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研究課題名（和文）The role of B cell-derived secretory autophagosome-like vesicles in immune regulation

研究課題名（英文）The role of B cell-derived secretory autophagosome-like vesicles in immune regulation

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研究成果の概要（和文）：オートファジーは、細胞の恒常性をサポートする細胞内分解システムとして知られています。但し、外部信号がオートファゴソームのような細胞外小胞（ALV）の分泌を誘発するかどうかは不明です。
ここでは、IL-4と抗CD40抗体が、B細胞でALVの分泌を誘導することを示しています。ALVの分泌はRab27aによって調節されており、さらに、B細胞由来のALVは、試験管内でCD8+T細胞の増殖を促進しますが、CD4+T細胞の増殖は促進しません。ALVはマウスとヒトの両方の血清に見られるため、外部信号がB細胞でALV分泌を誘導し、ALVがCD8+T細胞機能を調節する可能性があるという最初の証拠となります。

研究成果の学術的意義や社会的意義
After Yoshinori Ohsumi won the 2016 Nobel Prize for his discoveries of mechanisms for autophagy, fully understanding the functions of autophagy becomes emerging. Our findings extend the present knowledge of autophagy in regulating not only cellular homeostasis but also intercellular communication.

研究成果の概要（英文）：Autophagy is known as an intracellular degradation system to support the cellular homeostasis. Since an autophagosome (Ap) marker, LC3-II, could be found in the cell-derived extracellular vesicles (EVs) in vitro, autophagy may play a role during cell-to-cell communication. However, whether external signals induce the secretion of Ap-like EVs (ALVs) remains unknown. Here, we demonstrate that IL-4 plus anti-CD40 antibodies (IL-4/CD40), but not IL-4 plus LPS (IL-4/LPS) induces the secretion of LC3-II+ ALVs in B cells. Additionally, the secretion of ALVs by IL-4/CD40 signals was regulated by a small GTPase, Rab27a. Furthermore, B cell-derived ALVs enhance the proliferation of CD8+ T cells, but not CD4+ T cells, in vitro. Importantly, LC3-II+ ALVs can be found in both mouse and human sera. Thus, we provide the first evidence that external signals induce the ALVs secretion in B cells and the ALVs may regulate the CD8+ T cell functions in vivo.

研究分野：B cell biology

キーワード：Autophagy　autophagosome　extracellular　vesicles　CD40　IL-4　B cell

様　式　Ｃ－１９、Ｆ－１９－１、Ｚ－１９（共通）

１．研究開始当初の背景

Extracellular vesicles (EVs), which are secreted by eukaryotic cells, are small membrane-enclosed vesicles with the diameter ranging from 30 to 1000 nm (Colombo et al., 2014; Raposo and Stoorvogel, 2013). Different types of EVs are defined by their intracellular origins, such as exosomes from endosomal origin and microvesicles (MVs) from shed fragment of plasma membrane. Recent studies showed that transmembrane proteins and cell-derived cytosolic components including protein and small RNAs carried by EVs can be transferred between producing cells and target cells (Colombo et al., 2014), suggesting that EVs may play an important role in the intercellular communication.

Autophagy is known as an intracellular degradation system that contributes to cellular homeostasis (Clarke and Simon, 2019). In addition to autophagic degradation, autophagy also contributes to unconventional secretion of proteins (Ponpuak et al., 2015). The inhibition of autophagosome-lysosome fusion by treatment of inhibitors, such as Bafilomycin-A1 (Baf A1), induces the secretion of exosomes carrying autophagy-related proteins including LC3, p62 and NBR1 (Solvik et al., 2022). Drug-induced apoptosis also results in the secretion of LC3/p62^{+} exosomes in endothelial cells (Pallet et al., 2013). Since those LC3-II^{+} autophagosome-like EVs (ALVs) can be found in *in vitro* and *in vivo* conditions (Solvik et al., 2022), autophagy may play a role during cell-cell communication. However, whether the cells exposed to environmental stimuli secrete such ALVs is still unclear since those findings of ALVs secretion are depended on experimental conditions.

