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Attenuation of regulatory T cell function by type I IFN signaling in an MDA5 gain-of-function mutant mouse model

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ABSTRACT

Melanoma differentiation-associated gene 5 (MDA5) is an essential viral double-stranded RNA sensor to trigger antiviral immune responses, including type I interferon (IFN) induction. Aberrant activation of this viral sensor is known to cause autoimmune diseases designated as type I interferonopathies. However, the cell types responsible for these diseases and the molecular mechanisms behind their onset and development are still largely unknown. In this study, we revealed the attenuation of regulatory T cell (Treg) function by type I IFN signaling in a mouse model expressing a gain-of-function MDA5 G821S mutant. We found that experimental colitis induced by adoptive transfer of naïve T cells in *Rag2*$^{-/-}$ mice was rescued by simultaneous transfer of Tregs from wild-type but not from the MDA5 mutant mice. Type I IFN receptor deficiency in the MDA5 mutant mice recovered the suppressive function of MDA5 mutant Tregs. These results suggest that constitutive MDA5 and type I IFN signaling in Tregs decreases the suppressive function of Tregs, potentially contributing to the onset and exacerbation of autoimmune disorders in interferonopathies.

1. Introduction

Regulatory T cells (Tregs) play an essential role in the maintenance of self-tolerance and also the control of excessive immune responses [1]. The Forkhead-Box-Protein P3 (Foxp3) regulates Treg differentiation and immune suppressive functions. To control the inflammatory environment by suppressing the activation and expansion of a variety of immune cells, Tregs use several mechanisms, including anti-inflammatory cytokine secretion such as IL-10 and inhibition of costimulation by CTLA-4 [2,3].

Recent studies have investigated the effect of antiviral cytokine type I IFNs on Treg function by various experimental models of autoimmunity, viral infection and tumor [4]. One study demonstrated in an autoimmune mouse model that chronic IFN signature, due to loss of the DNA exonuclease Trex1, inhibits the proliferation and activation of Tregs. However, this inhibition does not contribute to the pathogenesis of inflammatory diseases [5]. In contrast, another study demonstrated that IFNAR signaling in Tregs also down-regulates the suppressive activity of Tregs, which is beneficial for antiviral and anti-tumor responses [6]. Type I IFNs are clearly shown to be potent in downregulating Treg function. However, the underlying mechanisms including effective timing and extent of type I IFN expression as well as the general or case-specific contribution of the reduced Treg activity to the pathogenesis, antiviral function and tumorigenesis remain to be clarified.

Previously, we reported a chronic IFN signature and various autoimmune disorders in mice with a constitutively active mutant of the viral sensor MDA5 (designated as MDA5$^{G821S/+}$ mice) [7–9], which had been obtained by ENU mutagenesis. We herein investigated the effect of chronic type I IFN production on Treg function in this autoimmune mouse model. Utilizing the experimental colitis model by adaptive transfer of naïve and regulatory T cells into RAG-2 knockout mice, we demonstrate the attenuation of Treg suppressive function in the mutant mice by type I IFN signaling.

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2. Materials and methods

2.1. Mice

MDA5$^{G821S/+}$ mice were generated and propagated by *in vitro* fertilization as reported [7]. *Rag2*$^{-/-}$ and *Ifnar1*$^{-/-}$ mice were purchased from B&K Universal. Mice used in these studies were 10–15 weeks old. All mice were maintained in the specific pathogen-free facility of the University of Kyoto and handled in accordance with the institutional guidelines for animal care and use.

2.2. Cell isolation

Single-cell suspensions from spleens were obtained by passing the organs through a 70 μm nylon mesh in phosphate buffered saline (PBS, Nacalai) containing 10%FBS (Gibgo) and 2 mM EDTA (Gibco). Then red blood cells were eliminated with ACK lysis buffer (Gibgo).

