Characterization of IL-17-producing T helper cells-like autoreactive T cells in aged mice

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Abstract: IL-17-producing T helper cells (Th17) are attracting attention as a new CD4-positive subset of T cells, reported to be responsible for various autoimmune diseases through stimulation of the release of inflammatory cytokines from target cells. However, most investigations of Th17 mediation of autoimmune diseases have focused on experimental autoimmune models derived from young animals, with few studies that have analyzed physiological factors such as aging. The present study analyzed autoreactive T cells established in a syngeneic mixed lymphocyte culture (sMLC) from aged mice and examined their similarity with Th17. IL-17-producing autoreactive CD4-intermediate T cells were observed in the sMLC; these expressed several stem cell markers or an immunosuppressive receptor PD-1 on the cell surface and so seemed to be different to typical Th17 cells. RT-PCR analysis revealed that purified Th17-like cells also expressed \( \text{Il}17a \), \( \text{Il}17f \), \( \text{Il}23r \), \( \text{Rorc} \) and \( \text{Tdt} \) mRNA, but not \( \text{Rag}1 \) or \( \text{Rag}2 \) mRNA. These findings that it is likely that Th17-like cells are involved in autoimmune responses in aged mice.

Key words: aging, autoimmunity, cytokines, T cells, Th17 cells

Introduction

IL-17 family members 17A and 17F have been implicated in the host defense against extracellular bacteria [9, 16]. Importantly, it has also been observed that IL-17 was overexpressed in a variety of autoimmune and autoinflammatory syndromes, in both mice and humans [4, 15]. The identification of an IL-17-producing T helper cell (Th17) was therefore of considerable interest. Recently, Th17 have been widely recognized as a new CD4-positive subset of T-cells, and they have been reported to be responsible for various autoimmune diseases through stimulation of the release of inflammatory cytokines from IL-17 receptor-expressing target cells [2, 11, 19, 20]. However, most reports on the mediation of autoimmune diseases by Th17 have focused on experimental autoimmune models derived from young animals, such as experimental autoimmune encephalomyelitis and collagen-induced arthritis, and there has been little study of physiological factors such as aging.

We previously reported that \( \alpha\beta \) and \( \gamma\delta \) T cells from aged BALB/c mice increased in number after syngeneic mixed lymphocyte culture (sMLC), and that these responder cells recognized syngeneic but not allogeneic spleen cells and a syngeneic Meth A fibrosarcoma [6, 12, 13, 18]. In present study, the contribution of Th17 to the differentiation and maturation of autoreactive \( \alpha\beta \) or \( \gamma\delta \) T cells in aged mice was investigated by analyzing the autoreactive T cells from aged mice established in sMLC, and examining their similarity with Th17.

The findings of the present study confirmed that there were IL-17-producing CD4-intermediate T cells in the

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peripheral lymph nodes of aged mice, but the expression of several molecular markers suggested these cells were different from typical Th17 cells. These Th17-like cells were found to be highly likely involved in autoimmune responses in the aged mice, suggesting that treatments targeting Th17-like cells may be a promising option for treating autoimmune diseases in the future.

Materials and Methods

Mice

Female young BALB/c mice and retired breeder BALB/c mice were obtained from Japan SLC (Hama-matsu, Japan) and were used for experiments when aged 10 or 40 weeks, respectively. These mice had been bred under specific pathogen-free conditions in the animal facility of Kitasato University Medical Center. All the experiments were approved by the Institutional Animal Care and Use Committee for Kitasato University Medical Center (approval number: H19-002A), and the animals were treated in accordance with the Regulations for Animal Experiments in Kitasato University.

Antibodies and reagents

Biotin-conjugated CD8a (clone: 53-6.7) monoclonal antibodies were purchased from BD Pharmingen (San Diego, CA, USA). The following monoclonal antibodies were purchased from Biolegend (San Diego, CA, USA): fluorescein isothiocyanate (FITC)-conjugated anti-Ly-6A/E (Sca-1, clone:D7), FITC-conjugated anti-CD279 (PD-1, clone:29F.1A12), phycoerythrin (PE)-conjugated anti-IFN-γ (clone:XMG1.2), PE-conjugated anti-IL-17A (clone:TC11-18H10.1), biotin-conjugated anti-Ly-6A/E (clone:D7), biotin-conjugated CD117 (c-kit, clone: 2B8), PE-Cy5 (PC5)-conjugated anti-CD4 (clone: GK1.5), and PE-Cy5 (PC7)-conjugated anti-CD3ε (clone: 145-2C11). Brefeldin A, phorbol 12-myristate 13-acetate (PMA) and ionomycin were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Lymphocyte culture

