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研究課題名（和文）The role of LILRB4 on MDSC-mediated immunosuppression in tumor environment

研究課題名（英文）The role of LILRB4 on MDSC-mediated immunosuppression in tumor environment

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研究成果の概要（和文）：骨髄由来抑制細胞(MDSC)は、腫瘍関連の免疫抑制に関与しており、腫瘍の転移を促進する。 この研究では、抑制性免疫受容体LILRB4（マウス相同分子gp49B、以下B4）がMDSCの極性化を調整し、腫瘍促進することを発見した。 B4欠損MDSCは、Treg細胞の活性化、癌細胞の遊走の促進、腫瘍血管新生の刺激などの腫瘍促進性免疫応答を阻害した。 野生型担癌マウスをB4欠損M-MDSCで治療すると、癌の転移が減少した。 また、B4をノックアウトすると、血漿エクソソーム由来の抗腫瘍miRNA発現量が増加した。 以上の結果は、B4がMDSCを調節し、抗腫瘍miRNAの分泌を抑制することを示した。

研究成果の学術的意義や社会的意義
Immune checkpoint inhibitors (ICIs) are used to enhance the antitumor immune response. Several patients do not respond to ICIs due to MDSC-mediated immune escape. Investigation of LILRB4 on how to contribute to MDSC-mediated immunosuppression could provide a new approach for ICI combination therapy.

研究成果の概要（英文）：Myeloid-derived suppressor cells (MDSCs) are involved in tumor-associated immunosuppression, and dominate tumor progression and metastasis. In this study, we report that the leukocyte immunoglobulin-like receptor subfamily B member 4 (LILRB4, murine ortholog gp49B) orchestrates the polarization of MDSCs to exhibit pro-tumor phenotypes. Gp49B－/－ MDSCs inhibited pro-tumor immune responses such as activation of Treg cells, promotion of cancer cell migration, and stimulation of tumor angiogenesis. Treatment of wild-type tumor-bearing mice with gp49B－/－ M-MDSCs reduced cancer metastasis. Furthermore, gp49B knockout affected plasma exosome composition in terms of increased anti-tumor microRNAs expression. Collectively, our findings reveal that LILRB4/gp49B promotes MDSC-mediated tumor metastasis by regulating the M2-polarization of MDSCs and suppressing the secretion of miR-1 family miRNAs, which facilitate tumor migration and invasion.

研究分野：Oncolmmunology

キーワード：MDSC　LILRB4　gp49B　immunosuppression　metastasis

１．研究開始当初の背景

Tumor metastasis, the spread of malignant cells to distant organs, is the main cause of death in cancer patients. The ability of tumor cells to modulate immune response and evade immune recognition is necessary for successful metastasis. In the tumor environment, myeloid cell-induced immunosuppression that inhibits anti-tumor immune responses is a pivotal requirement for cancer cells to grow and metastasize.

Myeloid-derived suppressor cells (MDSCs), a population of immune suppressive cells, strongly inhibit anti-tumor immune reactions mediated by T cells and enhance angiogenesis for metastatic formation. MDSCs consist of two major subsets in human and mice, monocytic-MDSC (M-MDSC) and polymorphonuclear MDSC (PMN-MDSC), also known as granulocytic MDSC (G-MDSC). Both MDSC subsets are found in the bone marrow, spleen, lung, peripheral blood and tumor tissue. MDSC-mediated immunosuppression limits the potency of cancer immunotherapy drugs such as anti-programmed death receptor-1 (PD-1), anti-programmed death-ligand 1 (PD-L1) and anti-cytotoxic T lymphocyte antigen 4 (CTLA4); therefore, targeting tumor-infiltrating MDSCs is an important issue in cancer therapy. Interestingly, the leukocyte immunoglobulin-like receptor subfamily B member 4 (LILRB4) is expressed on MDSCs and correlates with survival in human lung cancer patients. However, the biological function of LILRB4 for MDSC-mediated immunosuppression remains unclear.