2.3. Cell sorting and adoptive transfer experiments

For adoptive transfer experiments we sorted the CD4^{+}T cell subpopulation from WT mice. Splenocytes were first separated by using CD4 negative selection MicroBeads (Miltenyi Biotech). CD4^{+}T cells were stained with APC-*anti*-mouse CD4 (RM4-5, BioLegend) and PE-*anti*-mouse CD25 (PC61, BD bioscience). CD4^{+}CD25^{-} naïve T (Tn) cells and CD4^{+}CD25^{+} Treg cells were sorted using Sony SH800 sorters (purity>80%). *Rag2*$^{-/-}$ recipients were injected intravenously (IV) with 5×10^5 CD4^{+}CD25^{-} Tn cells and 5×10^5 CD4^{+}CD25^{hi} Treg cells and monitored.

2.4. Flow cytometry analysis

For surface staining, cells were resuspended in 100ul FACS buffer (10% FBS and 2 mM EDTA in PBS) and incubated with the respective antibodies for 15 min at 4 °C in the dark. Cell-surface staining was resuspended with the following conjugated anti-mouse Abs: CD4 (RM4-5, BioLegend), CD8 (53-6.7, BioLegend), CD25 (PC61, BD Bioscience). For intracellular staining of Foxp3, after the surface staining, cells were incubated in Transcription Factor Fixation and Permeabilization solution (Invitrogen) for 30 min at RT. Cells were then stained with Foxp3 antibody (MF-14, BioLegend) in the washing permeabilization buffer. Flow cytometry analyses were performed on FACS Verse and LSR Fortessa (BD Biosciences) and data were analyzed with FlowJo software (Tree Star, Inc).

2.5. Histological analysis

Tissues were fixed in 4% paraformaldehyde solution (Nacalai Tesque) and embedded in paraffin. Paraffin sections were cut into sections (3 μm) and stained with Hematoxylin-Eosin (Tissue-Tek). Histological analysis was performed on a BZ-X800E microscope (KEYENCE).

2.6. Statistical analysis

Statistical analyses were performed using the GraphPad Prism 9.0 software. Groups of data were compared using a Student T-test or an ANOVA. Data in bar and dot graphs means ± SDM. Significance is indicated as follows: *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001.

3. Result

3.1. Comparable Foxp3+ Treg population in CD4^{+}CD25^{+} cells between control wild-type and MDA5$^{G821S/+}$ mice

Previously, we reported various autoimmune disorders related to type I interferonopathy in mice with constitutively active MDA5 (designated MDA5$^{G821S/+}$ mice or GS), which had been obtained by ENU mutagenesis [7–9]. However, the contribution of individual cell types, especially T cells, to each autoimmune pathogenesis and the effect of constitutive MDA5 signaling in T cells still remain unclear. To investigate the possible alteration of the T cell population in MDA5$^{G821S/+}$ mice, we first analyzed the populations of CD4 and CD8 single or double positive splenic T cells. The percentages of CD8^{+} T cells, double positive and negative cells were comparable, while the percentages and cell numbers of CD4^{+} T cells were significantly reduced in the spleen of MDA5$^{G821/+}$ mice (Fig. 1A, B and C). CD25 expression is upregulated upon activation of CD4^{+} T cells, and is commonly used as a surface marker of Tregs. Interestingly, MDA5$^{G821S/+}$ mice exhibited a more than 4-fold increased percentage of CD4^{+}CD25^{+} cell population (Fig. 1D). Foxp3 is a transcription factor of Tregs that plays an important role in immune homeostasis and is a confirmed Treg marker [1]. To examine the Treg population in the CD4^{+}CD25^{+} cells, we analyzed the expression of Foxp3 in CD4^{+}CD25^{+} cells. The examined CD4^{+}CD25^{+} cells showed comparable Foxp3 positive population between wild-type (WT) and MDA5$^{G821S/+}$ mice (Fig. 1E). Although the total number of Foxp3^{+} Tregs were significantly reduced in MDA5$^{G821S/+}$ mice due to the decreased total numbers of CD4^{+} T cells (Fig. 1C and F). These data suggest the potential usage of these CD4^{+}CD25^{+} cells for the comparison of Treg population in both WT and MDA5$^{G821S/+}$ mice.

