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Interleukin-21 reduces *Listeria monocytogenes* secondary infection via CD8$^+$ effector memory T cells

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ABSTRACT

Background: Purpose: Interleukin-21 (IL-21) is mainly produced from CD4$^+$ T cells and has broad impact on immune responses. IL-21 isoform is a splicing variant of IL-21 and is functionally similar to conventional IL-21. We established IL-21 isoform transgenic mice (IL-21isoTg), which constitutively express IL-21 isoform specifically in T cells. IL-21isoTg possess high amount of CD8$^+$ T cells in normal physiological condition. The purpose of this study is to determine whether CD8$^+$ T cells in the IL-21isoTg work against intracellular bacteria infection.

Methods: Wild type mice (WT) and IL-21isoTg are orally inoculated *Listeria monocytogenes* (*L. monocytogenes*) on day 0, and 15 days after primary infection. Bacterial load in each organs, and T cell responses are analyzed.

Results: IL-21isoTg and WT had similar bacterial load after primary infection. On the other hand, after secondary infection, IL-21isoTg exhibited reduced bacterial load in some organs and showed higher levels of CD8$^+$ effector memory T (T_{EM}) cells than WT.

Conclusion: IL-21-induced CD8$^+$ T_{EM} cells might eventually reduce the bacterial load after secondary infection. To the best of our knowledge, this is the first study to show that IL-21 is a pivotal factor involved in eliminating intracellular bacteria, probably through CD8$^+$ T_{EM} cells.

Keywords: challenge infection, effector memory T cells, IL-21, IL-21 isoform, *Listeria monocytogenes*

Introduction

Interleukin 21 (IL-21) is a member of the common γ-chain cytokine family, comprising of IL-2, IL-4, IL-7, IL-9, and IL-15. This proinflammatory cytokine is produced by activated CD4$^+$ T cells, in particular follicular helper T cells, Th17, and activated NKT cells[1]. IL-21 acts on various targets of the immune system, including T cells, B cells, dendritic cells, and NK cells[2,3]. IL-21 enhances the proliferation and function of CD8$^+$ T cells in conjunction with IL-15[4], increases the antitumor activity of CD8$^+$ T cells[4,5], facilitates the maturation of memory CD8$^+$ T cells by the activation of STAT3[6], and controls chronic lymphocytic choriomeningitis viral infection in mice by triggering polyfunctional effector CD8$^+$ T cells[7-9].

CD8$^+$ T cell development and response are triggered by the recognition of presented antigen in addition to costimulatory signals and cytokines. A large number of CD8$^+$ effector T cells can be generated following the activation of naïve CD8$^+$ T cells, and these responses operate to eliminate

target cells; however, a large percentage of these cells are prone to apoptosis and lack the self-renewal capacity necessary to form the memory pool[10]. On the other hand, $CD8^+$ memory T cells are maintained over time following the peak of the response and contribute to long-lived immunity[11]. The memory cells can be subdivided into central memory T (T_{CM}) cell and effector memory T (T_{EM}) cell subsets[12]. T_{CM} cells reside in lymphoid tissues and mount rapid proliferative recall responses that help to amplify and replenish the response during secondary antigenic exposures[13),14)]. T_{EM} cells can migrate to nonlymphoid organs and immediately produce effector cytokines and cytotoxic proteins following reactivation but are less proliferative[15)-17)]. The ability of T_{EM} cells may be vital for the control of certain chronic pathogens, such as simian immunodeficiency virus and malaria, before the infection is fully established[18)-21)].

The intestinal mucosa acts as a major site for exposure to potential microbial invaders, and mucosal tissues are able to rapidly respond to insults by generating regulated and robust immunity. For most intracellular bacterial infections, generating appropriate T cell responses is ultimately necessary for successful elimination of the pathogen. For *Listeria monocytogenes* (*L. monocytogenes*) infections, sterilizing immunity requires a robust T cell response capable of providing common effector functions and facilitating lysis of infected cells. Intestinal resident memory $CD8^+$ T cells provide important protective functions in response to *L. monocytogenes* oral infection[22]. After infection, induction of a protective T cell response includes mobilization of effector cells to peripheral tissues resulting in elimination of any remaining bastions of infection.

Our group previously found an IL-21 splicing variant transcript, IL-21 isoform, which is present in humans and mice[23]. This isoform activates STAT1 and STAT3 similar to conventional IL-21. However, this isoform is present on the cell surface and is secreted into the culture supernatant at very low levels[23),24)]. Since IL-21 transgenic mouse could not be established, we have established IL-21 isoform transgenic mice (IL-21isoTg) that expresses mouse IL-21 isoform in T cells to evaluate the function of IL-21 *in vivo*[25]. IL-21isoTg were shown to possess high amount of $CD8^+$ T cells in normal physiological condition[25]. On the other hand, $CD4^+$ but not $CD8^+$ T cell abnormality has been reported in IL-21 gene knockout mice[26),27)]. In this study, we infected the IL-21isoTg with the intracellular bacteria *L. monocytogenes* by oral route to investigate the function of IL-21 and IL-21-induced $CD8^+$ T cells in intracellular pathogen control.