２．研究の目的

During infection or antigen immunization, antigen-experienced B cells migrate to the border between T-cell zone and the follicle and interact with T cells, which express CD40 ligand (CD40L) and other co-stimulatory molecules, to receive the help for their full activation (De Silva and Klein, 2015). It has been reported that T cells induced the secretion of exosomes carrying major histocompatibility complex (MHC) class II in B cells through CD40L-CD40 interaction(Muntasell et al., 2007; Saunderson et al., 2008). However, whether external signals induce the ALVs secretion in B cells is still unknown.

Autophagy is also important for the homeostasis of B cells (Clarke and Simon, 2019). Th2 cytokine, such as IL-4, has been reported to activate autophagy in B cells(Xia et al., 2018). Since B cells are thought to be a major *in vivo* source of EVs(McLellan, 2009), B cells may also secrete ALVs during autophagy induction by external stimulation. In agreement with this, our preliminary results showed that Epstein-Barr virus (EBV)-encoded latent membrane protein 1 (LMP1, mimics mimic CD40 signals partially)-expressing B cells increased the secretion of LC3-II^{+} exosomes in the presence of IL-4 (data not shown). Thus, this study is aimed to investigate whether IL-4/CD40 signals induce the ALVs secretion in B cells and whether ALVs play a role during intercellular communication.

３．研究の方法

1. To investigate whether external stimuli induce the ALVs secretion in B cells, the mouse splenic B cells are isolated and stimulated with cytokines combined with receptor activators, such as LPS and anti-CD40 antibodies (Abs). The fractions of EVs including MVs and exosomes are collected by ultracentrifugation. The exosome and autophagic markers in EVs are examined by western blotting. A mouse A20 B cell line is also used for this study.

2. The morphology of ALVs carrying GFP-LC3 protein is analyzed by transmission electron microscopy (TEM).

3. To study how ALVs are secreted, the GFP-LC3-expressing B cells isolated from GFP-LC3 transgenic mice (provided by Dr. Noboru Mizushima, the University of Tokyo, Japan) are used for monitoring the autophagosome-lysosome fusion by live-cell high-resolution confocal microscopy.

4. The gene expression levels, which are involved in either the control of autophagosome-lysosome fusion or the vesicle

transportation, in activated B cells are determined by quantitative reverse transcription PCR (RT-qPCR).

5. CRISPR/Cas9 technology is used to investigate what kinds of genes mediate the ALVs secretion in A20 cells.

6. To investigate whether LC3-II$^+$ ALVs can be found in vivo, B6 wild-type (WT) mice sera and human plasma form healthy donor are used. To examine whether *in vivo* B cell activation enhances the ALVs secretion, mice sera are collected from WT mice 7 days after NP-CGG-Alum immunization.

7. To investigate the physiological effects of B cell-derived ALVs on T cells, ALVs are co-cultured with purified naïve CD4$^+$ or CD8$^+$ T cells stimulated with anti-CD3/anti-CD28 Abs-conjugated Dynabeads. The cell proliferation is analyzed by flow cytometry.

8. The protein composition of EVs is analyzed by Mass spectrometry and the RNA composition of exosomes including message RNA (mRNA) and microRNA (miRNA) is analyzed by small RNA-sequencing (RNA-seq).

４．研究成果

Th2 signals induce the secretion of ALVs in B cells

We first demonstrated that IL-4 plus anti-CD40 Abs (IL-4/CD40) specifically induced the secretion of p62/LC3-II$^+$ MVs and exosomes in B cells compared to IL-4 plus LPS (IL-4/LPS) or IL-4 plus anti-IgM Abs (Figure 1A). Additionally, IL-4 induced the highest secretion of ALVs among other Th1 and Th2 cytokines (Figure 1B). Consistently, the secretion of ALVs was also higher in IL-4/CD40- than IL-4/LPS-stimulated A20 B cells (Figure 1C). We also observed the ALVs carrying GFP-LC3 proteins labeled with anti-GFP Abs-nanogold by transmission electron microscopy (TEM) (Figure 1D). Thus, our results suggest that Th2-dependent external stimulation is essential for the induction of LC3-II$^+$ ALVs secretion in B cells.