LILRB4, also known as ILT3, CD85k, LIR-5, or gp49B (mouse), contains two extracellular immunoglobulin domains, a transmembrane domain and three immunoreceptor tyrosine-based inhibitory motifs (ITIMs). Upon ligand engagement, the ITIMs of LILRB4 are phosphorylated by tyrosine kinases of the Src family, thereby recruiting phosphotyrosine phosphatases (SHP-1/2) or the inositol-phosphatase SHIP to inhibit the activation of molecules that contribute in cell signaling. Previous studies reported that activated leukocyte cell adhesion molecule (ALCAM/CD166/MEMD) and apolipoprotein E (APOE) are ligands of LILRB4, which mediate the growth of tumor cells and the development of acute myeloid leukemia, respectively. Herein, we hypothesized that LILRB4/gp49B may be involved in tumor metastasis, and that LILRB4/gp49B signaling may contribute to MDSC-mediated immunosuppression in the tumor environment.

２．研究の目的

The aims of this study are: (1) To explore the effect of LILRB4/gp49B on lung cancer/melanoma metastases. (2) To determine the potentiality of gp49B as an immune checkpoint on tumor therapy. (3) To clarify whether gp49B is involved in MDSC-stimulated pro-tumor responses, including activation of Treg cells and promotion of cancer cell migration. (4) To verify whether gp49B is involved in the recruitment of MDSC in tumor primary and metastatic sites.

３．研究の方法

Animals

C57BL/6 (B6, WT) mice were purchased from CLEA Japan (Tokyo, Japan). The generation of gp49B$^{-/-}$ mice with B6 background was described previously (24). All animals were bred and conducted in accordance with the animal guidelines of the Laboratory for Animal Resources of the Institute of Development, Aging and Cancer, Tohoku University. All protocols of animal experiments were approved by the Animal Studies Committee of Tohoku University. Male mice between 8 and 10 weeks of age were used.

Adoptive transfer experiment

For bone marrow transplantation (BMT), B6 mice were irradiated with 8.5 Gy total body irradiation (MBR-1520R-4, Hitachi Power Solutions Co., Ltd.). At 1-day post-irradiation, mice were transplanted with bone marrow cells from WT or gp49B$^{-/-}$ donor mice via tail vein injection (5×10^6 cells/mouse). Following adoptive transfer, mice were treated with gentamycin (1.2 mg/ml) for one month, and the total blood donor reconstitution was evaluated by flow cytometry at four weeks post-transplantation. For MDSC adoptive transfer, splenic MDSCs were isolated from WT or gp49B$^{-/-}$ tumor-bearing mice by flow sorting, and isolated MDSCs (2×10^5 cells/mouse) were then co-injected with LLC cells in a 1:1 ratio into WT mice on day 0. The adoptive transfer of splenic MDSCs was repeated 1 week later.

Mouse model of tumor metastases and therapeutic protocols

Regarding tumor metastases, LLC (1.5×10^6/mouse) and B16F10 (5×10^5/mouse) cells were inoculated into B6 and gp49B$^{-/-}$ mice by intravenous injection. After inoculation, LLC- and B16F10-bearing mice were sacrificed on day 30 and day 21, respectively, and lung and liver tissues from tumor-bearing mice were harvested for analysis of tumor metastasis by hematoxylin-eosin (H&E) staining or counting of surface nodules. For antibody treatment, the B6 mice were inoculated intravenously at a low dose of 5×10^5 LLC-Luc2 cells or 5×10^4 B16F10 cells. At 3 days post-tumor inoculation, mice were treated with anti-PD-1 (RPM1-14, Bio X Cell; 200 μg/mouse), anti-gp49 (H1.1-Amenian hamster IgG from hybridoma cells (26) or H1.1-mIgG1, 200 μg/mouse), or isotype matched control antibody (2A3 (Bio X cell), HTK888 (Biolegend) or MOPC-21 (Bio X Cell); 200 μg/mouse) by intraperitoneal injection six times at 3-day intervals. LLC-Luc2 tumor-bearing mice with antibody treatment were imaged using the IVIS Spectrum In Vivo Imaging System (Perkin Elmer) at three weeks after tumor inoculation, and sacrificed at five weeks after tumor injection for detection of tumor metastases in lung and liver by H&E staining.

Treg cell expansion by MDSCs

Splenocytes (2×10^6 cells/well) from OT-II mice were labeled with CFSE (Thermo Fischer Scientific) according to the manufacturer's instructions, and subsequently co-cultured with MDSCs (5×10^5 cells/well) from tumor-bearing mice in the presence of OVA peptides (1 μg/ml) for five days. The co-cultured cells were stained FITC-conjugated anti-CD4, APC-conjugated anti-CD25 and PE-conjugated anti-Foxp3 antibodies for flow cytometry analysis.