3.2. Tregs from MDA5$^{G821S/+}$ mice fail to prevent the colitis in Rag2$^{-/-}$ mice induced by adoptive transfer of naïve CD4^{+} T cells from WT mice

To examine the suppressive function of Tregs in MDA5$^{G821S/+}$ mice, we utilized the CD4^{+}CD25^{+} cell population as a source for Tregs and also an experimental colitis model that transfers naïve CD4^{+} T cells into *Rag2*$^{-/-}$ mice [12]. In this model, the adoptive transfer of CD4^{+}CD25^{-} naïve T cells (Tn) into *Rag2*$^{-/-}$ mice induces inflammatory colitis, while co-transfer of Tn and CD4^{+}CD25^{+} Tregs can prevent colitis [12]. As shown in Fig. 2A, we injected WT Tn into *Rag2*$^{-/-}$ mice in the presence or absence of sorted CD4^{+}CD25^{+} Tregs from WT or MDA5$^{G821S/+}$ mice with a purity of 80–90% (Fig. 2B). As reported previously, we confirmed that co-transfer of CD4^{+}CD25^{+} Tregs from WT mice prevented body weight loss related to the development of colitis (blue) (Fig. 2C). By contrast, in the case of co-transfer of WT Tn and CD4^{+}CD25^{+} Tregs from MDA5$^{G821S/+}$ mice (red), *Rag2*$^{-/-}$ recipient mice exhibited clinical signs of colitis, diarrhea, including hunching and showed a similar weight loss compared to mice with the transfer of only WT Tn (green) (Fig. 2C). Consistent with body weight data, histological analysis of colons revealed disrupted epithelial tissue in *Rag2*$^{-/-}$ recipient mice transferred with MDA5$^{G821S/+}$ Tregs (red) similarly to the case of the transfer of only WT Tn (green) (Fig. 2C and D). These results suggest that CD4^{+}CD25^{+} Tregs from MDA5$^{G821S/+}$ mice lost their suppressive function.

3.3. Type I IFN signaling is critical for the dysregulation of the Treg phenotype in MDA5$^{G821S/+}$ mice

To further investigate the contribution of the signaling from type I IFN receptor (IFNAR) to the dysfunction of MDA5$^{G821S/+}$ Tregs, we deleted IFNAR signaling by crossing MDA5$^{G821S/+}$ mice with

