Altered production of immunoregulatory cytokines by invariant Vα19 TCR-bearing cells dependent on the duration and intensity of TCR engagement

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Abstract

Cells bearing invariant Vα19-Jα33 TCR α chains are believed to participate in the regulation of inflammatory autoimmune diseases. In this study, the potential to produce immunoregulatory cytokines by these cells was characterized in order to find the mechanism underlying their immunoregulatory functions. Serum levels of IL-4, IL-10, transforming growth factor-β, IFN-γ and IL-17 increased in mice over-expressing an invariant Vα19-Jα33 TCR α transgene (Vα19 Tg) in response to anti-CD3 antibody injection. NK1.1+ Vα19 Tg+, but not NK1.1− Vα19 Tg− cells, promptly produced immunoregulatory IL-4, IFN-γ and IL-17 upon invariant TCR engagement with immobilized anti-CD3 antibody in culture. The activation of Vα19 Tg+ cells then triggered the production of pro-inflammatory cytokines by bystander cells. Interestingly, the ratio of Tα2 cytokines such as IL-4, IL-5 and IL-10, but not pro-inflammatory IL-17, to IFN-γ was increased when the intensity of the stimulation to invariant TCR was attenuated. Collectively, these findings suggest that invariant Vα19 TCR+ cells have the potential to participate in the regulation of inflammatory autoimmunity by producing Tα2-biased cytokines in certain circumstances.

Introduction

In adaptive immunity, antigen receptors expressed by T and B lymphocytes exhibit extensive diversity in their responses to different antigens. Besides those lymphocytes expressing diverse antigen receptors, several subsets of lymphocytes are characterized by a limited antigen receptor diversity, for instance, B1 B cells (1), certain lymphocytes are characterized by a limited antigen receptor diversity, for instance, B1 B cells (1), certain lymphocytes are characterized by a limited antigen receptor diversity, for instance, B1 B cells (1), certain lymphocytes are characterized by a limited antigen receptor diversity, for instance, B1 B cells (1) and that more than half of the hybrid line produced from NKT cells of CD1-deficient livers expressed this invariant TCR α chain (8). Recently, the localization of the invariant Vα19 TCR+ cells in gut lamina propria was demonstrated (9). These cells [designated as mucosal-associated invariant T (MAIT) cells] are suggested to control IgA production in the intestine (9). They are under restriction by one of the evolutionarily conserved MHC class Ib molecules, MHC-related protein 1 (MR1) (10). It is suggested that MAIT cell activation is ligand dependent, but the natural ligand associated with MR1 is not determined (11). It has been also reported that cells bearing invariant Vα7.2-Jα33 TCR α chains (corresponding to mouse Vα19-Jα33) but not Vα24-JαQ TCR α chains (corresponding to mouse Vα14-Jα18) accumulated in the lesions of multiple sclerosis patients (12).

Mice over-expressing an invariant Vα19-Jα33 TCR α Tg with a natural TCR α promoter generated invariant Vα19 Tg+ cells in gut lamina propria and other lymphoid organs including the liver (13–16). Invariant Vα19 TCR Tg+ cells...
produce immunoregulatory cytokines in response to TCR engagement (13–16). These cells are thought to regulate inflammatory autoimmune diseases (14). In the current study, cytokine production by Vα19 Tg+ cells was characterized in detail to find how these cells participate in the regulation of immune responses.

Materials and methods

Mice

C57BL/6 mice were purchased from Sankyo Service Co. (Tokyo, Japan). Beta2m-deficient mice, backcrossed with C57BL/6 mice for six generations, were obtained from Jackson Laboratory (Bar Harbor, ME, USA). CD1-deficient mice were provided by M. J. Grusby (Harvard University) (17). They were backcrossed with C57BL/6 mice for six generations, and mice with phenotypes H-2b, NK1.1+/CD1−/C0−/C0− were selected. TCR Ca-deficient mice, backcrossed with C57BL/6 mice for 10 generations (18), were provided by H. Ishikawa (Keio University) and M. Nanno (Yakult Co.).