Down-regulation of autophagosome-lysosome fusion activity increases ALVs secretion

To investigate how IL-4/CD40 signals induced LC3-II$^+$ ALVs secretion in B cells, GFP-LC3-expressing B cells were stimulated with IL-4/LPS or IL-4/CD40 supplemented with lysotracker and the fusion of autophagosome (GFP-LC3$^+$ dot) and lysosome (lysotracker$^+$ dot) was observed by high resolution live-cell confocal microscopy. Live imaging analysis showed that the autophagosome-lysosome fusion was down-regulated in B cells stimulated with IL-4/CD40 compared to IL-4/LPS (Figure 2A and movie not shown). TEM analysis also confirmed that low numbers of autolysosomes in IL-4/CD40-stimulated B cells (Figure 2B), suggesting that IL-4/LPS signals increased the autophagic flux and IL-4/CD40 signals reduced the autophagosome-lysosome fusion might contribute to the secretion of LC3-II$^+$ ALVs. As expected, suppression of autophagosome-lysosome fusion by Baf A1 enhanced the LC3-II$^+$ ALVs secretion in IL-4/LPS-stimulated B cells, but not in IL-4/CD40-stimulated B cells (Figure 2C and 2D). While autophagosomes were co-localized with lysotracker$^+$ lysosomes in IL-4/LPS-stimulated B cells, autophagosomes were partially co-localized with EEA1$^+$ early exosomes in IL-4/CD40-stimulated B cells (Figure 2E). Additionally, IL-4/CD40 signals induced higher EEA1 but lower LAMP2 (lysosome marker) expression compared to IL-4/LPS signals (Figure 2F). Together these results suggest that IL-4/CD40 signals decrease the autophagosome-lysosome fusion and promote the LC3-II$^+$ALVs secretion via an endosomal secretion pathway (Figure 2G).

Rab27a mediates the ALVs secretion

Considering the fact that the autophagosome-lysosome fusion was down-regulated in the IL-4/CD40-treated B cells, we next examined the gene expression involved in the autophagosome-lysosome fusion or vesicle transportation by RT-qPCR (Figure 3A). While IL-4/LPS signals induced the gene expression involved in the autophagosome-lysosome fusion, IL-4/CD40 signals preferentially enhanced the gene expression involved in the vesicle transportation including *Rab27a,*

Rab8b, Rab21 and *Rab35.* Western blotting also confirmed that Rab27a expression was higher in IL-4/CD40 than IL-4/LPS-stimulated B cells (Figure 3B). Additionally, Rab27a was not only localized in lysosomes as previous reported(Kariya et al., 2011), but also partially localized in autophagosomes (data not shown), suggesting that Rab27a may be a candidate for mediating the ALVs secretion (Solvik et al., 2022). To elucidate the function of Rab27a, we generated a Rab27a mutant A20 cell line by using CRISPR-Cas9 gene editing system (Figure 3C). Although loss of CXC motif of Rab27a decreased its expression in A20 cells (Figure 3D, WCE panel), overexpression of Rab27a mutant ($Rab27a^{m}$ #1-9) resulted in the evenly distribution inside the whole Hela cells (data not shown), confirming that CXC motif is required for the Rab27a translocation on the intracellular vesicle-membrane (Gomes et al., 2003). As expected, the GFP-LC3-II and endogenous LC3-II levels were decreased in both MVs and exosomes from Rab27a mutant A20 cell lines (Figure 3D and 3E). However, Rab27a lacking CXC motif was dispensable for the secretion of $Alix^{+}CD86^{+}$ EVs (Figure 3D). Taken together, IL-4/CD40 induced Rab27a expression mediates the LC3-II^{+} ALVs secretion.

