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学 位 論 文 題 名

The regulation mechanisms of immune balance through the activation of innate immunity

(自然免疫を介した免疫バランス制御機構の解明に関する研究)

学位論文内容の要旨

【Introduction】 T helper type 1 (Th1) cells regulate Type-1 immunity, has an essential function in the control of cellular immunity against tumor, intracellular bacteria and virus. On the other hand, Type-2 immunity, controlled by T helper type 2 (Th2) cells, plays a crucial role for humoral immunity against parasitic worms and allergens. Both Type-1/Type-2 immune systems, which are closely related with acquired immunity, inhibit the activation of each other and retain the homeostasis. It is thought that the increase of allergy, infectious diseases, tumor resulting from the excessively polarized Type-2 immunity and deteriorated Type-1 immunity. Many studies have reported that several types of Lactic acid bacteria (LAB) switch the Type-2 immunity-biased allergic patients towards a balanced Type-1/Type-2 immune profile, leading to amelioration of allergy. In this research, I attempted to elucidate how food-derived components, including LAB, regulated Type-1 immunity activation. I especially focused on innate immunity, which not only act as the first line of host defense but also play a critical role in the subsequent regulation of acquired immunity. Because dendritic cells (DCs), professional antigen presenting cells, have a critical role in innate and acquired immune responses, regulation of DC function is most important for Type-1/Type-2 immune balance. In addition, DCs effectively induce cytotoxic T lymphocytes (CTLs), which are essential for vaccination against virus, bacteria and tumor. Thus, I focused on regulation of DC function and investigated the precise mechanisms. Finally, I studied on application research for cancer immunotherapy by bone marrow-derived DCs (BMDCs) using a mouse tumor-implanted model.

【Methods and Results】 Mouse spleen cells were stimulated with *Lactobacillus pentosus* S-PT84. After 12-48h incubation, I determined IFN-γ and IL-12 production levels in the supernatant by ELISA. As a result, S-PT84 strongly induced production of IFN-γ and IL-12 from spleen cells. Next, I analyzed IL-12 and IFN-γ producer using techniques of intracellular staining and cell-depletion or isolation. IFN-γ and IL-12 are produced by $NK1.1^+$ cells (NK and NKT cells) and $CD11c^+$ DCs, respectively. DC-derived IL-12 is completely required for production of IFN-γ from $NK1.1^+$ cells. Moreover, direct interaction between $NK1.1^+$ cells and DCs is essential in the IFN-γ production by $NK1.1^+$ cells. To elucidate which receptor recognized S-PT84, I isolated DCs from wild-type (WT), Toll-like receptor $(TLR)2^{-/-}$, $TLR4^{-/-}$ and $TLR9^{-/-}$ mice. The productions of both IL-12 from DCs and IFN-γ from NK cells were significantly decreased in $TLR2^{-/-}$ or $TLR4^{-/-}$ DCs compared with those from WT mice.

I screened 57 lactic acid bacteria (LAB), isolated from Hokkaido vegetable pickles, by the individual IFN-γ, IL-12 and IL-10 production by spleen cells after the stimulation. I identified a novel *Lactobacillus sakei* strain, which was named 'Bio-S24'. Bio-S24 could stimulate spleen cells to induce the production of high levels of IL-12 and IFN-γ but negligible levels of IL-10. Bio-S24-mediated IL-12 induction is

completely dependent on TNF-α production. I examined IL-12 induction mechanisms by isolated spleen dendritic cells. As a result, DC-DC interactions through transmembrane TNF-α-TNFR-I/II and soluble TNF-α-TNFR-I signaling were required for maximum IL-12 production.

I examined whether the eight kinds of bean extract had a capability of inducing IFN-γ production in culture system with mouse spleen cells. Surprisingly, only the extract of *Glycine Max*, Kurosengoku, highly induced IFN-γ from spleen cells. Kurosenogku induced Type-1 cytokines through TLR2- and TLR4-signaling pathways as same as S-PT84. Finally, I revealed that Kurosengoku significantly enhanced IFN-γ production by human PBMCs stimulated with anti-CD3 mAbs.

Furthermore, I screened a new adjuvant from various extracts of agricultural products using BMDCs. As a result, I found that the extract of *Larix Leptolepis (Larix kaempferi)* (ELL) strongly activates BMDCs. Indeed, ELL induced antigen-specific CTLs in vivo through BMDC activation. I demonstrated that adoptive transfer of BMDCs with ELL and antigen remarkably inhibited tumor growth in the tumor-bearing mouse model. Thus, ELL would be useful for prevention of tumor and infectious diseases via effective induction of antigen-specific CTLs.

【Discussion】 In this research, I firstly found that S-PT84 activated Type-1 immunity. TLRs on DCs are key regulator in activation of Type-1 immunity. As well as LAB asuch as S-PT84 and Bio-S24, I demonstrated that extract of Kurosengoku effectively elevated Type-1 immunity by DC activation through TLRs. Interestingly, although TLR2 and TLR4 recognized these components, mechanism of cytokine production was very different. In the present experiments, I confirmed that the IFN-γ production by the Kurosengoku-stimulated spleen cells was partially blocked in the presence of galactomannan, mannan, or galactose, and completely blocked by the addition of EDTA, whereas IFN-γ production by LAB-stimulated spleen cells was not blocked. These findings suggest that the extract of Kurosengoku has at least sugar-related compounds, binding with a C-type lectin.

