whereas those from aged mice exhibited significantly lower numbers (Fig. 1C). The level of florescent LC3 was quantified using ImageJ 1.48 software. Unbiased quantification demonstrates that macrophages from young mice express high level of LC3, whereas those from aged mice fail to do so (Fig. 1D). Together, our data demonstrate that the expression of specific autophagy proteins and autophagy activity are reduced in macrophages derived from aged mice.

The methylation of promoter regions of autophagy genes is increased in macrophages from aged mice

Although accumulating studies, including our data above, have established a strong correlation between the reduction

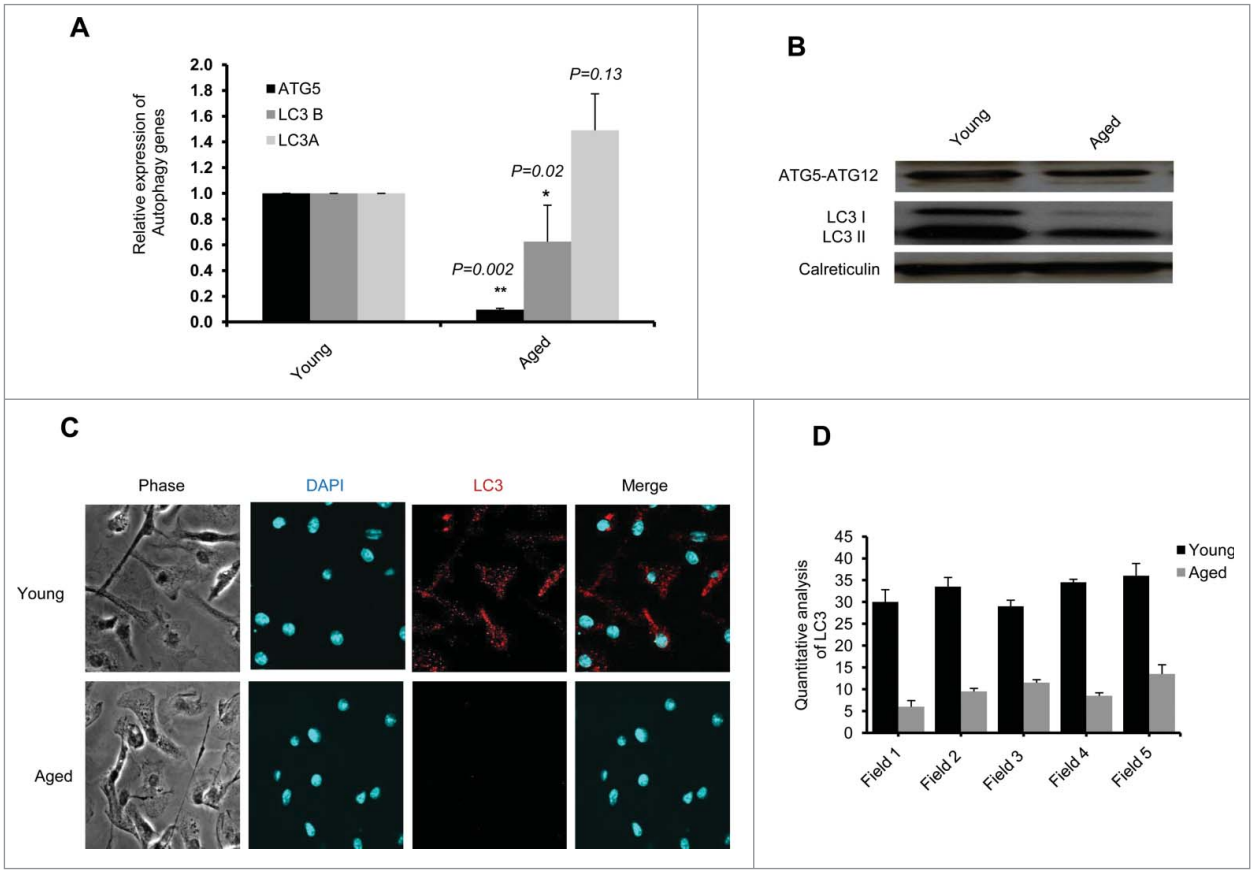

Figure 1. Autophagosome formation is reduced in macrophages derived from aged mice. (A) Relative expression of autophagy genes *Atg5, LC3A*, and *LC3B* in macrophages from young and aged mice. (B) Western blot analysis of Atg5-Atg12 protein complex and LC3 in the indicated macrophages. Calreticulin served as loading control. (C) Representative confocal images depicting macrophage from young and aged mice revealing the expression of LC3 (red) and DNA (blue). Student two-tailed test was used to determine the significance of autophagy related genes expression in aged macrophages (D) Quantitative analysis of detectable amount of LC3 using ImageJ software. Data is representative of three independent experiments. Error bars indicate the standard deviation (SD).

of autophagy activity and aging, the mechanism is still obscure. To understand the mechanism of low expression of autophagy molecules with aging, we investigated the expression of several microRNAs that are known to target autophagy molecules in young and aged mice and humans; however, we did not find significant differences (data not shown). On the other hand, when we examined the methylation of promoter regions of essential autophagy genes *Atg5, Atg12, LC3A*, and *LC3B*, we found that *Atg5* and *LC3B* genes were hypermethylated in macrophages derived from aged mice (Fig. 2A). We determined the expression of DNMT1, DNMT2, DNMT3a, DNMT3b, and DNMT3L in young and aging macrophages. Notably, we found that the expression of DNMT 2 was significantly increased in macrophages from aged mice, but not in their counterparts from young mice ($P = 0.005$) (Fig. 2B). By quantification using a confocal microscopy, macrophages from aged mice exhibited detectable amounts of DNMT2, whereas macrophages from young mice did not (Fig. 2C and D). Additionally, the expression level of DNMT2 protein was detectable by Western blot analysis in macrophages from aged mice, but not in macrophages from young mice (Fig. 2E). Taken together, these findings strongly suggest that reduction of autophagosome formation in aged mice is due to hypermethylation of promoters of autophagy genes and is accompanied by increased expression of DNMT2.