Transwell migration assay

LLC cells (1×10^4 cells/well) were seeded in the upper chamber of a 24-well 8-μm-pore transwell plate (Corning, NY, USA) with serum-free RPMI-1640 medium for 24 h. Subsequently, 1-day starved LLC cells were co-cultured with 1.2×10^5 of WT or gp49B$^{-/-}$ bone marrow-derived MDSCs (BM-MDSCs) from tumor-bearing mice plated in the lower chamber for another 72 h. In PC9 cell migration assay, WT or LILRB4$^{-/-}$ THP-1 cells (4×10^5 cells/well) were seeded on a 24-well culture plate for PMA (50 ng/ml) stimulation. At 48 h post-stimulation, the activated THP1 cells were co-cultured with 1×10^4 of starved PC9 cells plated on the upper chamber (8-μm-pore) for 36 h. Non-migrated cells on the upper side of the transwell membrane were removed with a cotton swab, and the migrated cells on the underside of the transwell membrane were stained with 0.5% crystal violet solution. Images of the migrated cells were captured with a Keyence BZ-9000 microscope (Keyence Corporation, Osaka, Japan). Cell number of migrated cells in 20 random fields was analyzed using ImageJ.

４．研究成果

(1) Genetic deficiency of gp49B impaired tumor metastases in tumor-bearing mice

To determine whether LILRB4/gp49B contributes to tumor metastases, wild-type (WT) B6 mice and gp49B$^{-/-}$ mice were intravenously injected with luciferase expressing-Lewis lung carcinoma cells (LLC-Luc2) and examined for the metastases 21 days later. We found that the metastatic ability of LLC-Luc2 was significantly inhibited in gp49B$^{-/-}$ mice. With adoptive bone marrow transplantation (BMT), the bone marrow cells of WT mice were reconstituted with that of gp49B$^{-/-}$ mice to confirm the gp49B-mediated tumor metastasis. In the comparison of WT BMT and gp49B$^{-/-}$ BMT mice, LLC-Luc2 tumor growth monitored by a non-invasive IVIS bio-image system was reduced in gp49B$^{-/-}$ BMT-tumor-bearing mice. We also inspected whether gp49B knockout could reduce the metastatic ability of melanoma B16F10. The numbers of pulmonary metastatic nodule and liver metastatic foci in B16F10-injected gp49B$^{-/-}$ mice were significantly decreased compared to those in B16F10-injected WT mice. Together, the above results indicated that LILRB4/gp49B is involved in the metastases of tumor cells.

(2) Gp49B blockade inhibited tumor metastases in tumor-bearing mice

An analysis of RNA expression data from The Cancer Genome Atlas (TGCA) database via the Xena plateform revealed a strong correlation between LILRB4 and PD-1 mRNA expression in LUAD and LUSC samples (r = 0.6064 and 0.7024, respectively). Since deficiency of gp49B reduced the tumor metastasis, we then assessed the effect of gp49B blockade on tumor metastases using anti-gp49 (H1.1) monoclonal antibody (mAb) or a combination of anti-gp49 with anti-PD-1 (RMP1-14) mAb. LLC-Luc2 cells-challenged B6 mice were intraperitoneally injected with isotype control IgG, anti-PD-1 mAb, anti-gp49 mAb, or a combination of anti-PD-1 mAb and anti-gp49 mAb six times. After antibody treatment, the metastatic growth by

bioluminescence image was decreased in LLC-Luc2 tumor-bearing mice treated with anti-gp49 mAb or anti-PD-1 mAb (p = 0.0124 and 0.011, respectively). In addition, a combination treatment of anti-PD-1 mAb and anti-gp49 mAb showed a modest synergistic effect on tumor metastatic growth in LLC-Luc2 tumor-bearing mice (p = 0.046). We also observed the metastatic foci in mAb-treated tumor-bearing mice by histological analysis. Blockade of gp49, as well as blockade of both PD-1 and gp49, indeed impaired tumor metastases in the lung and liver of LLC-Luc2 tumor-bearing mice. These results suggested that LILRB4 blockade inhibits tumor metastasis and that LILRB4 may serve as a potential therapeutic target for combination therapy in cancer diseases.