T cell-enriched lymphocyte populations were obtained by passing peripheral lymph node cells, which were obtained from young or aged mice, through a sterile nylon wool column (Wako Pure Chemical Industries, Osaka, Japan). T cell-enriched lymph node cells from BALB/c mice (5 × 10^6 cells/ml) were cultured in culture dishes (BectonDickinson, Oxnard, MA, USA) with the same number of 33 Gy-irradiated syngeneic spleen cells in RPMI 1640 medium (Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Life Technologies) and antibiotics (100 IU/ml penicillin and 100 μg/ml streptomycin; Sigma-Aldrich). The cultures were incubated at 37°C in 5% CO2 in air for 1, 3, 5, 7, and 10 days. To analyze the proliferative response, the same culture were prepared in flat-bottomed 96-well culture plates (Corning Glass Works, Corning, NY, USA), and 6 h before harvesting, 1.85 × 10^4 Bq of [3H]-thymidine (Japan Radioisotope Association, Tokyo, Japan) was added to each well. The incorporation of the [3H]- thymidine was analyzed using standard scintillation counting.

Flow cytometric analysis

Cells producing interferon (IFN)-γ or IL-17 were detected by intracellular cytokine staining. In brief, the cells were harvested 2 h after treatment with Brefeldin A (10 μg/ml) and were then fixed in saline containing 2% paraformaldehyde (Wako Pure Chemical Industries). The fixed cells were stained with fluorescent anti-CD8a, Ly-6A/E (Sca-1), CD279 (PD-1), CD117 (c-kit), CD4, and CD3ε. After washing, cells were also stained with fluorescent antibodies in 0.1% saponin permeabilization buffer (Wako Pure Chemical Industries). The antibodies used were anti-IFN-γ and IL-17 A. After staining, the biotinylated antibodies were visualized with streptavidin-energy-coupled dye (Beckman Coulter, Fullerton, CA, USA). The stained cells were analyzed with a Cy-tomics FC500 flow cytometer (Beckman Coulter) and Flowjo software (Tree Star, Ashland, OR, USA).

ELISA

Cell culture supernatants from the sMLC were assayed using specific ELISA kits for IL-2, IFN-γ, and IL-17 (Biolegend).

Positive panning of Sca-1+c-kit+ Th17-like cells

Sca-1+c-kit+ Th17-like cells included in a sMLC sample after 5 days were separated using a Dynabeads FlowComp Flexi kit (Dynal Inc., Oslo, Norway), and conjugated with biotin-labeled anti-Ly-6A/E (Sca-1) or anti-CD117 (c-kit) monoclonal antibodies, according to the manufacturer’s instructions. The separated Sca-1+c-kit+ cells were washed three times with phosphate-buffered saline (PBS) at pH 7.4, and then adjusted a concentration of 1 × 10^5 cells/ml using PBS; these cells were
used for RNA extraction. CD4+ T cells from peripheral lymph nodes of the aged mice were prepared for use as a negative control. As a positive control, CD4+ T cells stimulated for 16 h with PMA (50 ng/ml)/ionomycin (1 µg/ml) were used.

Reverse transcription polymerase chain reaction analysis
Total RNA was extracted from the cells using an eZ1 RNA Cell Mini Kit (QIAGEN, Valencia, CA, USA). Total RNA was reverse transcribed with a random primer using Superscript reverse transcriptase (Life Technologies), according to the manufacturer’s instructions. One-tenth of the cDNA was amplified with Th17-related factors specific primers by PCR with 2.5 U of Taq DNA polymerase (Takara Bio., Kusatsu, Japan) in a total volume of 50 µl, according to the manufacturer’s instructions. The PCR cycles were allowed to run for 30 s at 94°C, followed by 30 s at 60°C and 30 s at 72°C. Before the first cycle, a denaturation step of 1 min at 94°C was included; and after 40 cycles, the extension was prolonged for 3 min at 72°C. Then 10-µl aliquots of each PCR product were electrophoresed through 3% Nusieve 3:1 agarose gel (Takara bio) and visualized by ethidium bromide staining. The primers used were as follows: mouse Il17a, forward, 5'-CTC CAG AAG GCC CTC AGA CTA C-3', and reverse, 5'-TGG AGG GCA GAC AAT TCT GAA TC-3'; Il17f, forward, 5'-GAG GAT AAC ACT GTG AGA GTT GAC-3', and reverse, 5'-GCT TGG TGG ACA ATG GGC TTG-3'; Il23r, 5'-TCa GTG CTA CAA TCT TCA GAG GAC A-3', and reverse, 5'-CAA CAT TCC TAG AGG ACA TCT GTC A-3'; Rag1, forward, 5'-AGG CCT GTG GAG CAA GGT A-3', and reverse, 5'-GCT CAG GGG CTT CAC-3'; Rag2, forward, 5'-TGC CAA AAT AAG AAA GAG GAT TTC AC-3', and reverse, 5'-GGG ACA TTT TGAT GAT GAG AGG-3'; terminal deoxynucleotidyl transferase (Tdt), 5'-TGG GGA GAC ATC AGC TTG TT-3', and reverse, 5'-GCC AAG CCA AAC GGG TCA TCA-3', and reverse, 5'-CAT ATT TCT CGT GGT TCA CAC C-3'. The Universal ProbeLibrary Probes used were as follows: mouse Rag1, #46; Rag2, #4; Tdt, #62; and Gapgh, #29.