I found that DC-derived IL-12 was essential for IFN-γ production by $NK1.1^+$ NK cells and NKT cells in the present investigation. This data indicated that several innate immune cells cooperatively acted during Type-1 immune activation. Moreover, I revealed that not only NK-DC interaction but also TNF-α-mediated DC-DC interaction was very important for Type-1 immune activation. I speculated that different DC subsets such as $CD4^+$, $CD8^+$ and $CD4^-8^-$ conventional DCs and plasmacytoid DCs, which respectively exhibit different phenotypes and functions, were contributed to the production of TNF-α and IL-12.

The present data suggest that Bio-S24 and Kurosengoku would be a promising tool for activation of Type-1 immunity via oral intake. In the future, it is important to perform in vivo animal study and the clinical study to confirm Type-1 immuno-improving activity of Bio-S24 and Kurosengoku, which might prevent Type-2 immunity-dependent immune diseases including allergy as well as infectious diseases and cancers. In addition, I revealed that ELL effectively induced antigen-specific CTLs in vivo model, which might be useful as a TLR-mediated novel adjuvant for prevention of tumor and infectious diseases.

【Conclusion】 IL-12 production by DCs through TLR-signaling cascades is required for the subsequence induction of IFN-γ by $NK1.1^+$ cells. Moreover, cell-to-cell interaction among DCs through two types of TNF receptor signal cascades is essential for maximum IL-12 production. These data exhibited that hierarchical and successive reactions among innate immune cells play an important role in activation of Type-1 immunity.

学位論文審査の要旨

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(自然免疫を介した免疫バランス制御機構の解明に関する研究)

近年、衛生環境の変化などにより免疫バランスがType 2型に偏向し、そのことによりやアレルギーなどの様々な疾患が増加している可能性が危惧されている。乳酸菌は免疫バランスを改善することが既に数多くの企業や研究所により報告されているが、その詳細な活性化機構は明らかではなかった。今回の研究により、強い免疫バランス改善作用をもつ乳酸菌株である *Lactobacillus pentosus* S-PT84株が樹状細胞のToll-like receptor (TLR)-2およびTLR-4依存的に樹状細胞を活性化させること、それにより産生されたIL-12がNK1.1陽性の細胞群(NK細胞およびNKT細胞)を活性化させるということが明らかとなった。さらに抑制性サイトカインであるIL-10の産生を誘導しない乳酸菌株である *Lactobacillus sakei* Bio-S24株を同定し、この菌株を用いて乳酸菌によって誘導されるIL-12にTNF-αが重要であることも明らかにした。さらにその際に膜貫通型および可溶型の2種類のTNF-αを介した直接的相互作用を含めた樹状細胞間の相互作用が重要であることを明らかにした。また、乳酸菌と同様の成分をもつ農産物のスクリーニングを行い、北海道産黒大豆の一種である黒千石が、強いIFN-γ産生誘導能を示すことを明らかとし、その免疫賦活機構が乳酸菌と同様にTLR2およびTLR4を介した樹状細胞の活性化と、それによって産生されるIL-12を介したNK1.1陽性細胞の活性化であることを解明した。

審査会において、副査の今村教授より、過剰に免疫を活性化する可能性について質問を受け、今回実験に使用したものは既に食品として流通しているものであり、過度に免疫を活性化する可能性は低いものの、腸炎などを増悪させる危険は留意する必要があることを述べた。また、食品としての黒千石に関して質問を受け、長期摂取によるヒト介在性試験の重要性を述べた。副査の松本准教授より、乳酸菌株の免疫賦活活性の違いについて質問を受け、既知の報告として細胞壁の成分の厚さや分解酵素への抵抗性がIL-12の産生に大きな影響を与えることを述べた。主査の笠原教授より、免疫賦活活性の高い乳酸菌株の関連性について質問を受け、遺伝的にバックグラウンドが近い乳酸菌が比較的強い免疫賦活活性を示すことを述べた。また、遺伝子工学的手法によるより優れた乳酸菌株の開発の可能性に関して質問を受け、免疫学の観点からそのような試み

はされていないものの、遺伝子改変した乳酸菌株の研究は数多くされており、今後免疫学においてもその様な試みをしていく重要性があることを述べた。副査の西村教授より、*in vivo* において非常に長い時間がかかるような実験をもっと迅速に行なう方法はないかという質問を受け、三次元培養などを組み合わせた *in vitro* での生体環境の再構築が有用である可能性を述べた。また、黒千石における活性成分に関しての質問を受け、ガラクトマンナンやマンナンの添加や、C 型レクチン受容体を欠損した細胞で免疫賦活活性が減少することから、糖タンパク質や糖脂質がその活性成分の主体である可能性を述べた。

この論文は、乳酸菌による Type 1 免疫の賦活機構を解明したことで、乳酸菌の効果の科学的な根拠の確立に貢献したことに加え、そこで得られた実験データを基に新たな Type 1 免疫を活性化する食品として黒千石を同定し、その免疫賦活機構を解明したことで高く評価された。今後はヒト介在試験などを介して、これらの食品の摂取による免疫バランスの改善効果やアレルギー疾患の改善効果が得られることが期待される。

審査員一同は、これらの成果を高く評価し、大学院課程における研鑽や取得単位なども併せ、申請者が博士（医学）の学位を取得するのに十分な資格を有するものと判定した。