The level of DNMT2 is inversely proportional to LC3 expression in aged macrophages

The previous findings suggest that DNMT2 contributes to the methylation of autophagosomal markers *LC3* and *Atg5* in macrophages from aged mice. To investigate if LC3 and DNMT2 interact, fixed macrophages from aged and young mice were incubated with specific antibodies against LC3 and DNMT2. The localization of LC3 and DNMT2 was detected using an immunofluorescence microscope. Notably, LC3 protein co-localizes with DNMT2 in the cytoplasm of macrophages from aged mice accompanied with high expression level of DNMT2 compared to macrophages from young mice (Fig. 3A). Next, to further investigate if LC3 and DNMT2 interact, lysed macrophages were incubated with protein A agarose beads coated with LC3 primary antibody (Cell Signaling Technology). The immunoprecipitated protein complex from aged macrophages included LC3 accompanied with high expression level of DNMT2 when compared with young macrophages (Fig. 3B). These results suggested that DNMT2 interacts with LC3 either directly or

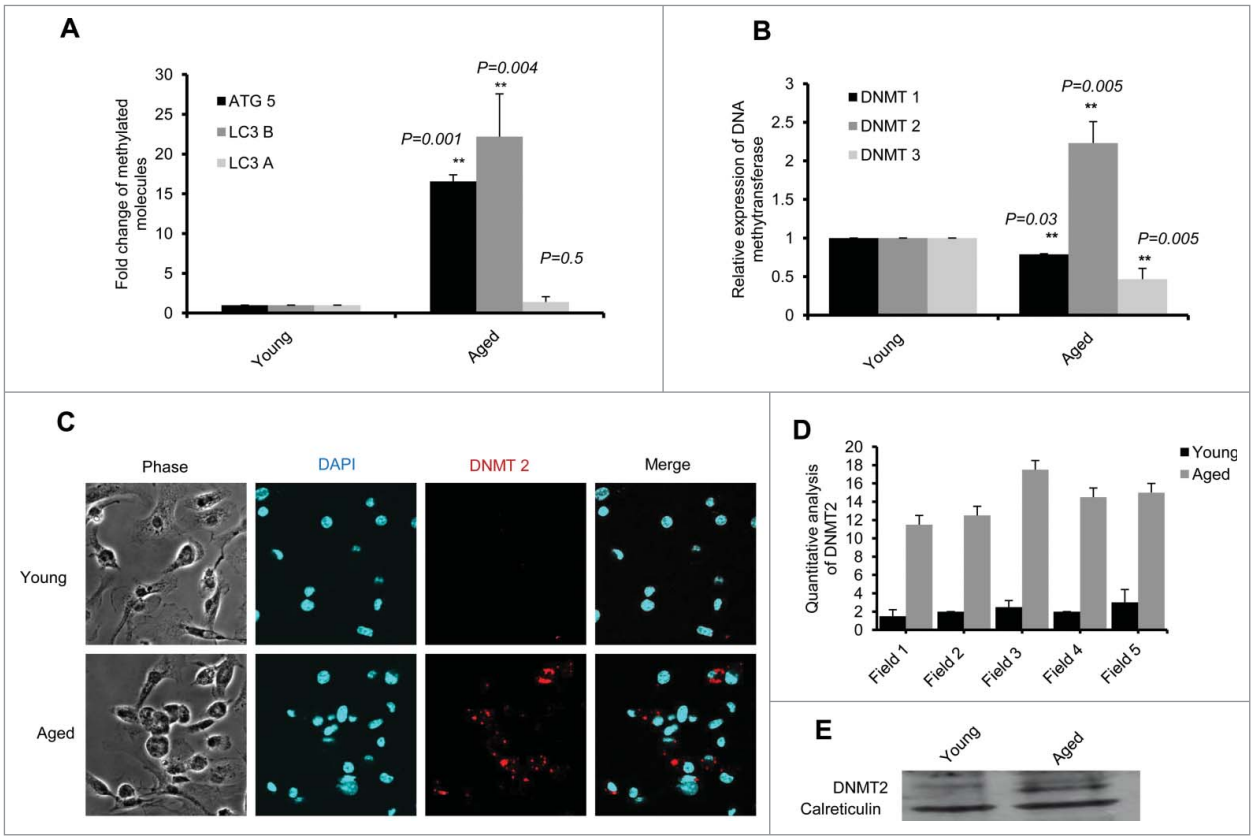

Figure 2. Hypermethylation of autophagy promoters is associated with elevated expression of DNMT2 in macrophages from aged mice. (A) Fold changes of methylated autophagy genes *Atg5, LC3A,* and *LC3B* in macrophages from young and aged mice quantified by qRT-PCR. (B) Relative gene expression of DNMTs in aged mice macrophages compared to macrophages from young mice. (C) Representative immunofluorescent images of macrophages from young and aged mice demonstrating the expression of DNMT2 (red) and DNA (blue). Student two-tailed test was used to determine the significance of methylated genes and DNMT expression in aged macrophages. (D) Quantitative analysis of detectable amount of DNMT2 using ImageJ software Data is representative of three independent experiments. Error bars indicate the SD. (E) Western blot analysis of DNMT2 expression level in the indicated macrophages using clareticluin level as internal loading control.