ALVs found in mice and human sera may play an important role in T cell regulation

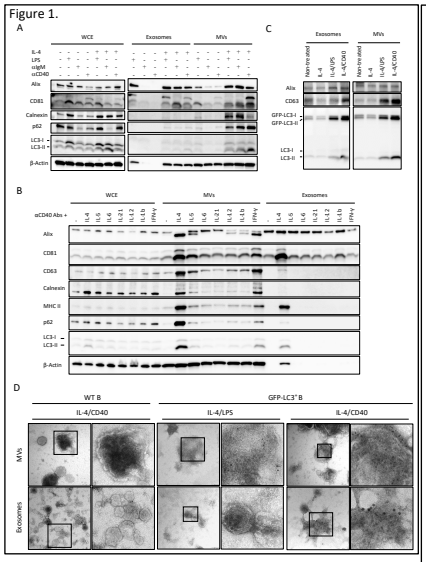
Whether autophagosomes were secreted directly or fused with endosomes is not clear. Two types of LC3-II^{+}ALVs were proposed (Figure 4A). Since EVs were secreted from activated B cells, surface IgG could be used as an isolation marker. If LC3-II^{+} autophagosomes are fused with IgG^{+}EVs, the LC3-II^{+} ALVs can be immunoprecipitated by anti-mouse IgG-conjugated beads (αMo IgG-beads) (Figure 4A). Interestingly, LC3-II was detected in both MVs and exosomes captured by αMo IgG-beads but not control αRabbit IgG-beads (Figure 4B), suggesting that LC3-II^{+} ALVs were fused-type EVs. To further investigate whether ALVs were secreted from activated B cells *in vivo*, GFP-LC3 transgenic mice were immunized with NP-CGG-Alum and sera EVs were purified from these animals. We observed increased sera LC3-II^{+} EVs secretion in immunized mice compared with unimmunized mice. Importantly, a proportion of LC3-II^{+} EVs from immunized mice sera were precipitated by αMo IgG-beads (Figure 4C), indicating that sera LC3-II^{+} EVs was partially secreted from activated B cells during immunization. Additionally, LC3-II^{+} EVs were also detected in the healthy human blood plasma (Figure 4D). Finally, we investigated the physiological effects of activated B cells-derived MVs by coculturing them with isolated naïve T cells stimulated with anti-CD3/CD28 antibodies-conjugated beads (Figure 4E). We found that cell proliferation of $CD8^{+}$, but not $CD4^{+}$, T cells was enhanced in the coculture of MVs derived from IL-4/CD40- compared to IL-4/LPS-stimulated B cells (Figure 4E). Thus, LC3-II^{+} ALVs may play an important role in T cell regulation *in vivo*.

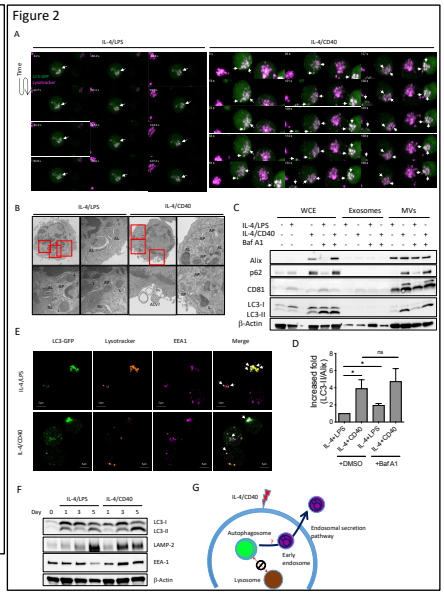
Small RNA sequencing and mass spectrometry analysis of ALVs

