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研究課題名（和文）Applying the anti-inflammatory phenotypes of macrophages to reverse salivary gland inflammation in mice with abnormal phosphatase SHP2 expression

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研究成果の概要（和文）：シェーグレン症候群（SS）は唾液腺に影響を及ぼし、腺の萎縮と口渇を引き起こします。 SSの治療法がないため、現在多くの細胞治療法が開発されていますが、細胞源の制限や侵襲的処置などの制限があります。 我々の結果は、これらの細胞（m2マクロファージ）が炎症性遺伝子発現を減少させることを示し、唾液腺炎症に効果的であることを示唆しています。 これらの細胞の取得は他の治療法に比べて簡単であるため、臨床研究に応用できる可能性があります。

研究成果の学術的意義や社会的意義
これらの結果は、m2マクロファージが唾液腺の炎症を抑制するのに効果的であり、唾液腺組織の破壊を減らし、萎縮と口渇を防ぐことができることを示唆しています。 口渇は、嚥下困難、虫歯、口腔粘膜の炎症など、多くの口腔合併症を引き起こします。したがって、この治療法は、シェーグレン症候群の患者の生活の質を大幅に改善することができます。 将来的には、この治療法は唾液腺の変化以外の他の炎症性疾患にも適用される可能性があります。

研究成果の概要（英文）：Sjogren’s syndrome (SS) affects the salivary glands leading to dry mouth and a reduced quality of life in many patients, therefore, many novel cell-based therapies to prevent or repair salivary gland function loss are being studied. The salivary glands of SS patients present chronic inflammation due to over production of inflammatory molecules. Many animal models are used to perform research on SS, including the MRL/lpr mouse and the non-obese diabetic (NOD) mouse. We have developed a therapy that consists of using anti-inflammatory cells called m2 macrophage-like cells. We aimed to study if M2 macrophage-like cell transplantation could reduce the inflammation in salivary glands in Sjogren’s syndrome mouse models. Our results showed that m2 macrophage-like cell transplantation in these mice reduced inflammatory gene expression. This suggests that m2 macrophage-like cells are an effective therapy for alterations in salivary glands caused by immune disorders such as Sjogren's syndrome.

研究分野：組織工学

キーワード：Sjogren's syndrome　Salivary glands　Chronic inflammation

１．研究開始当初の背景
(Background at the beginning of research)

At present, there is no cure for Sjogren's syndrome (SS), therefore, novel cell-based therapies to prevent or repair salivary gland function loss are being studied. Salivary gland or mesenchymal stem cell transplantation have shown promise in mouse models of irradiation or SS, (Nanduri LS et al., Radiother Oncol, 2013; Lombaert IM et al., PLoS One, 2008), however, these experimental therapies for salivary gland atrophy have significant limitations because harvesting these cells is difficult and invasive, complicating its clinical application. Our recently published study, (IT, Raudales JLM et al., Stem Cell Res Ther. 2019) shows a practical method of cell therapy for atrophic salivary glands. It consists of culturing the readily available peripheral blood mononuclear cells (PBMCs) for only 5-7 days in serum-free medium with 5 recombinant proteins: stem cell factor, thrombopoietin, vascular endothelial factor, IL-6 and flt-3 ligand. This culture results in the proliferation of CD11b/CD206+ cells (M2 macrophage-like cells) which have anti-inflammatory and tissue remodeling properties. Our study shows that M2 macrophage-like cell transplantation in radiogenic-injured salivary glands of mice restores saliva secretion. This therapy compared to others, is minimally invasive, safe, and proven to be effective for salivary gland regeneration.

Indeed, M2 macrophage-like cells recovered salivary gland function, yet, its mechanism needs elucidation. Aberrant production of T cell-derived cytokines promote salivary glands deterioration in SS. T cell activation is regulated by signaling cascades, for example, protein tyrosine phosphatases (PTPs) which act like on/off switches of signals involved in T cell activation (Castro-Sanchez et al., Front. Immunol. 2019). Recently, Wang J et al. (J Clin Invest. 2016) showed that the SH2 domain-containing PTP (SHP2) is overexpressed in MRL/lpr mice (a model for systemic lupus erythematosus (SLE) and SS. They showed that inhibition of SHP2 reduced symptoms of SLE, infiltration of T cells/macrophages in spleen and expression of cytokines related to SS such as, interferon-gamma (INF-γ) and interleukin-17 A/F (IL-17A/F). These mice are also known for developing salivary gland inflammation, with inflammatory cell infiltration as a hallmark of disease.

Although currently there is not much evidence between SHP2 and SS, SHP2 is related to autoimmune diseases, therefore this relationship could be explored and possibly in the future be treated by m2 macrophage-like cell therapy. M2 macrophage's anti-inflammatory potential might reduce SHP2-derived inflammation. Recently, Xioa P et al. (J Exp Med. 2019) showed a role for SHP2 during colon inflammation in mice and humans. SHP2 could desensitize IL-10-STAT3 signaling in macrophages, weakening IL-10's anti-inflammatory effect. Also, tumor necrosis alpha (TNF-α) upregulated SHP2 expression. This data underlines the role of SHP2 in inflammation by desensitization of IL-10 signaling and alteration of T cell activity.