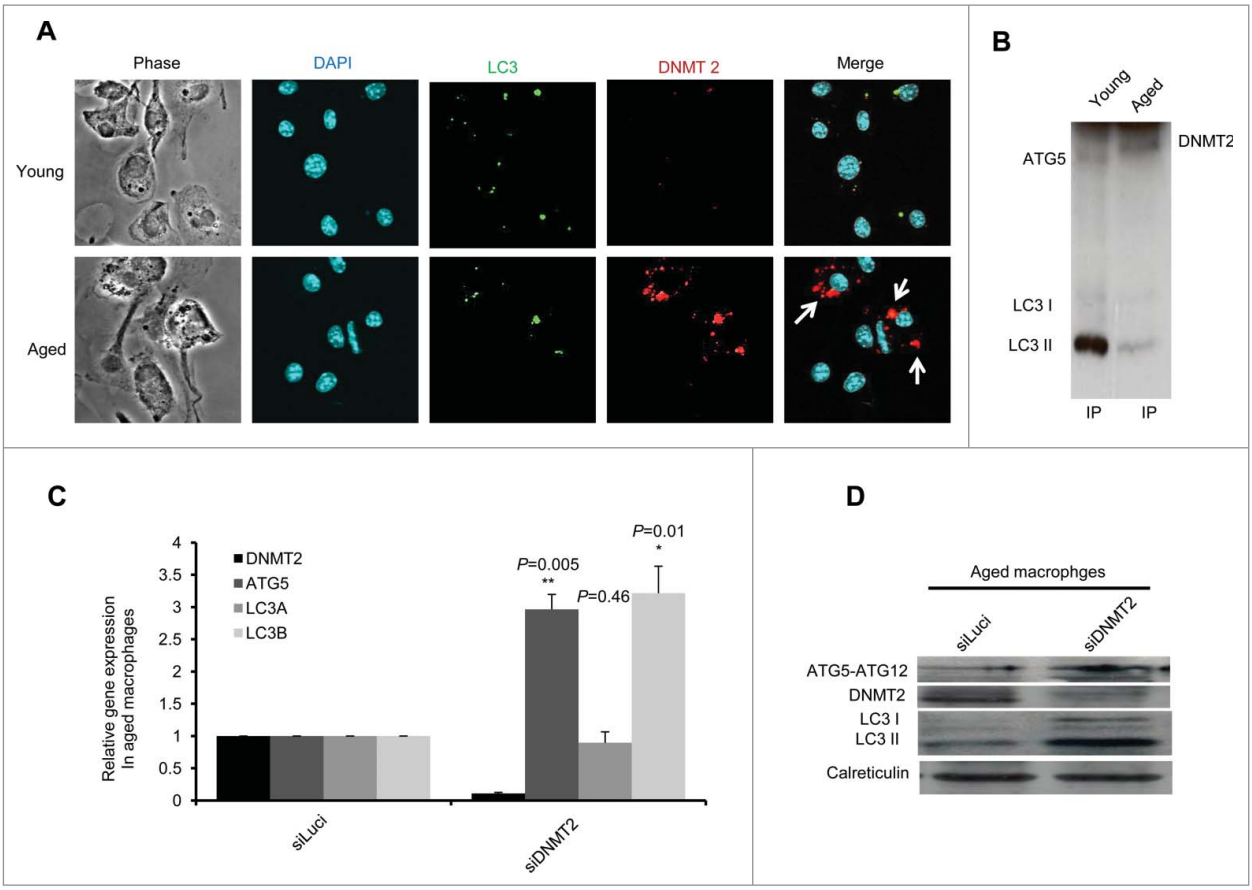

Figure 3. DNMT2 associates with LC3, (A) Representative of confocal images depicting macrophages from young and aged mice reveal the localization of autophagosomal marker LC3 (green) and methyltransferase DNMT 2 (Red). (B) Western blot analysis of immunoprecipitation samples prepared from aged and young mice macrophages reveal the expression level and protein interaction of LC3 and DNMT2. (C) Relative gene expression of *DNMT2, Atg5, LC3A*, and *LC3B* in aged mice macrophages that were transfected with indicated siRNA against DNMT2 or Luciferase gene as a control. Student two-tailed test was used to determine the significance of autophagy related genes expression in aged macrophages Error bars indicate the SD. (D) Immunoblotting analysis of conjugated Atg5-Atg12 protein complex, DNMT2 and LC3 in transfected macrophages from aged mice. Data is representative of two independent experiments.

indirectly in macrophages from aged mice and corroborates the idea that DNMT2 contributes to the repression of specific important autophagic molecules. To further confirm the involvement of DNMT2 in modulating the expression of autophagy molecules, macrophages from aged mice were transfected with specific DNMT2 siRNAs. Interestingly, depletion of DNMT2 resulted in high expression level of Atg5 and LC3 at both RNA and protein levels (Fig. 3C and D). Taken together, these data strongly suggest that the expression of DNMT2 is inversely proportional to the expression of two autophagosomal markers, LC3 and Atg5.

EGCG restores the expression of autophagy molecules in macrophages derived from senescent mice

To determine if hypermethylation of autophagy genes contributes to their low expression in aged macrophages, we reduced the methylation activity of DNMTs using (2)-epigallocatechin-3-gallate (EGCG). EGCG is a polyphenol compound extracted from green tea with anti-carcinogenic properties. Macrophages from aged mice were treated with 20 ug of either EGCG, genestien (another plant flavonoid), or 5-Azacytidine (5-Aza, a cytidine analog). Treatment of macrophages from aged mice with 5-Aza and EGCG increased the level of conjugated Atg5-Atg12 protein complex compared to DMSO-treated macrophages (Fig. 4A and B). Interestingly, treatment with EGCG showed strong reduction of DNMT2 expression compared to other indicated inhibitors (Fig. 4A). This result demonstrates that reducing the methylation activity with EGCG restores the expression of autophagy molecules in macrophages from aged mice. These findings suggest that the low expression of autophagy genes in aged macrophages is due to hypermethylation of their promoter regions that can be restored with the DNA methyltransferase inhibitor EGCG.