Statistical analysis
Statistical significance was evaluated with unpaired Student’s t-test using GraphPad Prism 6 (GraphPad Software Inc., La Jolla, CA, USA). P values <0.05 were considered statistically significant.

Results

Autoreactive T cells are present in the peripheral lymph nodes of aged mice
First, we established sMLC to clarify whether autoreactive cells were present in the peripheral lymph nodes of aged mice, examining the proliferation of autoreactive cells with the incorporation of [3H]-thymidine as an indicator. The [3H]-thymidine incorporation assay showed that lymph node cells derived from the peripheral lymph nodes of young mice did not proliferate in sMLC (Fig. 1A), but lymph node cells derived from aged mice proliferated in the time dependence as a peak during the fifth day from the start of the culture as the cells reacted to the animal’s own splenic cells (Fig. 1B). There was no sex difference and no influence from the bovine serum used for the cell culture. Our past research indicated that almost all the lymphocytes that proliferated after reacting to autologous splenic cells were T cells [6, 12, 13, 18]; thus, the present findings confirmed the presence of autoreactive T cells in the peripheral lymph nodes of aged mice.

The autoreactive cells are IL-17-producing CD4-intermediate T cells
Next, we analyzed the autoreactive cells amplified by the sMLC by intracellular cytokine staining using flow cytometric analysis. Characteristic IL-17-producing T cells with lower expression of CD4 than normal CD4-
positive T cells were detected as a peak during the seventh day after the start of sMLC (Fig. 2A). IFN-γ-producing T cells were also detected, as with IL-17-producing T cells. Furthermore, we detected IL-17 in cell culture supernatants from the sMLC together, with a large amount of IL-2 (Fig. 2B). Taken together, these findings showed that there were autoreactive IL-17-producing CD4-intermediate T cells amplified by sMLC in the peripheral lymph nodes of aged mice.

The autoreactive IL-17-producing T cells differ from typical Th17 cells

The distinctive nature of the reduced expression of CD4 in the IL-17-producing autoreactive T cells suggested that these cells might have the characteristics of immature T cells. We therefore analyzed the expression of Sca-1 and c-kit, which are known to be hematopoietic stem cell markers, in the responder cells with the most proliferative activity 5 days after the start of the sMLC. Flow cytometric analysis revealed that most of the autoreactive IL-17-producing T cells expressed Sca-1 and c-kit (Fig. 3). We also investigated the expression of PD-1, an immunosuppressive receptor which has attracted attention in recent years [10, 21], and found that it was expressed in a high proportion of the autoreactive IL-17-producing T cells (Fig. 3). These findings showed that autoreactive IL-17-producing T cells in the peripheral lymph nodes of aged mice had properties that differed from those of typical Th17 cells. We considered them to be “Th17-like cells”.

The autoreactive Th17-like cells also express terminal deoxynucleotidyl transferase

Using a positive panning method, Th17-like cells were isolated by the expression of Sca-1 and c-kit, which acted as an indicator, using magnetic beads with the appropriate antibodies. We then used reverse transcription PCR to examine the expression of Il17a, Il17f, Il23r, and Rorc mRNA, which were characteristic of Th17. This analysis demonstrated that the autoreactive Th17-like cells expressed Il17a, Il17f, Il23r, and Rorc mRNA, and that they showed almost the same characteristics as typical Th17 (Fig. 4A). Next, qPCR was used to investigate the expression of recombination activating gene-1 (Rag1), Rag2, and the terminal deoxynucleotidyl transferase gene (Tdt) in the Th17-like cells; no expression of Rag1 and Rag2 mRNA was observed, but the expression of Tdt mRNA was increased by about 18-fold as compared with the level in unstimulated CD4+ T cells (Fig. 4B).