Methods

Mice

C57BL/6J mice were purchased from CREA, Japan. IL-21isoTg were generated as described previously[25]. Mice were maintained in a 12-h light/dark cycle under specific pathogen-free conditions and had ad libitum access to a standard diet and water until reaching the desired age (6-10 weeks). All mice experiments were performed in accordance with the guidelines of the Laboratory Animal Center of the Yamagata University Faculty of Medicine and were approved by the animal experiment committee of the Yamagata University Faculty of Medicine (approval number: 31005).

Bacteria and bacterial stock preparation

L. monocytogenes strain EGDe expressing mutant internalin A (S129N and Y369S) (Lmo-$InIA^m$) was a kind gift from Prof. Dr. D. Heinz and Dr. Joop van den Heuvel[28]. To prepare the stock, bacteria were grown in Tryptic Soy broth for 17 hours. The bacteria were stored at −80℃ after the OD reached approximately 1.0. The bacteria were then grown on tryptic soy agar plate and the bacterial colonies were counted 24-48 hours after incubation. The desired inoculum of bacterial strain was mixed with PBS containing $CaCO_3$[28].

L. monocytogenes infection and determination of bacterial load in the organs

Mice, which were starved overnight and provided only with water, were intragastrically inoculated with the bacterial suspension using a 21-gauge feeding needle attached to a 1 mL syringe. Wild type (WT) and transgenic (Tg) mice were inoculated with a sublethal dose of bacteria (5×10^{11} CFU) for

primary infection. After 15 days of primary infection, mice were inoculated with bacteria (1×10^{12} CFU) for secondary challenge infection. After 3 days of primary or secondary infection, mice were sacrificed to collect spleen, liver, mesenteric lymph nodes (mLN) and small intestine. The small intestines were removed and incubated in PBS supplemented with 100 μg/mL gentamicin sulfate, to kill extracellular bacteria, for 2 hours at room temperature. Other organs were dissected under sterile conditions. Organs were homogenized and serial dilutions of the homogenates were plated onto tryptic soy agar plates. After 24-36 hours of incubation, colony numbers were counted to determine the bacterial load in each organ.

Cell staining and flow cytometry analysis

Single-cell suspensions of spleen and mLN were prepared using a glass homogenizer before and after 10 days of primary infection. RBCs in the spleen cells were lysed and then, spleen and mLN cells were washed twice with PBS. Spleen and mLN cells were incubated with V450-anti-mouse CD3 (BD Biosciences, Clone # 17A2), FITC-anti-mouse CD4 (BioLegend, Clone # RM4-5), PE/Cy7-anti-mouse CD8α (BioLegend, Clone # 53-67), APC-anti-mouse CD62L (BioLegend, Clone # 104411) and PE-anti-mouse CD44 (BD Pharmingen, Clone # IM7) antibodies on ice for 30 minutes in 3% FCS-PBS with 0.02 % NaN_3. Cells were washed with the same buffer and then analyzed by FACSCanto II flow cytometer (BD Biosciences). Data were analyzed by FlowJo software (version 7.6.1, Tree Star).

Statistical Analysis

The differences between the bacterial loads in the organs of WT and IL-21isoTg were analyzed by two-way ANOVA with Bonferroni post hoc test. The statistical significance of cell numbers in the organs of infected and non-infected mice was analyzed by one-way ANOVA with Tukey post hoc test. Data were analyzed using the GraphPad Prism Software version 5.03 (GraphPad Software, San Diego, CA, USA). Results with $p < 0.05$ were considered as statistically significant.

Results

Bacterial load was reduced in IL-21isoTg after secondary challenge infection

IL-21isoTg showed higher percentage of $CD8^+$ T cells in spleen, peripheral LN, and mLN than WT mice in normal physiological conditions[25]. $CD8^+$ T cells are important for removing intracellular microorganisms such as *L. monocytogenes* and protecting our body. To test the function of excessive, self-producing $CD8^+$ T cells, we infected WT and IL-21isoTg with *L. monocytogenes* orally. After 3 days of infection, mice were sacrificed and the bacterial loads of the spleen, liver, and mLN were evaluated. There were no differences in bacterial load in these organs between WT and IL-21isoTg (Figure 1a). This result indicates that the self-producing $CD8^+$ T cells in IL-21isoTg are not effective in controlling *L. monocytogenes* during primary infection. After 15 days of primary infection, we challenged the mice with *L. monocytogenes*. In secondary infection, the IL-21isoTg exhibited significantly reduced bacterial load in these organs compared to WT mice (Figure 1b).