In vivo administration of EGCG restores the expression of autophagy molecules in aged mice

To test if treating live, aged mice with EGCG compound will restore the expression of autophagy genes *in vivo*, three aged mice (64 weeks) were treated intra-tracheally with 300 μg EGCG daily for three consecutive days. As controls, another set of three aged mice and three young mice (8 weeks) were treated with the same volume of DMSO, the vehicle for EGCG. Lungs were then homogenized and the expression profiles of DNMT2, LC3B, and Atg5-Atg12 were examined. EGCG treatment dramatically reduced DNMT2 expression, while significantly elevating the expression of

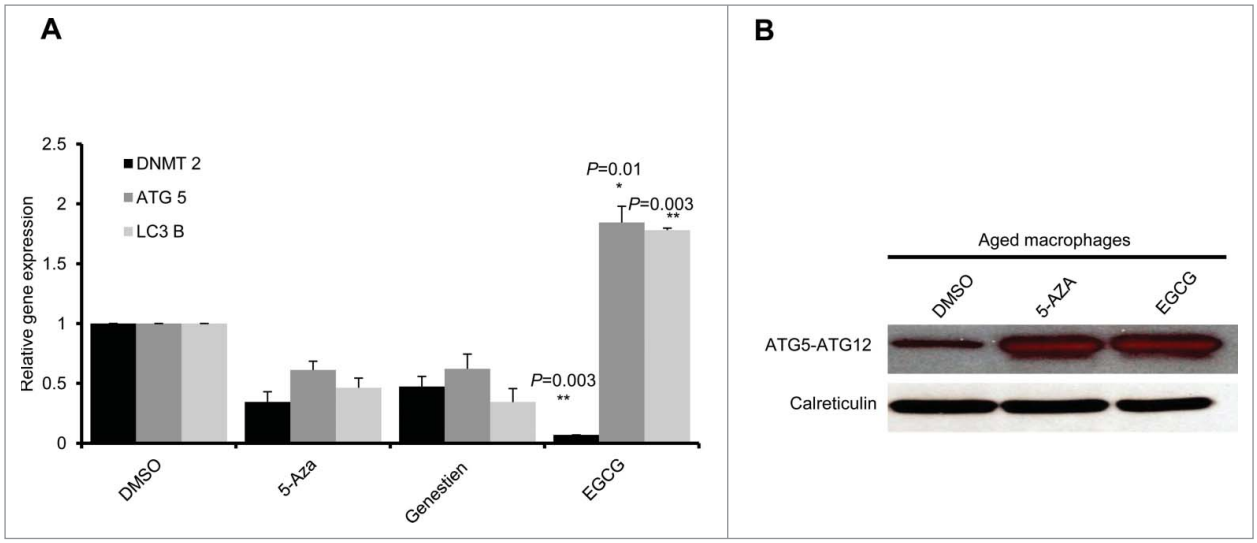

Figure 4. Restoration of ATG5 and LC3 expression in macrophages from aged mice in response to EGCG. (A) Relative gene expression of *DNMT2, Atg5* and *LC3B* in aged mice macrophages that were pretreated with the indicated methylation inhibitors compared to macrophages pretreated with DMSO. Student two-tailed test was used to determine significance. Error bars indicate the SD. (B) Immunoblotting analysis of Atg5-Atg12 protein complex in macrophages from aged mice upon treatment with the indicated methylation inhibitors. Calreticulin served as loading control.

both LC3B and Atg5 in aged mice at the RNA level (Fig. 5A). Additionally, the protein levels of the Atg5-Atg12 complex and LC3 were also increased compared to DMSO treated mice (Fig. 5B). This finding demonstrates that *in vivo* treatment with EGCG restores autophagy expression in aged mice via reduction of DNMT2 gene expression.

Discussion

Aging is associated with reduced autophagy activity of unknown etiology.[2,3,16-18] Compromised autophagy has been linked to several age related disorders, such as Alzheimer's, Parkinson's, Huntington's diseases, cancer, and susceptibility to specific infections. In the current study we investigated the mechanism of reduced autophagy activity associated with aging. We found that the expression of important autophagy genes, such as *Atg5* and *LC3*, is reduced in macrophages from aged mice. Three human LC3 isoforms exist: LC3A, LC3B, and LC3C. They undergo post-translational modifications during autophagy.[19,20] LC3B is a protein involved in ATG8-conjugation system, one of two evolutionarily conserved conjugation systems crucial for autophagosome formation. Interestingly, the findings here indicate that methylated promoters of *Atg5* and *LC3B*, but not *LC3A*, are associated with reduction of expression of essential autophagy proteins in aged mice and, subsequently, reveal an essential mechanism for defective autophagy in macrophages from senescent mice.

