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研究課題名（和文）小腸自然免疫のリンパ系細胞と肥満細胞の機能的な相互関係の解明

研究課題名（英文）Identification of functional relationship between the ILCs and the mast cells in the small intestine

研究代表者
李　英愛（LEE, YOUNGAE）

大阪大学・免疫学フロンティア研究センター・特任研究員(常勤)

研究者番号：６０６１０６８１

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研究成果の概要（和文）：Lin-c-Kit+NKp46-CD4-細胞のうちIL-7Rα+ 細胞群(CD4-LTi様細胞)は、IL-17FとIL-22の発現による細菌感染防御を解明した。IL-7Rα-CD34-β7int細胞群のDEFA発現は組織恒常性維持に重要である。IL-7Rα-CD34+β7intとIL-7Rα-CD34intβ7hi細胞群は、MCPT5とCPA3の発現で炎症反応を制御するマスト細胞の前駆細胞である。

研究成果の概要（英文）：We found that the IL-7Ra+ population (CD4- LTi-like cells) within Lin-c-Kit+NKp46-CD4- cells has a protective role against bacterial infection through high expression of IL-17F and IL-22. The strong expression of DEFAs by the IL-7Ra-CD34-b7int population in the steady-state condition may play an important role in maintaining tissue homeostasis with commensal bacteria. IL-7Ra-CD34+b7int and IL-7Ra-CD34intb7hi cells are MCPs, which can differentiate into MCs that may regulate inflammatory responses by expressing high levels of MCPT5 and CPA3.

研究分野：　Medicine, dentistry and pharmacology

科研費の分科・細目：　Basic medicine, Immunology

キーワード：　IL-22　IL-17F　DEFAs　Innate lymphoid cells　Mast cells

１．研究開始当初の背景

Innate lymphoid cells (ILCs) are a novel family of hematopoietic effectors that play protective roles in innate immune responses to infectious microorganisms, in lymphoid tissue formation, and in tissue remodeling (Spits *et al*., *Nature Immunol*. 2011). ILCs localized much more in the small intestinal lamina propria (LP) than in the mesenteric lymph node, inguinal lymph node, spleen, liver, and bone marrow (Sawa *et al*., *Science* 2010). However, the lineage relationship between the ILCs and myeloid cells or lymphoid cells remains unknown. In previous study, we did a microarray experiment to analyze gene expression profiles of small intestinal ILCs ($Lin^-c\text{-}Kit^+Sca\text{-}1^+$ cells), and detected that ILCs highly express c-Kit, Csf2rb2, mast cell makers, and ILCs makers compared with non-ILCs ($Lin^-c\text{-}Kit^-Sca\text{-}1^+$ cells). We also detected that ILCs ($Lin^-c\text{-}Kit^+Sca\text{-}1^+$ cells) can differentiate into mast cells under the culture conditions together with SCF and IL-3 for 4 weeks. Mast cells in the small intestine involve in food allergy and parasitosis. Mast cell-deficient mice are less efficient in removing bacteria and more susceptible to infection (Sutherland *et al*., *J Immunol*. 2008 and Lin *et al.*, *J Immunol*. 2002). Until recently, the functions of mast cells and mast cell precursors in the small intestine are not clear. We have experimental plans to investigate the ILCs as potential mast cell precursors and the relationship between the intestinal mast cells and the intestinal ILCs and to investigate the function of intestinal mast cells.

２．研究の目的

(1) Identify the lineage relationship between mast cells and innate lymphoid cells in the small intestinal lamina propria: We confirmed that $Lin^-c\text{-}Kit^+Sca\text{-}1^+$ cells in the small intestinal LP highly express RAR-related orphan receptor gamma (RORc) mRNA and IL-22 mRNA, which are ILCs markers, and these cells also express c-Kit, Csf2rb2, mast cell markers such as mast cell proteases (Mcpts). In addition, we detected that $Lin^-c\text{-}Kit^+Sca\text{-}1^+$ cells can differentiate into mast cells in the presence of recombinant protein IL-3 and SCF. Therefore, we need further study to identify the ILCs as potential mast cell precursors.