$CD8^+$ T_{EM} cells are increased after L. monocytogenes infection in IL-21isoTg

Before infection, we confirmed that the percentage of $CD8^+$ T cells in spleen and mLN were significantly higher in IL-21isoTg than WT mice (Figures 2a and b).

The percentage of $CD44^+CD62L^-CD8^+$ T_{EM} cells and $CD44^-CD62L^+CD8^+$ naïve T cells were not different in these organs between WT and IL-21isoTg. On the other hand, percentage of $CD4^+$ T cells was lower in mLN of IL-21isoTg than WT mice. After 10 days of primary infection, lymphocytes were isolated from spleen and mLN. The percentage of $CD8^+$ T cells in these organs of IL-21isoTg was still higher than that of WT mice. Interestingly, the percentage of $CD44^+CD62L^-CD8^+$ T_{EM} cells was significantly increased in IL-21isoTg after infection and was considerably higher than that in WT mice. On the other hand, the percentage of $CD4^+$ T_{EM} cells tended to increase after infection in both organs in both mice; however, these changes were not significant.

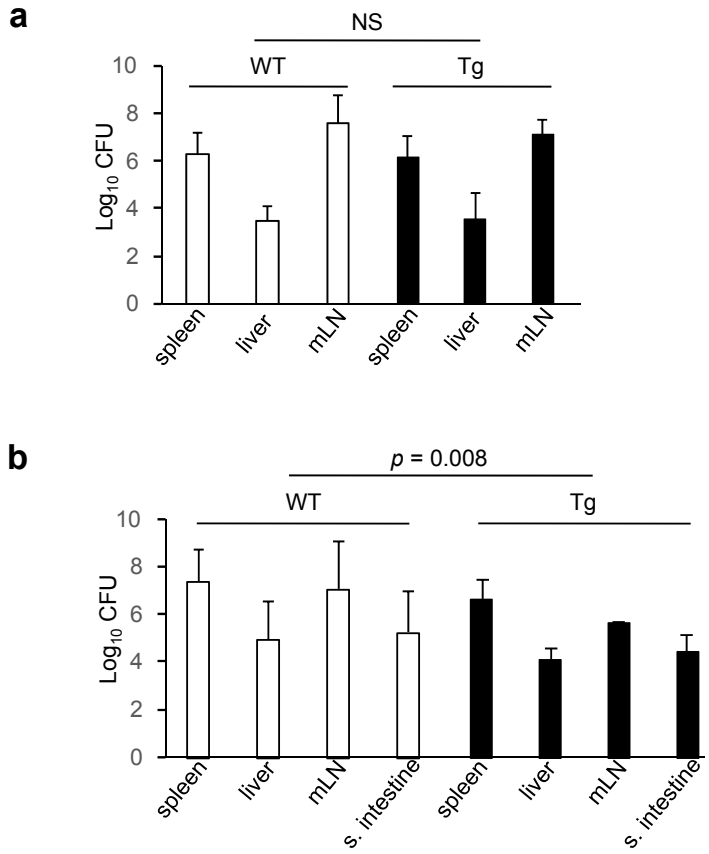

Figure 1. IL-21isoTg exhibited reduced bacterial load after *L. monocytogenes* challenge infection
Bacterial loads of each organ after primary (a) and secondary infection (b) are shown. Values are represented as mean ± SD (n=5 for WT and n=7 for Tg (a) and n=7-9 for WT and n=5-9 for Tg (b)). Data were analyzed by two-way ANOVA with Bonferroni post hoc test where $p < 0.05$ is statistically significant. NS: not significant.

Discussion

The functional significance of IL-21 in regulating $CD8^+$ T cell responses is highlighted by its essential role in sustaining antiviral $CD8^+$ T cells during chronic lymphocytic choriomeningitis viral infection[7)-9)]. Additionally, IL-21 cooperates with IL-10 to promote the maturation of memory $CD8^+$ T cells via the transcription factor STAT3[6)]. During certain infections, IL-21 is also required for the generation of effector $CD8^+$ T cells[29)] and for the optimal recall responses of memory $CD8^+$ T cells[30)-32)].