Establishment of Vα19 Tg mice

A Vα19-Jα 33 Tg with the endogenous TCR α promoter and enhancer was injected into C57BL/6 and TCR Ca-deficient fertilized eggs, and Tg mouse lines were established (15). A Vα19Tg+ CD1−/− mouse line was established from one of the three Tg lines with the C57BL/6 background by crossing it with CD1-deficient mice. Vα19 Tg mice were compared with non-Tg mice in the same litter or with non-Tg mice with an appropriate genetic background (C57BL/6, 129/Sv or BALB/c) possibly included in each Tg line.

The experiments using mice were permitted by the Animal Experimental Committee of Mitsubishi Kagaku Institute of Life Sciences and performed along the guidelines of the committee.

In vivo stimulation of Vα19 Tg lymphocytes by TCR engagement

Mice of Vα19Tg+ CD1−/− and CD1−/− from the same litter and C57BL/6 genetic background (8 weeks of age) were intravenously injected with anti-CD3 antibody (2C11; PharMingen, San Diego, CA, USA; 1.5 μg per mouse) in 200 μl PBS. They were bled at various times after the injection, and the cytokine levels in serum were determined by ELISA. Cytokines in the supernatants were determined by ELISA.

In vitro stimulation of Vα19 Tg cells through TCR

Plastic culture plates were pre-coated with anti-CD3 antibody (2C11, PharMingen) at the indicated concentration in PBS, at 4°C for 16 h, and washed three times with DMEM. Mononuclear cells (MNCs) were isolated from the spleens and livers of mice (8–10 weeks of age) by density gradient centrifugation using Lymphosepar II (IBL, Gunma, Japan; d = 1.090) or Percoll (Pharmacia, Uppsala, Sweden) as described previously (19). They were cultured on the plates in DMEM (10% FCS), and cytokines in the culture supernatants...
were analyzed by ELISA. In some experiments, MNCs were separated according to the expression of NK1.1 and/or TCR \( \alpha \beta \) by a cell sorter (EPICS-Altra, Coulter Co., Hialeah, FL, USA) before culture. The purity of NK1.1\(^*\), TCR \( \alpha \beta \)\(^*\) and NK1.1\(^+\), TCR \( \alpha \beta \)\(^+\) fractions were in the range of 91–95% and 92–96%, respectively.

**Intracellular cytokine staining**

Spleen MNCs were cultured on a plate pre-coated with anti-CD3 antibody for 1–2 days. They were stimulated with phorbol myristate acetate (50 ng ml\(^{-1}\), Sigma) and ionomycin (750 ng ml\(^{-1}\), Sigma) in the presence of Golgi plug at the recommended concentration (PharMingen) for the next 5 h. Cells were stained with anti-NK1.1 and anti-TCR C\(\beta\) antibody injection, while this prompt cytokine production was accompanied with total TCR engagement. Liver MNCs were prepared from V\(\alpha\)19 Tg mice (V\(\alpha\)19 Tg mice) were produced (16). The development of NK1.1\(^+\) Tg\(^*\) [NK1.1\(^+\) V\(\alpha\)19-J\(\alpha\)33 invariant TCR \( \alpha \) cell (V\(\alpha\)19 NKT cell)] cells predominated in the liver and other lymphoid organs. For instance, V\(\alpha\)19 NKT cells share 29.5, 7.5 and 3.6% of MNCs in the liver, bone marrow and spleen of V\(\alpha\)19 Tg mice with TCR \( \alpha \)-deficient genetic background. Cells from these mice were used to characterize cytokine production by V\(\alpha\)19 Tg cells.