(2) Compare the functional heterogeneity by stimulation with TLRs agonists between small intestinal LP-derived mast cells (LPMCs) and bone marrow-derived mast cells (BMMCs): The bone marrow is sterile tissue conditions; however, the small intestine is non-sterile environment. Until now, it is not clear that those mast cells from the bone marrow and small intestinal LP induce how different immune responses by microbial products. In this study, we compared cell differentiation, mast cell degranulation, and the expression of mast cell proteases, TLRs, and cytokines between LPMCs and BMMCs.

３．研究の方法

(1) Identify the lineage relationship between mast cells and innate lymphoid cells in the small intestinal lamina propria: We isolated $Lin^-c\text{-}Kit^+Sca\text{-}1^+$ cells in the small intestinal LP of female, 8 week-old of BALB/c mice by FACS sorting. Isolated $Lin^-c\text{-}Kit^+Sca\text{-}1^+$ cells were cultured in the presence of recombinant IL-3 (10 ng/ml) and SCF (50 ng/ml) for 4 weeks. Bone marrow-derived MCs from femoral bone marrow cells of BALB/c mice were generated in the

presence of recombinant IL-3 and SCF for 4 weeks. Mast cell morphology and mucin expression levels were detected by cytospin and Alcian blue staining methods, respectively. The expressions of mast cell markers, c-Kit and FcεRI, were analyzed by FACS analysis. For detail and further study, we isolated $Lin^-c\text{-}Kit^+Sca\text{-}1^-$ cells divided into 4 subgroups in the small intestinal LP using FACS Aria. To investigate whether which type of these cells (4 subgroups) can differentiate into mast cells, each type of cells are cultured in the presence of recombinant IL-3 and SCF for 4 weeks. To detect whether differentiated cells have similar phenotypes as mast cells, the expression of c-Kit and FcεRI is analyzed by FACS analysis and cell morphology is detected by May-Grünwald-Giemsa staining.

(2) Compare the functional heterogeneity by stimulation with TLRs agonists between small intestinal LP-derived mast cells and bone marrow-derived mast cells: We generated LPMCs and BMMCs by cultivation in the presence of IL-3 (10 ng/ml) and SCF (50 ng/ml) for 4 weeks. We then compared cell morphology and granules by cytopsin and checked the gene expression for mast cell proteases, TLRs, and cytokines between LPMCs and BMMCs.

4．研究成果

(1) Identify the lineage relationship between mast cells and innate lymphoid cells in the small intestinal lamina propria.

① The $IL\text{-}7R\alpha^-CD34^+\beta7^{int}$ and $IL\text{-}7R\alpha^-CD34^{int}\beta7^{hi}$ populations within the $Lin^-c\text{-}Kit^+NKp46^-CD4^-$ cells represent MCPs, and the $IL\text{-}7R\alpha^+CD34^-\beta7^{int}$ population is $CD4^-$ LTi-like cells: We investigated what percentage of cells within the $Lin^-c\text{-}Kit^+NKp46^-CD4^-$ population expresses CD127 (IL-7Rα), CD34, and β7 integrin by FACS analysis, because IL-22-producing ILCs express IL-7Rα, and mouse intestinal MCPs express CD34 and $\beta7^{hi}$. We found that 95.0% of the $Lin^-c\text{-}Kit^+NKp46^-CD4^-$ population was $IL\text{-}7R\alpha^+CD34^-\beta7^{int}$ cells (Subset IV), and the rest of the $Lin^-c\text{-}Kit^+NKp46^-CD4^-$ population consisted of $IL\text{-}7R\alpha^-CD34^+\beta7^{int}$ (Subset I), $IL\text{-}7R\alpha^-CD34^-\beta7^{int}$ (Subset II), and $IL\text{-}7R\alpha^-CD34^{int}\beta7^{hi}$ (Subset III) cells.

May-Grünwald-Giemsa staining showed that the $IL\text{-}7R\alpha^-CD34^+\beta7^{int}$ (I) and $IL\text{-}7R\alpha^-CD34^{int}\beta7^{hi}$ (III) cells had few or no granules. We cultured the four subsets in a cytokine cocktail for 1 week and found that the $IL\text{-}7R\alpha^-CD34^+\beta7^{int}$ (I) and $IL\text{-}7R\alpha^-CD34^{int}\beta7^{hi}$ (III) cells differentiated almost entirely into MCs ($c\text{-}Kit^+Fc\varepsilon RI^+$ cells), whereas a small percentage of the $IL\text{-}7R\alpha^+CD34^-\beta7^{int}$ cells (IV) differentiated into MNPs ($Mac\text{-}1^+Gr\text{-}1^-$ cells). The $IL\text{-}7R\alpha^-CD34^-\beta7^{int}$ cells (II) showed low viability and did not differentiate into other cell types.

