京都大学	博　士（薬　科　学）	氏名	竹中　美佐子
論文題目	Development of macrophage-targeted therapy using peptide/protein-loaded extracellular vesicles（ペプチド及びタンパク質搭載細胞外小胞を利用したマクロファージを標的とする疾患治療法の開発）		

Macrophages are cells critically involved in the onset and progression of various diseases such as cancer and autoimmune diseases, therefore disease-related macrophage is an important therapeutic target. Extracellular vesicles (EVs) are membrane vesicles naturally released by almost all cell types. Among three groups of EVs, small extracellular vesicles (sEVs), 50-200 nm vesicles, and large extracellular vesicles (lEVs), 200-1000 nm vesicles, are both known to mediate communication between cells by carrying biological cargos. Owing to their characteristics as natural delivery vehicles in the body, they are expected to be a novel platform for drug delivery systems (DDS). As systemically administered sEVs are mostly taken up by macrophages through the recognition of negative charges of sEV membrane, I considered that they can be a promising drug delivery carrier to macrophages. Although the cellular uptake of lEVs had not been studied, considering their negative membrane charges and size, it was expected that lEVs would also be efficiently taken up by macrophages.

Therefore, in this thesis, I attempted to develop of safe and effective macrophage-targeted therapy using EVs including both sEVs and lEVs. In chapter 1 and chapter 2, I developed sEVs loaded with cytokine or peptides, and investigated their anti-inflammatory effect by targeted delivery of cargoes to inflammatory macrophages. In chapter 3, I first established an efficient peptide/protein loading method into lEVs. Thereafter, I constructed peptide-carrying lEVs, and verified their modulatory effect on gene and protein expression in tumor associated macrophages (TAMs).

Chapter 1. Development of synovial macrophage-targeted, anti-arthritis therapy based on IL-4 delivery by sEVs

In inflammatory diseases including rheumatoid arthritis, it has been reported that M1-polarized macrophages, inflammatory macrophages, are dominant whereas M2-polarized macrophages, anti-inflammatory macrophages, are decreased. Interleukin-4 (IL-4), a Th2-type cytokine that can drive M2 macrophage polarization, has been expected to be used as an anti-inflammatory therapy agent. However, several problems, such as unsatisfactory efficacy and side effects, have hampered the clinical application. I attempted to develop a safe and effective anti-arthritis therapy by delivering IL-4 to synovial macrophages, main target cells in IL-4 therapy, using sEVs. As a result, compared to IL-4 protein, IL-4 loaded sEVs significantly inhibited the progression of arthritis symptom and inflammation in the synovium in rheumatoid arthritis mice by inducing M2 macrophages.

Chapter 2. Intracellular delivery of anti-inflammatory peptides to macrophages using sEVs

Peptide drugs are one of the drug modalities that have gained attention due to their high affinity and specificity to the target molecules, and low production cost. However, when peptides target the intracellular molecules, their membrane impermeability and poor stability often require intracellular delivery systems. Previously, my laboratory reported that cargos which were internally-loaded to sEVs were delivered into the cytosol more efficiently than those externally loaded on sEVs. Therefore, for developing a peptide

based anti-inflammatory therapy, I constructed sEVs internally loaded with NF-κB inhibitor peptide (NBD peptide), and evaluated their anti-inflammatory effect on M1 macrophages through the efficient delivery of NBD peptides into the macrophage cytosol. Moreover, I also evaluated the extent of anti-inflammatory effect of different amounts of cargo loaded into sEVs, and optimized the quantity of NBD peptides. NBD-loaded sEVs showed inhibitory effect on expression of proinflammatory mediators in M1 macrophages, by delivering NBD peptides into their cytosol. Also, it was found that there was positive correlation between the loading number of peptides in sEVs and the magnitude of anti-inflammatory effect.

Chapter 3. Development of lEV-based, tumor associated macrophage (TAM)-targeted anti-cancer therapy

lEVs had not been widely studied as DDS carrier, however given their physicochemical properties, I assumed that lEVs also can be used for drug delivery to macrophages. Based on the fact found in chapter 2 that the quantity of cargoes in vehicles are important in the establishment of EV-based therapeutics, I initially developed a new efficient method of loading peptide/protein into lEVs. The biotinylation reaction in archaeon *Sulfolobus tokodaii* is known as unique in that the enzyme, biotin protein ligase, forms a tight complex with the product, biotinylated biotin carboxyl carrier protein. I utilized this stable protein complex for the establishment of a peptide/protein loading method, and it was found that this method can approximately 5-times more efficiently load cargoes into lEVs than the conventional method. In the tumor microenvironment, immunosuppressive M2-TAMs create a favorable environment for tumor progression. The immunosuppression by M2-TAMs is considered to be a factor of insufficient efficacy of immune checkpoint inhibitors. Thus, by using the cargo loading method developed in this study, I constructed lEVs efficiently carrying inhibitor peptides of protein kinase A (PKA) or signal transducer and activator of transcription 3 (STAT3), both which contribute to M2 polarization of TAMs, and investigated anti-cancer effect of peptide-loaded lEVs with PD-1 antibody, an immune checkpoint inhibitor. Intratumorally administered lEVs loaded with PKA inhibitors (PKI-MVs) suppressed M2 polarization and facilitate M1 polarization of TAMs. Furthermore, combination therapy of PKI-MVs and PD-1 inhibitor showed greater anti-tumor activity in breast cancer mice than anti-PD-1 monotherapy.

In conclusion, I developed two different, M1 macrophage-targeted anti-inflammatory therapeutics using cytokine or peptide loaded sEVs. Next, aiming for the development of lEV-based therapeutics, I first established novel method of peptide/protein loading into lEVs. Moreover, I successfully developed anti-cancer therapy via peptide delivery into TAMs by lEVs. The findings in this thesis provide critical information that would contribute to the development of EV-based therapies targeting macrophages.

（論文審査の結果の要旨）

In this thesis, the applicant attempted to develop of safe and effective macrophage-targeted therapy using extracellular vesicles (EVs) including both small extracellular vesicles (sEVs), 50-200 nm vesicles, and large extracellular vesicles (lEVs), 200-1000 nm vesicles. In chapter 1 and chapter 2, the applicant developed sEVs loaded with cytokine or peptides, and investigated their anti-inflammatory effect by targeted delivery of cargoes to inflammatory macrophages. In chapter 3, the applicant first established an efficient peptide/protein loading method into lEVs. Thereafter, the applicant constructed peptide-carrying lEVs, and verified their modulatory effect on gene and protein expression in tumor associated macrophages (TAMs).

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In conclusion, the findings in this thesis provide critical information that would contribute to the development of EV-based therapies targeting macrophages.

以上、本論文は博士（薬科学）の学位論文として価値あるものと認める。また、令和５年２月13日、論文内容とそれに関連した事項について試問を行った結果、合格と認めた。なお、本論文は、京都大学学位規程第１４条第２項に該当するものと判断し、公表に際しては、（令和８年３月２３日までの間）当該論文の全文に代えてその内容を要約したものとすることを認める。

要旨公表可能日：　　年　　月　　日以降