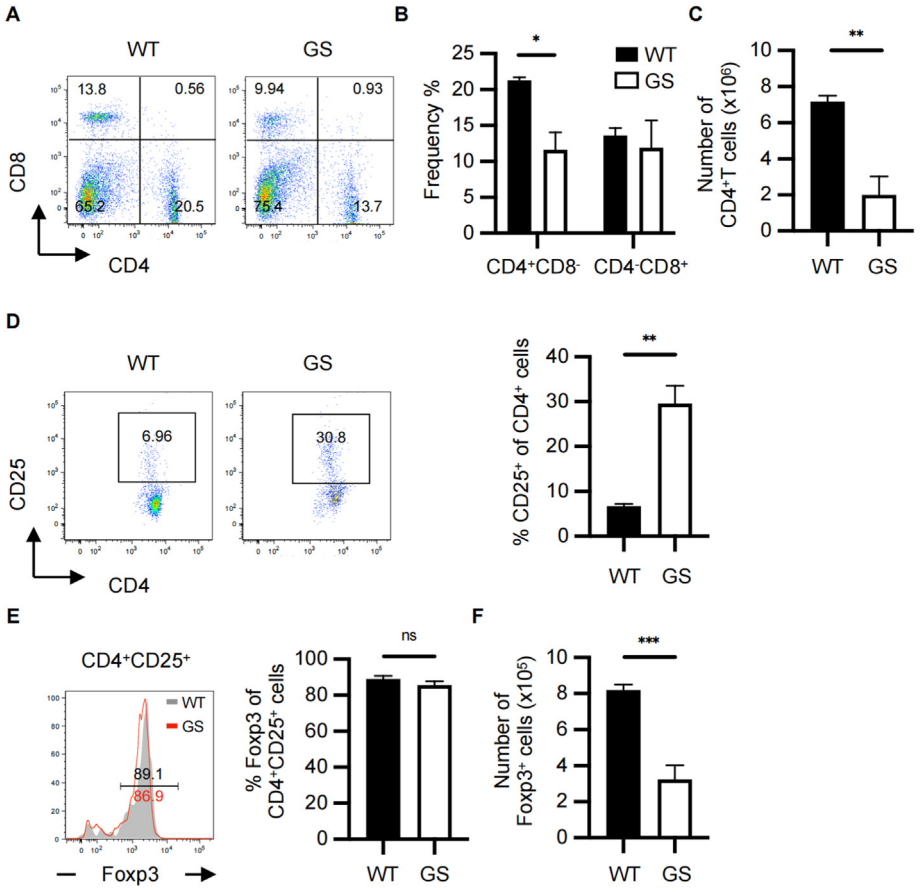

Fig. 1. Analysis of Treg population and total number in MDA5$^{G821S/+}$ mice
(A) Representative flow cytometric analysis of CD4 and CD8 expression of WT or MDA5$^{G821S/+}$ (GS) splenocytes. (B) Statistical analysis of (A) (n = 3). (C) Total CD4$^+$ T cell number of WT or GS splenocytes (n = 3). (D) Flow cytometric and statistical analysis of CD25 expression of CD4$^+$ T cells of WT or GS splenocytes (WT n = 4, GS n = 6). (E) Flow cytometric and statistical analysis of Foxp3 expression of WT or GS CD4$^+$CD25$^+$ T cells (n = 7). (F) Total number of CD4$^+$CD25$^+$Foxp3$^+$ cells. Bar graphs represent the mean ± SEM. Statistical significance was analyzed by the Student's *t*-test. *P < 0.05; **P < 0.01; ***P < 0.001.