In addition, the $IL\text{-}7R\alpha^-CD34^+\beta7^{int}$ (I) and $IL\text{-}7R\alpha^-CD34^{int}\beta7^{hi}$ (III) populations strongly expressed Mcpts mRNA, whereas the $IL\text{-}7R\alpha^-CD34^-\beta7^{int}$ population (II) highly expressed α–defensins mRNA, and the $IL\text{-}7R\alpha^+CD34^-\beta7^{int}$ population (IV) predominantly expressed Il22 and Rorc mRNA.

We next compared the mRNA expressions of Mcpt family members in the $IL\text{-}7R\alpha^-CD34^+\beta7^{int}$

(I), IL-7Rα^{-}CD34^{int}β7^{hi} (III) cells, and mature MCs (samples at 1 week). The IL-7Rα^{-}CD34^{int}β7^{hi} (III) cells expressed the highest levels of Mcpt1 and Mcpt2 mRNA among the four groups, whereas the mature MCs induced high levels of Mcpt5 and Cpa3 mRNA.

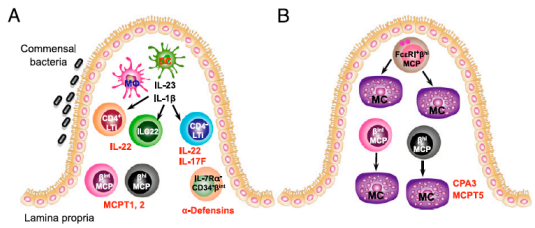
Our results suggest that the IL-7Rα^{-}CD34^{+}β7^{int} and IL-7Rα^{-}CD34^{int}β7^{hi} populations represent MCPs, and the IL-7Rα^{+}CD34^{-}β7^{int} population represents CD4^{-} LTi-like cells.

② The IL-7Rα^{+}CD34^{-}β7^{int} population is the main source of IL-17F and IL-22 after stimulation with recombinant IL-1β and IL-23: We next compared the induction of Il17f and Il22 transcripts in the IL-7Rα^{-}CD34^{+}β7^{int} (I), IL-7Rα^{-}CD34^{-}β7^{int} (II), IL-7Rα^{-}CD34^{int}β7^{hi} (III), and IL-7Rα^{+}CD34^{-}β7^{int} (IV) populations under co-stimulation with IL-1β and IL-23 for 16hr. We found that the co-stimulation induced the Il17f and Il22 mRNA levels only in the IL-7Rα^{+}CD34^{-}β7^{int} population (IV). Also, intracellular staining showed IL-7Rα^{+}CD34^{-}β7^{int} (IV) populations highly produced IL-17F and IL-22 after co-stimulation with IL-1β and IL-23 for 24hr compared with NKp46^{+}ILC22. These data indicate that the main source of IL-17F and IL-22 production after stimulation with IL-1β and IL-23 is the IL-7Rα^{+}CD34^{-}β7^{int} population (CD4^{-} LTi-like cells).

③ The Lin^{-}c-Kit^{+}NKp46^{-}CD4^{-} population contains more immature MCPs than the Lin^{-}CD45^{+}CD34^{+}FcεRI^{+}β7^{hi} population: A previous study reported that intestinal MCPs, the Lin^{-}CD45^{+}CD34^{+}FcεRI^{+}β7^{hi} cells, contain a few scattered metachromatic granules and differentiate into pure mast cell colonies. We sorted the Lin^{-}CD45^{+}CD34^{+}FcεRI^{+}β7^{hi} population from small intestinal LP, and cultured this population in the presence of a recombinant cytokine cocktail for 0-2 weeks, and then analyzed the cell phenotype by cytospin and FACS. Our results agreed with the previous report: that is, the sorted Lin^{-}CD45^{+}CD34^{+}FcεRI^{+}β7^{hi} population (at 0 weeks) already had a few granules in their cytoplasm, showed higher SSC compared with the IL-7Rα^{-}CD34^{+}β7^{int} (I) and IL-7Rα^{-}CD34^{int}β7^{hi} (III) populations, which express no FcεRI, and could differentiate into pure MCs. These results suggest that the Lin^{-}CD45^{+}CD34^{+}FcεRI^{+}β7^{hi} population is relatively more mature MCPs than the IL-7Rα^{-}CD34^{+}β7^{int} and IL-7Rα^{-}CD34^{int}β7^{hi} populations.