Serum levels of cytokines in V\(\alpha\)19 Tg\(^+\) and non-Tg mice were compared upon stimulation with anti-TCR antibody to identify the immunoregulatory cytokines in activated V\(\alpha\)19 TCR\(^*\) cells (Fig. 1). V\(\alpha\)19 Tg\(^*\) CD1\(^{1-}\), CD1-deficient (both lacking V\(\alpha\)19 NKT cells) and normal mice were injected with anti-CD3 antibody and bled at various time points. Production of T\(\gamma\)2-biased cytokines (IL-4, transforming growth factor-\(\beta\) and IL-10) was found in the C57BL/6 mice at 2 h after antibody injection, while this prompt cytokine production was reduced in CD1\(^{1-}\) mice. The reduction was eliminated by the introduction of V\(\alpha\)19 Tgs, as demonstrated by the cytokine levels in V\(\alpha\)19 Tg\(^*\) CD1\(^{1-}\) mice. In these mice, the restoration of T\(\gamma\)2 cytokine levels was more significant than that of IFN-\(\gamma\) levels. In addition, the kinetics of the decline in T\(\gamma\)2 cytokine levels was faster than that of IFN-\(\gamma\) levels in V\(\alpha\)19 Tg\(^*\) CD1\(^{1-}\) serum. Thus, over-generated V\(\alpha\)19 Tg\(^*\) cells in Tg mice may take the place of V\(\alpha\)14 NKT cells in C57BL/6 mice and are responsible for the prompt production of T\(\gamma\)2-biased immunoregulatory cytokines in response to TCR engagement.

The T\(\gamma\)2 cytokine production found in CD1\(^{1-}\) mice may be partially attributable to invariant V\(\alpha\)19 TCR\(^*\) cells.

**Results**

**Prompt T\(\gamma\)2-biased cytokine production and the subsequent pro-inflammatory cytokine production by V\(\alpha\)19 Tg cells upon TCR engagement**

An invariant V\(\alpha\)19-J\(\alpha\)33 TCR gene segment, isolated from a hybrid line (7), was cloned into a TCR \( \alpha \) vector containing the TCR \( \alpha \) endogenous promoter and enhancer, and Tg mice (V\(\alpha\)19 Tg mice) were produced (16). The development of NK1.1\(^+\) Tg\(^*\) [NK1.1\(^+\) V\(\alpha\)19-J\(\alpha\)33 invariant TCR \( \alpha \) cell (V\(\alpha\)19 NKT cell)] cells predominated in the liver and other lymphoid organs. For instance, V\(\alpha\)19 NKT cells share 29.5, 7.5 and 3.6% of MNCs in the liver, bone marrow and spleen of V\(\alpha\)19 Tg mice with TCR \( \alpha \)-deficient genetic background. Cells from these mice were used to characterize cytokine production by V\(\alpha\)19 Tg cells.

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**Cytokine determination by ELISA**

Cytokines were determined by ELISA using antibodies from PharMingen.

**Prompt T\(\gamma\)2-biased cytokine production by V\(\alpha\)19 Tg cells**

was followed by pro-inflammatory-biased cytokine production as shown by the serum levels of IFN-\(\gamma\) and IL-17 at 6 h and the later time points. The rise in IL-17 levels was more significant in V\(\alpha\)19 Tg than in non-Tg mice.

The temporal change in the cytokine profiles of V\(\alpha\)19 Tg\(^+\) cells was further demonstrated in the analysis of those cells in vitro upon TCR cross-linking (Fig. 2). NK1.1\(^+\) and NK1.1\(^-\) T cells prepared from V\(\alpha\)19 Tg\(^+\) TCR \( \alpha \)-/\( \beta \)- and normal mouse livers accompanied with total TCR \( \alpha \beta \)\(^+\) cells from \( \beta \)2m\(^{-}\) livers (lacking both V\(\alpha\)19 and V\(\alpha\)14 NKT cell population) were cultured in wells pre-coated with anti-CD3 antibody (10 \( \mu \)g ml\(^{-1}\)) and the cytokine production by them was determined. NK1.1\(^+\) T cells, but not NK1.1\(^-\) T cells, isolated from both strains of mice promptly produced a significant amount of IL-4, while the cells in each fraction continuously produced IFN-\(\gamma\) until the second day of culture. Similar profiles of cytokine production were observed in the culture of NK1.1\(^+\) and
NK1.1$^+$ spleen cells from Va$^+$ Tg$^+$ and non-Tg mice (data not shown). These observations strongly suggest that NK1.1$^+$ invariant Va$^+$ TCR$^+$ cells in Tg mice as well as Va$^{14}$ NKT cells in wild-type mice (20) are responsible for early-phase T$_r$2-dominant and subsequent pro-inflammatory cytokine secretion following antigenic stimulation.