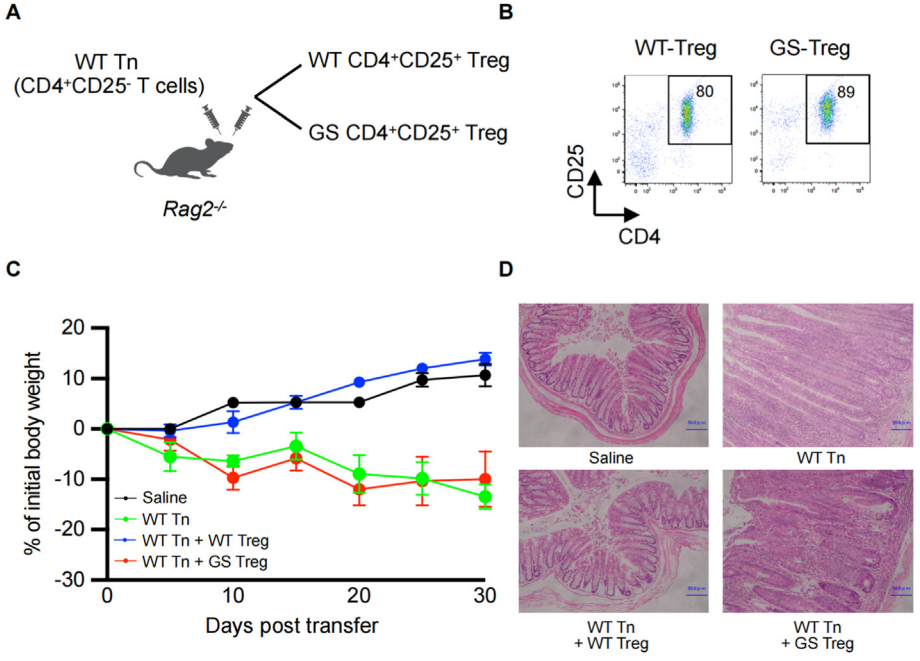

Fig. 2. CD4$^+$CD25$^+$ Tregs from MDA5$^{G821S/+}$ mice fail to prevent weight loss and colitis in *Rag2*$^{-/-}$ mice with adoptive transfer of Tn cells
(A) Diagram of the experimental design. CD4$^+$CD25$^-$ Tn cells from WT mice were transferred into *Rag2*$^{-/-}$ mice along with CD4$^+$CD25$^+$ Treg cells from WT or MDA5$^{G821S/+}$ mice. Control mice were injected with saline or Tn from WT mice. (B) Flow cytometric analysis of CD4$^+$CD25$^+$ expression in Tregs after sorting (purity 80–95%). (C) Body weight loss curves of *Rag2*$^{-/-}$ recipients. (Saline n = 3, WT Tn n = 4, WT Tn + WT Tregs n = 5, WT Tn + GS Tregs n = 5). (D) Representative H&E staining of the colon 30 days after adoptive transfer. Scale bar 50 μm.