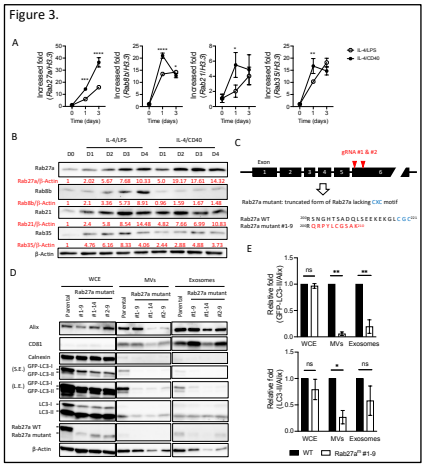
To further explore how ALVs enhanced the proliferation of $CD8^{+}$ T cells, we first analyzed the small RNA composition in the exosomes derived from IL-4/CD40- (IL-4/CD40-Exos) and IL-4/LPS-stimulated B cells (IL-4/LPS-Exos) by small RNA sequencing. Among them, miRNA was 50.5% in the IL-4/CD40-Exos and 39.5% in the IL-4/LPS-Exos (Figure 5A). Since miRNAs are short and only 22–24 nucleotides long, MVs may share the same composition of miRNA with exosomes. Thus, up-regulation of mir128 expression may contribute to the enhanced $CD8^{+}$ T cell proliferation (Figure 5B) (Zhang and Zhang, 2020). Additionally, mass spectrometry (MS) also showed the differential protein expressions between the MVs derived from IL-4/CD40- (IL-4/CD40-MVs) and IL-4/LPS-stimulated B cells (IL-4/LPS-MVs) (Figure 5C). Among them, upregulation of MHC class I H2-K^{b} chain in IL-4/CD40-MVs may induce the nonspecific enhancement of $CD8^{+}$ T cell proliferation (Wooldridge et al., 2010). However, the molecular mechanism of how ALVs enhance $CD8^{+}$ T cell proliferation needs further investigation.

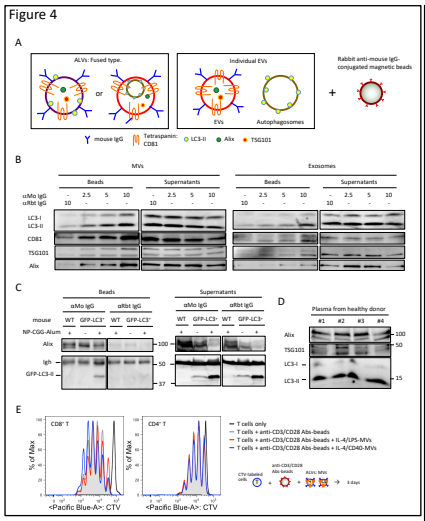
Conclusion

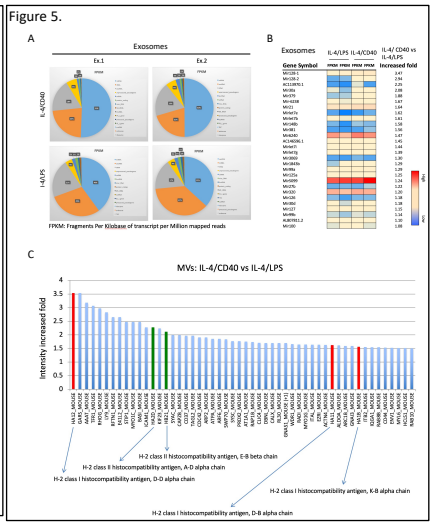
In this study, we demonstrate the first evidence that external stimulation can induce the ALVs secretion in B cells *in vitro* and *in vivo*. Additionally, a small GTPase, Rab27a, induced by IL-4/CD40 signals mediates the ALVs secretion. Since it has been reported that follicular $CD8^+$ T cells were expanded during HIV infection (Leong et al., 2016) and ALVs can be found in human plasma, activated B cells-secreted ALVs may regulate the function of $CD8^+$ T cells during chronic HIV infection.











５．主な発表論文等

〔雑誌論文〕　計0件

〔学会発表〕　計1件（うち招待講演　0件／うち国際学会　0件）

１．発表者名 Kuan Yu-Diao
２．発表標題 The molecular mechanism and physiological role of the secretion of autophagosome-like vesicles in B cells
３．学会等名 第50回日本免疫学会学術集会
４．発表年 2021年

〔図書〕　計0件

〔産業財産権〕

〔その他〕

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６．研究組織

	氏名 （ローマ字氏名） （研究者番号）	所属研究機関・部局・職 （機関番号）	備考

７．科研費を使用して開催した国際研究集会

〔国際研究集会〕　計0件

８．本研究に関連して実施した国際共同研究の実施状況

共同研究相手国	相手方研究機関