Next, Tg and non-Tg cells were analyzed for intracellular cytokine production to identify the producer of each cytokine after TCR engagement. Intracellular cytokine staining patterns in spleen TCR$^{ab}$+ cells of Tg and non-Tg mice are shown in Fig. 3. IL-4, IL-17 and IFN-$\gamma$ were mainly produced by NK1.1$^+$ cells on day 1 of culture, whereas these cytokines were produced by NK1.1$^+$ cells on day 2 of culture in both Tg and non-Tg cells. These findings support the findings in Fig. 2 that T$_r$2-dominant cytokine production by the TCR engaged NK1.1$^+$ Va$^{19}$ TCR$^+$ (Va$^{19}$ NKT) cells in Tg mice or presumably Va$^{14}$ NKT cells in non-Tg mice is followed by the pro-inflammatory cytokine secretion by conventional T cells.

**Altered profiles of cytokine production by Va$^{19}$ Tg$^+$ cells depending on the intensity of TCR engagement**

Although the potent immunoregulatory function of Va$^{19}$ Tg$^+$ cells toward inflammatory diseases is accounted for by their early-phase T$_r$2-biased cytokine production, it is not in accord with the subsequent pro-inflammatory-dominant cytokine production. To address this question, we examined cytokine production of Va$^{19}$ Tg$^+$ cells during different intensities of TCR engagement. MNCs were isolated from the livers of Va$^{19}$ Tg$^+$ TCR$^{a/-}$, C57BL/6 and $\beta$2m$^{-/-}$ mice (NK1.1$^+$ TCR$\alpha\beta^+$ cells share ca. 30, 26 and 0.5% and NK1.1$^+$ TCR$\alpha\beta^+$ cells share 19, 20 and 25% of liver MNCs of these mice) (16). These cells were cultured on a plate pre-coated with different concentrations of anti-CD3 antibody, and the cytokine production by these cells was determined. The profiles of cytokine production dependent on the dose of anti-CD3 antibody were observed (Fig. 4A). The Tg$^+$ and non-Tg cells, but not the $\beta$2m$^{-/-}$ cells, produced a significant amount of IL-4 on day 1 of culture, while the cells of all the strains produced larger amounts of cytokines other than IL-4 on the second day of culture. These observations are well in accord with those found in Fig. 2 and suggest that the major cytokine producers in both Tg$^+$ and non-Tg cells are NKT cells and T cells on day 1 and day 2 of culture, respectively. In addition, the Tg$^+$ and non-Tg cells continuously produced T$_r$2 cytokines (IL-4, IL-5 and IL-10) more than $\beta$2m$^{-/-}$ cells until day 2 of culture; the production was saturated before reaching the maximum dose of anti-CD3 antibody. The relative production of each cytokine by Va$^{19}$ Tg$^+$ and B6 cells to the production by $\beta$2m$^{-/-}$ cells was plotted along with the dose of anti-CD3 antibody to encompass the contribution by Va$^{19}$ and Va$^{14}$ NKT cells (Fig. 4B). In addition to the prompt IL-4 production proportional to the intensity of TCR engagement by Va$^{19}$ as well as Va$^{14}$ NKT cells, it is also suggested that both NKT cells, especially Va$^{19}$ NKT cells, have the potential to continuously produce T$_r$2-dominant cytokines after TCR engagement within an appropriate range of intensity.
Fig. 4. Continued.
Discussion