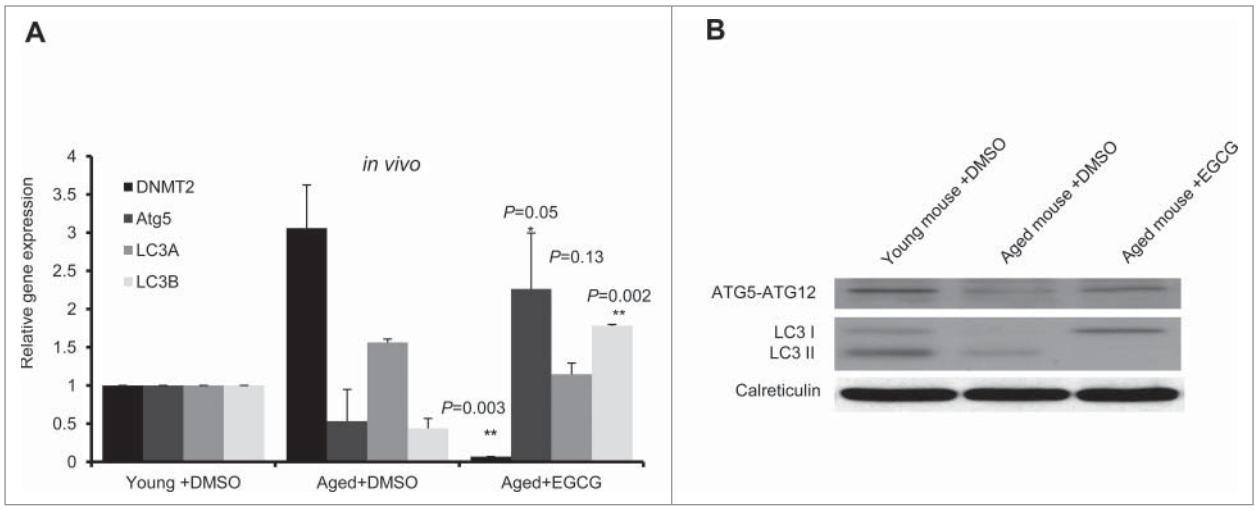

Figure 5. The expression of *Atg5* and *LC3* is restored in lungs of live aged mice in response to EGCG. (A) Relative gene expression of *DNMT2, Atg5, LC3A*, and *LC3B* in lung homogenates of aged mice treated by intratracheal installation of EGCG (300 μg/day) in comparison to young and aged mice treated with DMSO (30 μl/day). Student two-tailed test was used to determine the significance of DNMT2 and autophagy related genes expression in aged macrophages. Error bars indicate the SD. (B) Immunoblotting analysis of Atg5-Atg12 protein complex and LC3 in aged mice treated with EGCG. Calreticulin served as loading control. n = 3.

Epigenetics has become the center of many studies because it can help explain the relationship between an individual's genetic background, the environment, aging, and disease.[21] DNA methylation is mediated by DNMTs. Here, we found that DNMT2 is significantly expressed in macrophages derived from aging mice in association with hypermethylation of the promoter region of *Atg5* and *LC3B*. Several studies have implicated the biological role and molecular interaction of DNMTs in cancer and medical disorders. The expression of DNMT1 is increased in breast cancer and induces DNA methylation of various tumor suppressor genes. Beside some DNA methylation activities, DNMT2 catalyzes cytosine methylation in RNA substrates and protects tRNA from cleavage under stress conditions.[22] The expression of DNMT2 has been detected in oocytes, embryos, spermatocyes, and spermatids. Upregulation of DNMT2 was reported to activate viability and fertility of null mutants in *Drosophila, Arabidopsis*, and mice. Additionally, DNMT2-modulated cellular signaling pathways have been demonstrated in health and human disease conditions.[23,24] Importantly, recent evidence indicates the crucial and essential role of DNMT2 in modulation of genomic DNA methylation in *Drosophila* and demonstrates the ability of DNMT2 to bind to DNA with affinity similar to DNA methyltransferase.[25,26] Here, we demonstrate a novel role for DNMT2 in autophagy regulation and, subsequently, aging-associated, autophagy-related diseases and strongly suggest the contribution of DNMT2 to DNA methylation.

EGCG is a plant flavonoid with protective anti-cancer properties against lung, colon, liver, and breast cancer. Both plant flavonoids, EGCG and genistein, have been recognized as direct inhibitors of DNMTs, leading to restoration of tumor suppressor genes in carcinogenic cells.[27,28] However, it seems that EGCG is more effective at targeting DNMT2 in macrophages from aged mice when compared to genistein (Fig. 3). Others have shown that EGCG promotes autophagic degradation of endotoxin-induced HMGB1, a late inflammatory factor.[29] The autophagy-promoting effect of EGCG also occurs in bovine aortic endothelial cells, and accounts for reduction of lipid accumulation.[30] Although EGCG is also protective against liver injury, it is not known whether it regulates hepatic autophagy to mediate these effects. EGCG inhibits the activity of any DNMT; therefore, it is possible that the positive effect is due to inhibition of DNMT1 or 3. Yet, their expression is very low compared to the expression of DNMT2, which is significantly elevated in macrophages from aged mice.

In summary, although this is not the first report to demonstrate improvement of autophagy with EGCG, it is the first study to provide evidence that autophagy genes are hypermethylated in macrophages derived from aged mice and respond to EGCG treatment, demonstrating a potential new therapeutic target for age-associated conditions provoked by weak autophagy activity. Additionally, the effect of DNMT2 on the methylation of two distinctive autophagy genes promoters supports other studies suggesting that DNMT2 mediates distinctive DNA methylation motif that are different from the ones mediated by DNMT1 and DNMT3A.