In short, our data show that IL-7Rα^{+} population (CD4^{-} LTi-like cells) within Lin^{-}c-Kit^{+}NKp46^{-}CD4^{-} cells may contribute to tissue homeostasis and have a protective role against bacterial infection through high expression of IL-17F and IL-22. IL-7Rα^{-}CD34^{-}β7^{int} population strongly expressing α–defensins in the steady state condition may play an important role in maintaining tissue homeostasis with commensal bacteria. IL-7Rα^{-}CD34^{+}β7^{int} and IL-7Rα^{-}CD34^{int}β7^{hi} cells are MCPs, which can differentiate into MCs that may regulate inflammatory responses by expressing high levels of MCPT5 and CPA3 (Fig.1).

Figure 1.



(2) Compare the functional heterogeneity by stimulation with TLRs agonists between small intestinal LP-derived mast cells and bone marrow-derived mast cells.

LPMCs highly express TLR3 mRNA compared with BMMCs: LPMCs and BMMCs were differentiated from each progenitor cells by cultivation in the presence of IL-3 and SCF for 1-4 weeks. We then compared cell morphology by May-Grünwald-Giemsa stain. LPMCs were more rapidly differentiated from their progenitors, were bigger in cell size, and had more granules in their cytoplasm compared with BMMCs. We then compared the expression levels of TLRs mRNA and protein between LPMCs and BMMCs by real-time PCR and FACS analysis, respectively, because the small intestine is continuously exposed to exogenous antigens such as dietary and microbes. LPMCs highly expressed TLR3 mRNA than the other TLRs mRNA and showed higher expression levels of TLR3 mRNA and protein than BMMCs.

Then, we investigated cytokine expression in LPMCs and BMMCs after stimulation with TLR2, 3, 4, 7 agonists. These cells were stimulated with 1 or 10 μg/ml of Pam3CSK4, 1 or 10 μg/ml of Poly(I:C), 0.1 or 1 μg/ml of LPS, 1 or 10 μg/ml of imiquimod, respectively, for 24 hrs. Th1, Th2, and Th17 cytokine levels in cell culture supernatants were measured by Bio-Plex. Only IL-6 production in LPMCs and BMMCs was induced by treatment with TLR2 or TLR4 agonists and BMMCs produced higher levels of IL-6 compared with LPMCs. Although LPMCs did not produce the other cytokines by stimulation with TLRs agonist in vitro, they may involve in the small intestinal pathogenesis in vivo. A study showed that dsRNA from rotavirus induces severe mucosal injury in the small intestine (Zhou *et al., J Immunol.,* 2008). Another study reported that TLR3-induced immune responses contribute to suppress rotavirus replication (Pott *et al., PLOSpathogens,* 2012).

５．主な発表論文等

（研究代表者、研究分担者及び連携研究者には下線）

〔雑誌論文〕（計　1 件）

Intestinal $Lin^{-}c\text{-}Kit^{+}NKp46^{-}CD4^{-}$ Population Strongly Produces IL-22 upon IL-1β Stimulation. Lee Y, Kumagai Y, Jang MS, Kim JH, Yang BG, Lee EJ, Kim YM, Akira S, Jang MH. *J Immunol.* Peer-reviewed 109: 5296-5303, 2013.

doi: 10.4049/jimmunol.1201452

〔学会発表〕（計　2 件）

① The American Association of Immunologists (AAI) May 3-7th, 2013, Hawaii, USA. Intestinal $Lin^{-}c\text{-}Kit^{+}NKp46^{-}CD4^{-}$ population highly produces IL-22 upon IL-1β stimulation. Y Lee, Y Kumagai, MS Jang, JH Kim, BG Yang, S Akira, and MH Jang.

② The American Association of Immunologists (AAI) May 4-8th, 2012, Boston, USA. Intestinal mast cell progenitors function as innate lymphoid cells. Y Lee, Y Kumagai, S Akira, and MH Jang.

６．研究組織

(1)研究代表者

李　英愛

大阪大学・免疫学フロンティア研究センタ

一．特任研究員

研究者番号：60610681