２．研究の目的
(Purpose of research)

We have recently shown that M2 macrophage-like cells have a positive effect in the recovery of salivary secretion in irradiated C57BL/6 mice. These cells had a significant higher expression of the anti-inflammatory cytokine IL-10. Therefore, these cells may also assist inflammatory regulation, since much of the damage in SGs from SS comes from inflammatory cytokines.

Recently, there have been important studies in relation to SHP2 and SLE manifestations and inflammation (Wang J et al., J Clin Invest. 2016; Xioa P et al. (J Exp Med. 2019) Attention has come into inhibiting aberrant expression of PTPs. Therefore, if the anti-inflammatory and regenerative potential of E-MNCs, could be applied, soon we may find a way to not only regenerate damages in SGs but also to prevent its initiation.

Therefore, we aim to study if M2 macrophage-like cell transplantation can reduce inflammation in salivary glands of SS mouse models such as MRL/lpr mice with overexpressed SHP2. Perhaps the anti-inflammatory activity of M2 macrophage-like cells

can inhibit TNF-α, via IL-10 secretion and normalize SHP2 expression or indirectly suppress SHP2 by enriching salivary glands with M2 macrophage-like cell that stably express SHP2. This study will be the first to analyze the relationship between SHP2, SS and salivary gland inflammation and test if cell therapy affects SHP2 expression, opening new directions for the use of E-MNCs.

３．研究の方法
(Research methods)

(1) Preliminary stage:

Determination of the best timepoint for E-MNC transplantation in SGs of MRL/lpr mice: Firstly, the peak expression of candidate genes in salivary glands of in SS mice will be determined. Starting with TNF-α, IL-1β, INF-γ (detected in salivary glands of 8-week-old MRL/lpr mice (von Blokland et al., Clin Immunol. 2002)) and SHP2. The mice will be sacrificed at 6, 12, 18 and 24 weeks of age. After harvesting the salivary glands of each mouse, gene expression with RT-QPCR will be analyzed. Also, the number of inflammatory cell infiltrates and percentages of T cells, B cells and macrophage in the infiltrates will be counted (same timepoints). For this, H&E staining and immunohistochemistry (IHC) for antibodies of these cell types will be used.

Donor mouse culture screening: Background matched C3H/He (SLC/Japan) mice will be used as donors for transplantation and $5x10^6$ PBMCs/well (6-well plate) will be used for M2-macrophage-like cell culture. M2-macrophage-like cells are going to be prepared by culturing donor mouse PBMCs for 7 days in serum-free medium with 5 recombinant proteins: SCF, TPO, VEGF, IL-6 and FLT-3L. To confirm if M2 macrophage-like cells proliferated, flow cytometry will be used to detect $CD11b^+$ and $CD206^+$ cells (M2 macrophage markers). As an alternative donor, the congenic strain of MRL/lpr mice, MRL/Mp (SLC, Japan) could be used.

(2) Transplantation stage:

M2 macrophage-like cells will be transplanted at $1x10^5$ cells/salivary gland in each SS model mouse (treatment group), 1 week prior to peak inflammatory gene expression. For controls we will leave salivary glands untread or transplant them with PBMCs. To analyze the involvement of m2 macrophages in tissue recovery, the $CD11b^+$ portion of cells will be depleted from the total cultured cells with an automated cell separator and transplant the remaining portion of cells into the salivary glands of SS mice (m2 macrophage group). For most analyses, salivary glands will be harvested after 2 weeks of transplantation.

(3) Post-transplantation analyses:

Tracking of transplanted cells: To track m2 macrophage-like cells in salivary glands we will stain the cells with PKH staining (membrane staining) 1 week after transplantation and can be detected with in sections of fixed tissues.

Inflammatory gene expression analysis: Analyze if there is a reduction in the expression of the genes established in the preliminary stage using the methods stated above.

Histological analysis: Asses the number of inflammatory infiltrates and percentages of T, B cells and macrophages by H&E and IHC as described in the preliminary stage. Finally, the data will be analyzed and prepared for dissemination.

４．研究成果
(Research results)

As we aim to study if m2 macrophage-like cell transplantation can reduce inflammation in salivary glands of Sjogren's syndrome mouse models, such as the MRL/lpr mice. Firstly, we have examined the potential of the anti-inflammatory effect of m2-macrophage-like cell transplantation in a well-established model of Sjogren's disease, the non-obese diabetic (NOD) mouse. Our rationale behind this decision is due to our previous experience with this model and we could use it as a base for the study of the MRL/lpr later on.