Material and methods

Bone-marrow-derived macrophages

All animal experiments were performed according to protocols approved by the Animal Care Use Committee of the Ohio State University College of Medicine. Wild-type (WT) C57BL/6 aged (62-64 weeks old) and young (8 weeks old) mice were obtained from Jackson laboratories. Mice were housed in the OSU vivarium. Bone marrow-derived macrophages (BMDMs) were isolated as previously described.[13,14,31-33]

DNA isolation and epigenetic analysis

Genomic DNA from macrophages was isolated using DNA purification kit (Qiagen) according to the manufacture protocol. To investigate the methylation of autophagy genes, specific methylated primers for *Atg5* and *LC3* (Qiagen) were used to detect the methylated fold changes in aged macrophages compared with macrophages from young mice. EpiTect Methyl II DNA Restriction Kit (Qiagen) was used to digest autophagy genomic DNA based on the methylation sensitive and dependent restriction sites. Fold changes of methylated molecules was calculated according the manufacture protocol.

Methyltransferase inhibitor treatment in vitro and in vivo

Macrophages were derived from aged mice and seeded in 6 well plates at a concentration of 5 million cells per well. After 24 h, cells were treated with 20 μg of EGCG dissolved in 20 μl of DMSO and were incubated for 24 h. Other sets of macrophages were treated with the same concentration of 5-Aza or genistein. In addition, macrophages were also treated with 20 μl of DMSO as negative control. For the *in vivo* experiment, three aged mice were treated by intra-tracheal installation of 300 μg of EGCG dissolved in 30 μl of DMSO for three consecutive days. Three other aged mice and three young mice were treated with the same volume of DMSO (30 μl) and served as control mice.

RNA interference

Macrophages delivered from aged mice were transfected with siRNA sequences targeting DNMT2 (DNMT2 siRNA Set I, SignalChem, T352-911) or anti-Luciferase (human-unrelated siRNA AACUUACGCUGAGUACUUCGA), used as a control. RNAiFect transfection kit (Qiagen, 301605) was used to transfect the macrophages. Two days post-transfection, cells were lysed and total RNA and total proteins were isolated. Efficiency of gene silencing was validated using real-time PCR. The knockdown efficiency of siDNMT2 and siLuciferase, indicated by relative gene expression of the sequences targeted, were almost 90%.

RNA isolation and qRT-PCR

Total RNA from macrophages and murine lung tissue were isolated and cDNA was generated from total RNA using QuantiTech Reverse Transcriptase Kit (Qiagen) according to the manufacturer's protocol. The relative expression of

LC3, Atg5, and DNMTs 1, 2 and 3 were detected using the QuantiTect SYBR Green PCR Kit (Qiagen) and oligonucleotides specific for each individual gene (Table 1). Levels of GAPDH were amplified using specific oligonucleotides, and used for normalization. The results were analyzed using ΔΔCt equations.

Immunoprecipitation

Macrophages from aged and young mice were seeded in 6-well plates then scraped and collected using cell lysis buffer (Promega). Total proteins were prepared from macrophages and were incubated with rabbit monoclonal anti-LC3 (Santa Cruz) over night at 4°C. Protein A agarose beads (30 μl; Cell Signaling Technology, 9863) was added and followed by 3 h incubation on ice. Samples then were centrifuged and pellets were resuspended in 20 μl 3X SDS sample buffer and were prepared for Western blot analysis.

Western blot

Protein electrophoresis was carried out using the vertical Biorad Mini-Protean II electrophoresis system. Equal amounts of protein were subjected to 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The separated proteins were next transferred onto PVDF membranes (Biorad). The membranes were then incubated with rabbit monoclonal anti-LC3 (Santa Cruz) or rabbit monoclonal anti-Atg5-Atg12 complex (Santa Cruz) or rabbit polyclonal anti-DNMT2. Proteins were detected with donkey anti-rabbit secondary antibody conjugated with horseradish peroxidase and enhanced chemiluminescence system (ECL, Amersham). Calreticulin was used as a loading control. To investigate immunoprecipitated samples, the separated samples onto PVDF membrane were incubated overnight at 4°C with the rabbit primary antibodies (Santa Cruse) against LC3, Atg5, and DNMT2. Indicated proteins were detected using donkey anti-rabbit secondary antibody conjugated with horseradish peroxidase and enhanced chemiluminescence system (ECL, Amersham).

Table 1. Oligonucleotide sequences used for detection of indicated genes at RNA level.

Name	Primer sequences 5′–3′
DNMT1-For	CCCATGCATAGGTTCACTTCCTTC
DNMT1-Rev	TGGCTTCGTCGTAACTCTCTACCT
DNMT2-For	CATACAATGCCCGTGTGAGTTCTTAAGG
DNMT2-Rev	CGTGTGTCTAAATGGCTTGAGTACAGT
DNMT3-For	TGCAATGACCTCTCCATTGTCAAC
DNMT3-Rev	GGTAGAACTCAAAGAAGAGGCGG
Atg5-For	CGTGTATGAAAGAAGCTGATGC
Atg5-Rev	ACGAAATCCATTTTCTTCTGGA
LC3B-For	CGTCCTGGACAAGACCAAGT
LC3B-Rev	CCATTCACCAGGAGGAAGAA
LC3A-For	AGACCAAGTTTTTGGTCCCGG
LC3A-Rev	AAGATGAACCACACAGCAGG
GAPDH-For	TGG CAT TGT GGA AGG GCT CA
GAPDH-Rev	TGG ATG CAG GGA TGA TGT TCT

Fluorescence confocal microscopy

Macrophages from aged and young mice were seeded onto cover slips in 24-well plates at a density of 5×10^5 cells per well and incubated overnight. Then, cells were fixed using 4% paraformaldehyde for 25 min at RT, permeabilized with cold methanol for 10 sec and then incubated overnight at 4°C with a primary LC3 antibody or rabbit polyclonal DNMT2 antibody (Santa Cruz). Cells were then incubated with Alexa flour monoclonal secondary antibody (Abcam). Lastly, cells were stained with the fluorescent DNA dye DAPI at 1 μg/ml for 15 min. Images were captured using a laser scanning confocal fluorescence microscope with a 60X objective (Olympus Fluoview FV10i).