(3) Knockout of gp49B impairs the infiltration of MDSCs in the tumor environment

Since MDSCs play an important role in tumor-associated immunosuppression, we considered whether gp49B regulates MDSC infiltration in the tumor environment. To this end, we confirmed that gp49B is expressed on MDSCs in WT mice, but not in gp49B$^{-/-}$ mice. To measure the population of tumor-infiltrated MDSCs, lung tissues from WT and gp49B$^{-/-}$ LLC-Luc2 tumor-bearing mice were dissociated for flow cytometry analysis. The result showed that lung-infiltrated monocytic (M)-MDSCs were reduced in gp49B$^{-/-}$ tumor-bearing mice, while lung-infiltrated polymorphonuclear (PMN)-MDSCs were comparable in WT and gp49B$^{-/-}$ tumor-bearing mice. With LLC subcutaneous inoculation, the population of tumor-infiltrating M-MDSCs were reduced in gp49B$^{-/-}$ tumor-bearing mice. These results suggested that LILRB4/gp49B regulates M-MDSC differentiation and infiltration in the tumor environment.

(4) Gp49B$^{-/-}$ MDSCs reduce immunosuppressive cytokine secretion, Treg activation and tumor cell migration.

Immunosuppressive cytokines, such as IL-10 and TGF-β, are secreted by activated MDSCs to down-regulate IL-12 secretion by macrophages, block cytotoxic T lymphocyte activity, and activate Treg cells. To determine the expression level of immunosuppressive genes in MDSCs from tumor-bearing mice, the splenic MDSCs from WT and gp49B$^{-/-}$ tumor-bearing mice were isolated for gene expression analysis by qRT-PCR. The expression of TGF-β and IL-10 was downregulated, while the expression of TNF-α was upregulated in gp49B$^{-/-}$ splenic MDSCs, suggesting that gp49B$^{-/-}$ MDSCs exhibit an M1 phenotype. In comparison with WT splenic MDSCs, gp49B$^{-/-}$ splenic MDSCs secreted low levels of TGF-β and IL-10. To verify whether gp49B could regulate the MDSC-mediated expansion and activation of Treg cells, MDSCs from WT or gp49B$^{-/-}$ tumor-bearing mice were co-cultured with OT-II splenocytes with OVA peptides stimulation. Upon co-culture with gp49B$^{-/-}$ MDSCs, the expansion of Treg cells decreased, whereas the proliferation of CD4 Teff increased, suggesting that the knockout of gp49B affects the immunosuppressive function of MDSCs. Since MDSCs also contribute in the migration of tumor cells, we therefore examined the effect of gp49B knockout on MDSC-regulated tumor cell migration. *In vitro* transwell migration assay revealed a significantly decreased migration of murine LLC cells co-cultured with gp49B$^{-/-}$ BM-MDSCs as well as human PC9 cells co-cultured with activated LILRB4$^{-/-}$ THP-1 cells. The above results suggested that gp49B knockout affects the immunosuppressive function of M-MDSCs.

(5) Treatment of tumor-bearing mice with gp49B$^{-/-}$ MDSCs suppresses tumor metastasis.

In order to verify whether tumor-primed gp49B$^{-/-}$ MDSCs have anti-tumor activity, WT LLC-Luc2 tumor-bearing mice were intravenously injected with tumor-primed WT or gp49B$^{-/-}$ MDSCs twice, and the bioluminescence activity was monitored to assess tumor metastatic growth. Indeed, gp49B$^{-/-}$ MDSC treated tumor-bearing mice revealed a significant reduction of LLC-Luc2 metastasis compared to WT controls. With a lower expression level of gp49B, lung-infiltrated M-MDSCs were also reduced in gp49B$^{-/-}$ MDSC treated WT tumor-bearing mice as well as in gp49B$^{-/-}$ tumor-bearing mice. In contrast, gp49B$^{-/-}$ MDSC adoptive transfer did not affect the population frequency of PMN-MDSCs resident in lung tissue. These data suggested that gp49B knockout in MDSCs triggers anti-tumor activity, and gp49B might be involved in M-MDSCs recruitment in the tumor environment.

５．主な発表論文等

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２．論文標題 LILRB4 promotes tumor metastasis by regulating MDSCs and inhibiting miR-1 family miRNAs	５．発行年 2022年
３．雑誌名 OncoImmunology	６．最初と最後の頁 e2060907
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〔図書〕　計0件

〔出願〕　計1件

産業財産権の名称	発明者	権利者
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産業財産権の種類、番号	出願年	国内・外国の別
特許、PCT/JP2020/030175	2020年	国内

〔取得〕　計0件

〔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