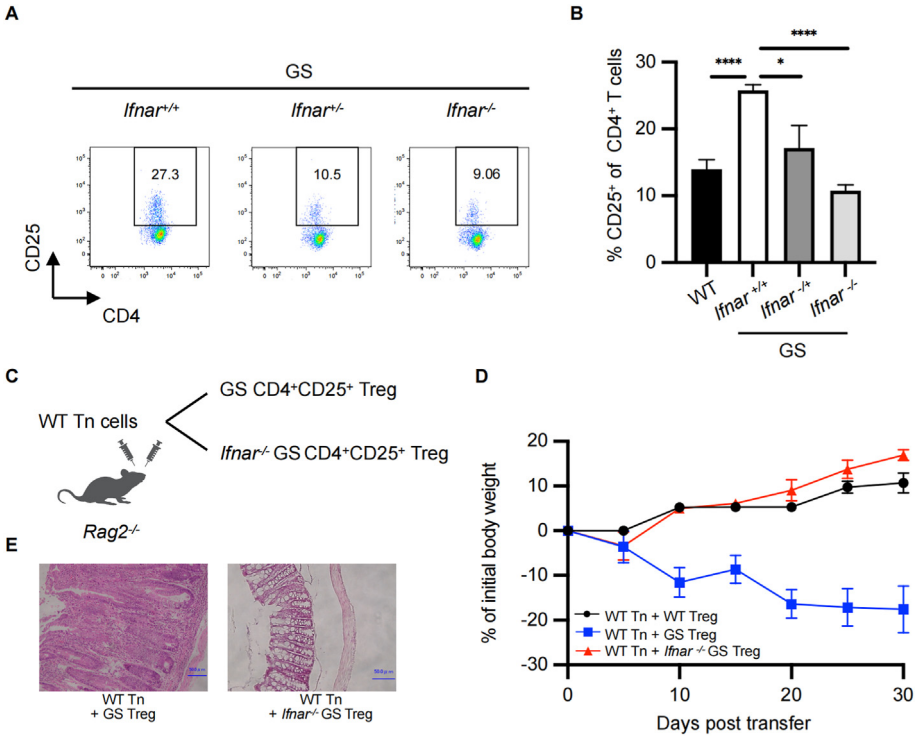

Fig. 3. Type I IFN signaling is critical for the dysregulation of Treg function in MDA5$^{G821S/+}$ mice.
(A) Representative flow cytometric analysis of CD25 expression of CD4$^+$ splenocytes isolated from *Ifnar*$^{+/+}$ MDA5$^{G821S/+}$, *Ifnar*$^{+/-}$ MDA5$^{G821S/+}$ and *Ifanr*$^{-/-}$ MDA5$^{G821S/+}$ mice (10-week-old). (B) Statistical analysis of (A) (*Ifnar*$^{+/+}$ WT n = 6, *Ifnar*$^{+/+}$ MDA5$^{G821S/+}$ n = 6, *Ifnar*$^{+/-}$ MDA5$^{G821S/+}$ n = 3 and *Ifanr*$^{-/-}$ MDA5$^{G821S/+}$ n = 3). (C) Diagram of the experimental design. CD4$^+$CD25$^-$ Tn from WT mice were injected into *Rag2*$^{-/-}$ mice along with sorted CD4$^+$CD25$^+$ Tregs from WT, *Ifnar*$^{+/+}$ MDA5$^{G821S/+}$ or *Ifanr*$^{-/-}$ MDA5$^{G821S/+}$ mice. (D) Body weight loss curves of *Rag2*$^{-/-}$ recipients of (C). (WT Tn + WT Tregs n = 3, WT Tn + GS Tregs n = 3, WT Tn + *Ifanr*$^{-/-}$ MDA5$^{G821S/+}$ Tregs n = 3) (E) Representative H&E staining of the colon of mice with the indicated cells 30 days after adoptive transfer. Scale bar 50 μm. Bar graphs represent the mean ± SEM. Statistical significance was analyzed by the Student's *t*-test. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001.

Ifnar$^{-/-}$ mice. Increased percentage of CD4$^+$CD25$^+$ Treg population in CD4$^+$ cells of MDA5$^{G821S/+}$ mice was normalized in *Ifnar*$^{-/-}$ MDA5$^{G821S/+}$ mice to the level of WT mice (Fig. 3A and B). To investigate the suppressive activity of *Ifnar*$^{-/-}$ MDA5$^{G821S/+}$ Tregs, we transferred CD4$^+$CD25$^+$ Tregs from *Ifnar*$^{+/+}$ MDA5$^{G821S/+}$ or *Ifnar*$^{-/-}$ MDA5$^{G821S/+}$ mice into *Rag2*$^{-/-}$ mice. Importantly, co-transfer of *Ifnar*$^{-/-}$ MDA5$^{G821S/+}$ Tregs (red) but not of *Ifnar*$^{+/+}$ MDA5$^{G821S/+}$ Tregs (blue) with WT Tn prevented the development of colitis and weight loss (Fig. 3C–E). Altogether, these data indicate that IFNAR signaling in Tregs of MDA5$^{G821S/+}$ disrupts the suppressive function and suggest that this potentially augments autoimmune symptoms.

4. Discussion

Utilizing an experimental colitis model, we demonstrate that Tregs in MDA5$^{G821S/+}$ mice have attenuated suppressive function compared to those in WT mice. Considering that this dysfunction of Tregs in mutant mice can be rescued by IFNAR deficiency, constitutive type I IFN signaling is responsible for the malfunction of Tregs. It is tempting to speculate that malfunction of Tregs contributes to excessive immune function of the patients of interferonopathy in general. In this regard, the clinical effect of JAK inhibitor could be partly restoring the normal function of Tregs.

MDA5 signaling is well known to induce not only type I IFN but also other inflammatory cytokines such as IL-6 and TNF-α. Although type I IFN is clearly the major factor, the contribution of other inflammatory cytokines to the Treg dysregulation in MDA5$^{G821S/+}$ mice is not strictly ruled out. In addition, in our mouse system, the sole contribution of MDA5 signaling was not investigated because of constitutive IFN production and the inflammation of the donor mouse (MDA5$^{G821S/+}$). Treg-specific expression of gain of function MDA5 mutant will delineate importance of the signaling by MDA5 or IFNAR for the Tregs inactivation.

Declaration of competing interest

The authors declare no competing interests.

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