During the preliminary stage, the peak of inflammatory gene expression (TNF-α, IL-1β, and INF-γ) and signaling molecules involved in lymphocyte infiltration in salivary glands of in SS mice was determined to be around 8 weeks. Through H&E staining of salivary gland tissue, lymphocyte infiltrates were detected from this stage. IHC staining showed that these infiltrates were composed in part by $CD4^+$ T cells.

Since the NOD mouse model was selected at this point, the donor mouse to be used was adjusted and the CB6F1 mouse strain was selected. Then we tested if our method of PBMC culture from CB6F1 mice led to the proliferation of m2 macrophage-like cells. We could confirm that after 7 days of culture the morphology of the cells after culture, they presented a round shape resembling macrophages, with a larger size compared to the initial PBMC culture. Then, to confirm that these cells were indeed m2 macrophage-like cells, we used flow cytometry analysis. The results confirmed the proliferation of m2 macrophage-like cells after 7 days of culture.

In an effort to validate if the transplantation of these cells in the salivary glands was feasible, we first confirmed the presence of the transplanted cells in the salivary gland tissues through histochemical analyses. After harvesting the m2 macrophage-like cells we labeled them with PKH-26 just before transplantation. PKH-26 labels the cell membrane and is detected as a red signal using fluorescence microscopy. We could detect the transplanted cells as grouped or scattered formations in the tissue confirming the effectiveness of the transplantation in the tissues even after 1 week post transplantation. Furthermore, to confirm if the transplanted cells maintained their phenotypical characteristics after transplantation and were correlated to the phenotype shown by flow cytometric analysis, the donor cells in the salivary gland tissue were stained with m2-macrophage markers. Our results showed positive staining of donor cells with m2 macrophage markers confirming that these cells preserve their phenotypes even after 1 week of transplantation. This confirms that M2 macrophages maintained their phenotype in the tissue and did not change to an inflammatory m1 type.

To assess the anti-inflammatory effect of m2 macrophage-like cells, these cells were transplanted in the salivary glands of the SS model mouse at 8 weeks of age. To prevent damage to the salivary gland tissue after transplantation at $1x10^5$ cells were transplanted using the minimum volume possible (5 ml). After harvesting the salivary glands of each mouse after 1 week of transplantation, RT-QPCR was used to analyze gene expression of inflammatory cytokines. Our data showed that within 1 week of transplantation in salivary glands of 8-week-old NOD mice, expression of inflammatory genes such as TNF-α, IL-1β, INF-γ were lower compared to untreated controls.

Having favorable results using the NOD mouse, we continued our investigations using this model to see if m2 macrophage-like cell transplantation could reduce the expression of inflammatory genes in salivary glands in Sjogren's syndrome. As described above, M2 macrophage-like cell transplantation in salivary glands reduced inflammatory gene expression in these mice.

Since m2 macrophage-like cell transplantation led to a decrease of inflammatory gene expression we proceeded to investigate if transplantation of m2 macrophage-like cells could also affect signaling molecules involved in lymphocyte infiltration, particularly T lymphocytes. For this we transplanted m2 macrophage-like cells in the salivary glands of NOD at around 9 weeks of age, since at this time point it showed high expression. Then, after 1 and 3 days we measured the gene expression of theses signaling molecules. Especially after 3 days their expression was significantly reduced. In addition, histological analysis with H&E staining of diseased submandibular glands showed a decrease in the size and number of lymphocyte infiltration.

While initially we aimed at evaluating if the anti-inflammatory phenotypes of macrophages could reduce inflammatory gene expression in the MRL/Lpr mouse model, which has over expression of SHP2, we found that the NOD mouse was also a suitable and well-established model of SS and our laboratory had experience in its delicate handling, since it also presents inflammation of the salivary glands, therefore our strategy shifted towards this model. One limitation of using this model is that we couldn't analyze the effect of m2 macrophage-like cells on the SHP2 expression since it is not documented in the literature.

However, the positive effects from the m2 macrophage-like cell transplantation may provide indirect evidence that the SHP2 pathway was positively affected. INF-γ expression is regarded as a signature signal in SLE and SS since SS patients present elevated plasma levels of INF-γ, in addition several studies using SS mouse models have documented chronic expression INF-γ in salivary glands. Moreover, research has shown that inhibition of SHP2 reduced INF-γ cytokine production. Nonetheless, we will

have to confirm this in further studies using the conditions we established in this study.

In summary, these results suggest that our m2 macrophage-like culture method is easily reproducible across different mouse strains and has a great flexibility in its use making it a very feasible therapy to test in *in vivo* research and subsequently clinical research. M2 macrophage-like therapy also showed it is effective for inhibiting salivary gland inflammation by inhibiting the expression of TNF-α, IL-1β, and INF-γ. The inhibition of INF-γ, in particular, demonstrates that m2 macrophage-like cell therapy can possibly normalize aberrant T cell activity in these glands. This therapy could be applicable to several inflammatory conditions aside from alterations in salivary glands caused by immune disorders such as Sjogren's syndrome.

５．主な発表論文等

〔雑誌論文〕　計0件

〔学会発表〕　計0件

〔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