Cells in Vα19 Tg* mice promptly secrete T\(_{\text{h}2}\)-dominant and then pro-inflammatory cytokines following TCR stimulation \textit{in vivo} (Fig. 1). The altered cytokine profiles of NK1.1\(^+\) Vα19 Tg* cells along with the duration of TCR stimulation become clear when these cells are isolated and stimulated in culture with anti-TCR-CD3 antibody (Fig. 2). Similar kinetics of cytokine production by NK1.1\(^+\) Vα19 Tg* cells was suggested by the intracellular cytokine staining experiments (Fig. 3) and the bulk culture of liver MNCs (Fig. 4). However, it remains possible that the delayed pro-inflammatory-dominant cytokine production found in the Tg liver cells in Fig. 4 is also ascribable to NK1.1\(^+\) Tg* following their own TCR engagement and/or the bystander cells other than NK1.1\(^+\) Tg* cells since pro-inflammatory-dominant cytokines from β2m\(^{-/-}\) cells were observed after prolonged TCR stimulation (Fig. 2).

Recently, Sakuishi \textit{et al.} (21) reported that the ratio of IL-5 to IFN-γ production by cells of human Vα24 invariant TCR-bearing lines was increased with a decrease in concentration of the anti-CD3 antibody used for stimulation. In accord with this report, we found T\(_{\text{h}2}\)-dominant cytokine production by invariant Vα19 TCR Tg* cells with the prolonged, attenuated stimulation to the invariant TCR (Fig. 4B).

The intensity of TCR engagement in invariant Vα19 TCR* cells may be controlled by antigen-presenting cells under the physiological conditions; these cells regulate the density of MR1 expression and select the antigens that they present. In Vα19 Tg mice, the disease progress of experimental autoimmune encephalomyelitis, an animal model for multiple sclerosis, was suppressed (15). On the other hand, accumulating evidence suggests that T\(_{\text{h}17}\) cells mediate certain inflammatory autoimmune diseases (22). In fact, invariant Vα19 TCR Tg* cells, especially those of the NK1.1\(^+\) subset, preferably produced IL-10, but not IL-17, after priming of mice with a partial peptide of myelin oligodendrocyte glycoprotein (15). Presumably, the intensity of TCR engagement in the Vα19 TCR* cells of these mice may be in the range suitable for the induction of regulatory rather than pro-inflammatory-dominant immune responses. In the present study, enhanced production of IL-17 was observed in cells from Vα19 Tg mice in response to polyclonal stimulation of TCR-bearing cells using anti-CD3 antibody as a TCR stimulator. Further studies were thus required to elucidate how Vα19 TCR* cells contribute to preventing the progress of inflammatory autoimmune diseases.

We have found that certain α-mannosylated glycolipids have the potential to specifically activate invariant Vα19 TCR* cells in an MR1-dependent manner (23, 24) and that some of these induce T\(_{\text{h}2}\)-biased immune responses (23). Taking into account these findings, it is possible to speculate that certain natural ligands have potential to induce T\(_{\text{h}2}\)-dominant immune responses from invariant Vα19 TCR* cells.

The production of immunoregulatory cytokines by a novel NK1.1\(^+\) T cell subset, Vα19 NKT cells, was characterized in the current study. Vα19 and Vα14 NKT cells partially share similar properties despite being subjected to the independent control of different antigen-presenting molecules. In addition, these two subsets are possibly involved differently in some immune responses. Thus, these NKT cell subsets are...
possible targets for immunotherapies using activators specific to each subset.

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Conflict of Interest: The authors have no financial conflicts of interest in this study.

Abbreviations
MAIT mucosal-associated invariant T
MNC mononuclear cell
MR1 MHC-related protein 1
Tg transgene or transgenic
Vα14 NKT NK1.1+ Vα14-Jα18 invariant TCR αβ
Vα19 NKT NK1.1+ Vα19-Jα33 invariant TCR αβ

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