Discussion

Disruption of the immune tolerance to autologous cells can result in the elicitation of an autoimmune response; eventually, this may lead to the onset of an autoimmune disease. It has been assumed that autoimmune diseases are associated with several factors, including genetic predisposition, lifestyle habits, and the physiological stress that accompanies aging; it is also clear that the emergence of immune cells with acquired autoreactiv-
ity is deeply involved in the pathogenesis. Pathogenic T cells that acquired autoreactivity with aging, as a result of the breakdown of immune homeostasis, have been observed in vivo [1, 5, 7, 8]. Such autoreactive T cells are known to recognize heat shock protein, type-II collagen, and glucose-6-phosphate isomerase as autoantigen epitopes [22, 23, 25]. However, it is unclear why, despite their autoreactivity, these cells did not eliminate themselves via a host immune mechanism.

In this study, we found that IL-17-producing T cells...
Fig. 3. Autoreactive CD4-intermediate T cells amplified by a syngeneic mixed lymphocyte culture (sMLC) express the stem cell markers Sca-1 or c-kit and the immunosuppressive receptor PD-1. Cell surface expression of various molecular marker in autoreactive CD4-intermediate T cells from 5 days after starting the sMLC sample. The cells were stained with anti-CD3ε, anti-CD4, anti-IL-17, anti-Sca-1, anti-c-kit, and anti-PD-1 monoclonal antibodies, and were analyzed by flow cytometry. Data are representative of two independent experiments.

Fig. 4. Purified Sca-1+c-kit+ cells are Th17-like cells. Reverse transcriptase PCR analysis of Th17-related factor mRNA (A) and quantitative PCR analysis of Rag1, Rag2, and Tdt mRNA (B) in Sca-1+c-kit+ cells. Total RNA was extracted from Sca-1+c-kit+ cells purified by positive panning, and the expression of the target gene mRNA was detected by reverse transcriptase PCR or quantitative PCR. Data are presented as the means ± SD of triplicates, and are representative of two independent experiments. **P<0.05 by Student’s t-test.

Fig. 5. Schematic illustration showing in this study. IL-17-producing CD4-intermediate T cells amplified by a sMLC are present in the peripheral lymph nodes of aged mice. The autoreactive Th17-like cells express the stem cell markers Sca-1 or c-kit and the immunosuppressive receptor PD-1.
with autoreactivity were present in the peripheral lymph nodes of aged mice. It was considered that these cells are likely to be a “double producers” that produce not only IL-17 but also IFN-γ. These autoreactive IL-17-producing T cells were similar to typical Th17 cells in that they expressed Il17a, Il17f, Il23r; and Rorc at the mRNA level, as well as Sca-1, c-kit, PD-1, and Tdt (Fig. 5). Generally, on accumulation of oxidative stress that accompanies aging, self-antigens that do not exist on the cell surface in young individuals began to appear [3]. In addition, aging qualitatively and functionally changes all immune cells. Because the T cell producing function of the thymus declines at an early time-point, age-related changes are most likely to be exhibited by T cells. For this reason, the possibility of appearance of autoreactive T cells appearing which would have been eliminated in a younger individual also increases. The Th17-like cells that we discovered may be an example of this.

Previous reports have shown that natural helper cells, a type of innate lymphoid cells, also expressed Sca-1 or c-kit [14, 17]. Natural helper cells exist only in the fat-associated lymphoid clusters of the mesenterium, which have been shown to increase in number and size with aging. Similarities between the natural helper cells in the fat-associated lymphoid clusters and the autoreactive Th17-like cells will be investigated in future studies. Conversely, it has been reported that CD4+PD-1+ T cells, which completely lose their acquired immune responses with aging, increased in vivo [24]. Because CD4+PD-1+ T cells express the transcription factor C/EBPα, they are considered to be so-called “bad cells” that produce osteopontin, a known inflammatory cytokine. Immunosenescence is not caused by a decrease in the rate of mature T cells or a decrease in their function, but it may be caused by dilution of the function of the normal immune system through an increase in the proportion of these bad cells.

In summary, this study found Th17-like cells with autoreactivity in the peripheral lymph nodes of aged mice. These Th17-like cells may be a promising target for the treatment of autoimmune diseases that develop with aging.

Conflicts of Interest

The authors have no financial conflicts to declare.

Acknowledgments

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