The IL-21isoTg express significantly high levels of $CD8^+$ T cells. However, these cells are neither effective nor adequate to reduce *L. monocytogenes* primary infection compared to WT mice. Interestingly, we found that $CD8^+$ T_{EM} cells were significantly increased after *L. monocytogenes* primary infection in IL-21isoTg compared to WT mice. After secondary challenge infection, the IL-21isoTg exhibited significantly reduced bacterial loads in organs compared to WT mice. It could be possible that the significantly high level of $CD8^+$ T_{EM} cells, generated after primary infection, efficiently reduced the bacterial load during secondary infection in IL-21isoTg, as reported in certain acute pathogenic infections[30),31)]. Factors other than $CD8^+$ T_{EM} cells may also play a role in controlling *L. monocytogenes* infection. Further studies are required to clarify the function of IL-21 in the intracellular bacterial infection. In this study, we demonstrated for the first time that IL-21 and IL-21-induced $CD8^+$ T_{EM} cells have an important function for controlling *L.*

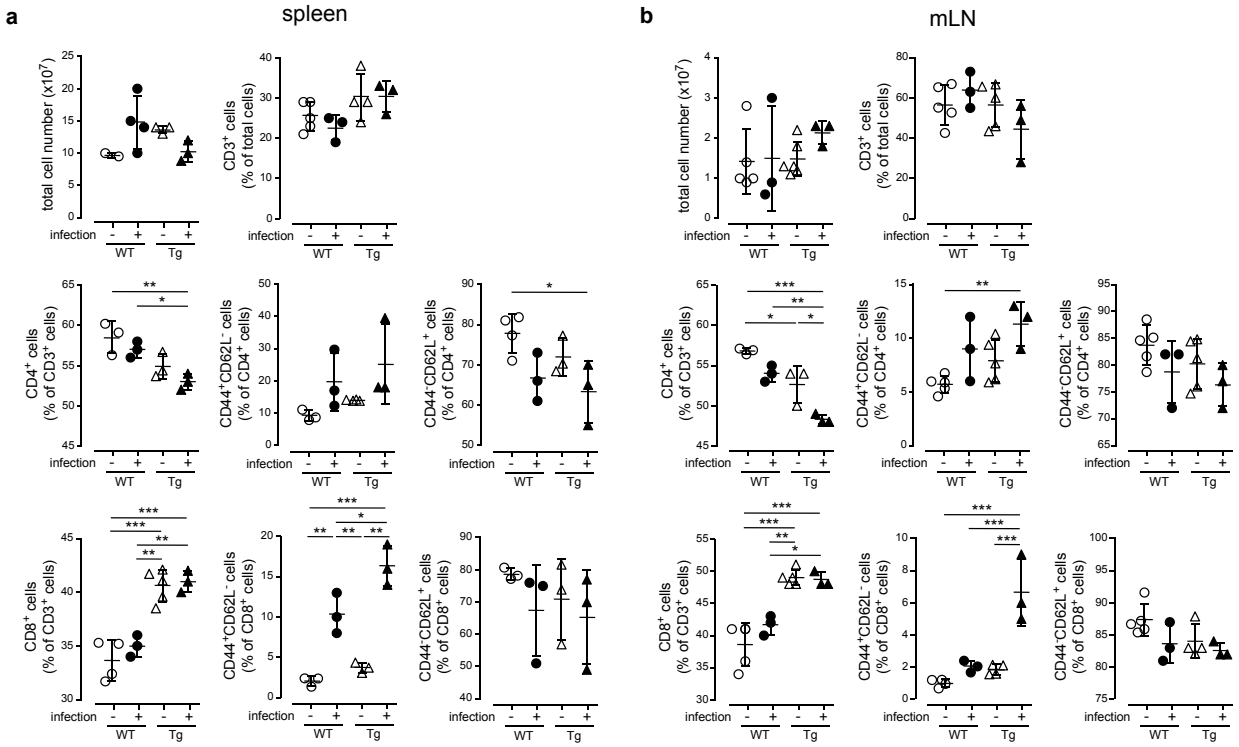

Figure 2. IL-21isoTg express high numbers of $CD8^+$ T_{EM} cells after *L. monocytogenes* infection
IL-21isoTg and WT mice were sacrificed before and 10 days after *L. monocytogenes* infection. Spleen (a) and mLN (b) were collected and cells were stained for detecting the expression of lymphocyte markers. Scatter plots showing the total cell number or percentage of each cell population (n= 3-5 for WT and n=3-5 for Tg). Data were analyzed by one-way ANOVA with Tukey post hoc test where $p < 0.05$ is statistically significant. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

monocytogenes infection.

Ethics

We don't use human materials or information.

Conflicts of interest

The authors declare that they have no competing interests.

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Abbreviations

IL-21, Interleukin-21; IL-21isoTg, IL-21 isoform transgenic; *L. monocytogenes, Listeria monocytogenes;* mLN, mesenteric lymph nodes; T_{CM}, central memory T cell; T_{EM}, effector memory T cell; WT, wild type.

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