Statistical analysis

Microsoft Excel was used for statistical calculation, graphs, and histograms. SDS 2.2.2 software was used to analyze the qRT-PCT data to drive the ΔΔCt values using the following equations; (ΔCt) = Ct-sample – Ct-control. (ΔΔCt) = ΔCt – ΔCt-normalized control. Finally, the relative gene expression is equal $2^{-\Delta\Delta ct}$ of the final values.[34] ImageJ 1.48 software was used for quantitative analysis of detectable protein amount indicated by immunofluorescence microscopy. Based on detection and calculation of mean and integrated density of cells, the number of corrected density was calculated by ImageJ software dependent on selected background. Excel sheet was then used to performe the indicated diagrams for quantitative analysis of each image. Student two-tailed t-test was used to determine significance of indicated genes expression in aged mice in comparison to young mice.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

Acknowledgments

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References

1. Choi AJ, Ryter SW. Autophagy in inflammatory diseases. Int J Cell Biol 2011; 2011; 732798; PMID:22190939; http://dx.doi.org/10.1155/2011/732798
2. Martinez-Lopez N, Athonvarangkul D, Singh R. Autophagy and aging. Adv Exp Med Biol 2015; 847:73-87; PMID:25916586; http://dx.doi.org/10.1007/978-1-4939-2404-2_3
3. Stranks AJ, Hansen AL, Panse I, Mortensen M, Ferguson DJ, Puleston DJ, Shenderov K, Watson AS, Veldhoen M, Phadwal K, et al. Autophagy Controls Acquisition of Aging Features in Macrophages. J Innate Immun 2015; 7:375-91; PMID:25764971; http://dx.doi.org/10.1159/000370112
4. Abdelaziz HAD, Khalil H, Cormet-Boyaka E, Amer A. The cooperation between the autophagy machinery and the inflammasome to implement an appropriate innate immune response: Do they regulate

each other? Immunol Rev 2015; 265:194-204; PMID:25879294; http://dx.doi.org/10.1111/imr.12288
5. Ryter SW, Nakahira K, Haspel JA, Choi AM. Autophagy in pulmonary diseases. Annu Rev Physiol 2012; 74:377-401; PMID:22035347; http://dx.doi.org/10.1146/annurev-physiol-020911-153348
6. Barnett A, Brewer GJ. Autophagy in aging and Alzheimer's disease: pathologic or protective? J Alzheimers Dis 2011; 25:385-94; PMID:21422527; http://dx.doi.org/10.3233/JAD-2011-101989
7. Alirezaei M, Kemball CC, Lindsay Whitton J. Autophagy, inflammation and neurodegenerative disease. Eur J Neurosci 2011; 33:197-204; PMID:21138487; http://dx.doi.org/10.1111/j.1460-9568.2010.07500.x
8. Klionsky DJ. Crohn's disease, autophagy, and the Paneth cell. N Engl J Med 2009; 360:1785-6; PMID:19369659; http://dx.doi.org/10.1056/NEJMcibr0810347
9. Huang J, Klionsky DJ. Autophagy and human disease. Cell Cycle 2007; 6:1837-49; PMID:17671424; http://dx.doi.org/10.4161/cc.6.15.4511
10. Robertson KD, Jones PA. DNA methylation: past, present and future directions. Carcinogenesis 2000; 21:461-7; PMID:10688866; http://dx.doi.org/10.1093/carcin/21.3.461
11. Prendergast GC, Ziff EB. Methylation-sensitive sequence-specific DNA binding by the c-Myc basic region. Science 1991; 251:186-9; PMID:1987636; http://dx.doi.org/10.1126/science.1987636
12. Feinberg AP. Phenotypic plasticity and the epigenetics of human disease. Nature 2007; 447:433-40; PMID:17522677; http://dx.doi.org/10.1038/nature05919
13. Abdulrahman BA, Khweek AA, Akhter A, Caution K, Kotrange S, Abdelaziz DH, Newland C, Rosales-Reyes R, Kopp B, McCoy K, et al. Autophagy stimulation by rapamycin suppresses lung inflammation and infection by Burkholderia cenocepacia in a model of cystic fibrosis. Autophagy 2011; 7:1359-70; PMID:21997369; http://dx.doi.org/10.4161/auto.7.11.17660
14. Abdulrahman BA, Khweek AA, Akhter A, Caution K, Tazi M, Hassan H, Zhang Y, Rowland PD, Malhotra S, Aeffner F, et al. Depletion of the ubiquitin-binding adaptor molecule SQSTM1/p62 from macrophages harboring cftr DeltaF508 mutation improves the delivery of Burkholderia cenocepacia to the autophagic machinery. J Biol Chem 2013; 288:2049-58; PMID:23148214; http://dx.doi.org/10.1074/jbc.M112.411728
15. Akhter A, Caution K, Abu Khweek A, Tazi M, Abdulrahman BA, Abdelaziz DH, Voss OH, Doseff AI, Hassan H, Azad AK, et al. Caspase-11 promotes the fusion of phagosomes harboring pathogenic bacteria with lysosomes by modulating actin polymerization. Immunity 2012; 37:35-47; PMID:22658523; http://dx.doi.org/10.1016/j.immuni.2012.05.001
16. Vellai T, Takacs-Vellai K, Sass M, Klionsky DJ. The regulation of aging: does autophagy underlie longevity? Trends Cell Biol 2009; 19:487-94; PMID:19726187; http://dx.doi.org/10.1016/j.tcb.2009.07.007
17. Yen WL, Klionsky DJ. How to live long and prosper: autophagy, mitochondria, and aging. Physiology (Bethesda) 2008; 23:248-62; PMID:18927201; http://dx.doi.org/10.1152/physiol.00013.2008
18. Cuervo AM. Autophagy and aging–when "all you can eat" is yourself. Sci Aging Knowledge Environ 2003; 2003(36):pe25; PMID:12968056
19. Kirisako T, Ichimura Y, Okada H, Kabeya Y, Mizushima N, Yoshimori T, Ohsumi M, Takao T, Noda T, Ohsumi Y. The reversible modification regulates the membrane-binding state of Apg8/Aut7 essential for autophagy and the cytoplasm to vacuole targeting pathway. J Cell Biol 2000; 151:263-76; PMID:11038174; http://dx.doi.org/10.1083/jcb.151.2.263
20. Tanida I, Ueno T, Kominami E. LC3 conjugation system in mammalian autophagy. Int J Biochem Cell Biol 2004; 36:2503-18; PMID:15325588; http://dx.doi.org/10.1016/j.biocel.2004.05.009
21. Feinberg AP. Epigenetics at the epicenter of modern medicine. JAMA 2008; 299:1345-50; PMID:18349095; http://dx.doi.org/10.1001/jama.299.11.1345
22. Schaefer M, Pollex T, Hanna K, Tuorto F, Meusburger M, Helm M, Lyko F. RNA methylation by Dnmt2 protects transfer RNAs against stress-induced cleavage. Genes Dev 2010; 24:1590-5; PMID:20679393; http://dx.doi.org/10.1101/gad.586710
23. Schaefer M, Lyko F. Solving the Dnmt2 enigma. Chromosoma 2010; 119:35-40; PMID:19730874; http://dx.doi.org/10.1007/s00412-009-0240-6
24. Kiani J, Grandjean V, Liebers R, Tuorto F, Ghanbarian H, Lyko F, Cuzin F, Rassoulzadegan M. RNA-mediated epigenetic heredity requires the cytosine methyltransferase Dnmt2. PLoS Genet 2013; 9:e1003498; PMID:23717211; http://dx.doi.org/10.1371/journal.pgen.1003498
25. Kunert N, Marhold J, Stanke J, Stach D, Lyko F. A Dnmt2-like protein mediates DNA methylation in Drosophila. Development 2003; 130:5083-90; PMID:12944428; http://dx.doi.org/10.1242/dev.00716
26. Takayama S, Dhahbi J, Roberts A, Mao S, Pachter L, Martin D, Boffelli D. Genome methylation in D. melanogaster is found at specific short motifs and is independent of DNMT2 activity. Genome Res 2014; 24:821-30; PMID:24558263; http://dx.doi.org/10.1101/gr.162412.113
27. Nandakumar V, Vaid M, Katiyar SK. (-)-Epigallocatechin-3-gallate reactivates silenced tumor suppressor genes, Cip1/p21 and p16INK4a, by reducing DNA methylation and increasing histones acetylation in human skin cancer cells. Carcinogenesis 2011; 32:537-44; PMID:21209038; http://dx.doi.org/10.1093/carcin/bgq285
28. Gros C, Fahy J, Halby L, Dufau I, Erdmann A, Gregoire JM, Ausseil F, Vispe S, Arimondo PB. DNA methylation inhibitors in cancer: recent and future approaches. Biochimie 2012; 94:2280-96; PMID:22967704; http://dx.doi.org/10.1016/j.biochi.2012.07.025
29. Li W, Zhu S, Li J, Assa A, Jundoria A, Xu J, Fan S, Eissa NT, Tracey KJ, Sama AE, et al. EGCG stimulates autophagy and reduces cytoplasmic HMGB1 levels in endotoxin-stimulated macrophages. Biochem Pharmacol 2011; 81:1152-63; PMID:21371444; http://dx.doi.org/10.1016/j.bcp.2011.02.015
30. Kim HS, Montana V, Jang HJ, Parpura V, Kim JA. Epigallocatechin gallate (EGCG) stimulates autophagy in vascular endothelial cells: a potential role for reducing lipid accumulation. J Biol Chem 2013; 288:22693-705; PMID:23754277; http://dx.doi.org/10.1074/jbc.M113.477505
31. Caution K, Gavrilin MA, Tazi M, Kanneganti A, Layman D, Hoque S, Krause K, Amal AO. Caspase-11 and caspase-1 differentially modulate actin polymerization via RhoA and Slingshot proteins to promote bacterial clearance. Scientific Reports 2015; 5:18479; http://dx.doi.org/10.1038/srep18479
32. Abu Khweek A, Kanneganti A, Guttridge D, AO A. The sphingosine-1-phosphate lyase (LegS2) contributes to the restriction of Legionella pneumophila in murine macrophages. Plos One 2016; 5:18479: In press; PMID:26686473; http://dx.doi.org/10.1371/journal.pone.0146410.
33. Khweek AA, Caution K, Akhter A, Abdulrahman BA, Tazi M, Hassan H, Majumdar N, Doran A, Guirado E, Schlesinger LS, et al. A bacterial protein promotes the recognition of the Legionella pneumophila vacuole by autophagy. Eur J Immunol 2013; 43:1333-44; PMID:23420491; http://dx.doi.org/10.1002/eji.201242835
34. Schmittgen TD, Jiang J, Liu Q, Yang L. A high-throughput method to monitor the expression of microRNA precursors. Nucleic Acids Res 2004; 32:e43; PMID:14985473; http://dx.doi.org/10.1093/nar/gnh040