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Potential of Th17 cytokines in aging process contributes to the development of colitis

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Summary

Th17 cells, which produce IL-17 and IL-22, promote autoimmunity in mice and have been implicated in the pathogenesis of autoimmune/inflammatory diseases in humans. However, the Th17 immune response in the aging process is still not clear. In the present study, we found that the induction of IL-17-producing CD4⁺ T cells was significantly increased in aged individuals compared with young healthy ones. The mRNA expression of IL-17, IL-17F, IL-22, and RORC2 was also significantly increased in aged people. Similar to humans, Th17 cells as well as mRNAs encoding IL-17, IL-22 and ROR γ t were dramatically elevated in naïve T cells from aged mouse compared to young ones. In addition, CD44 positive IL-17-producing CD4⁺ T cells were significantly higher in aged mice, suggesting that memory T cells are an important source of IL-17 production. Furthermore, the percentage of IL-17-producing CD4⁺ T cells generated in co-culture with dendritic cells from either aged or young mice did not show significant differences, suggesting that dendritic cells do not play a primary role in the elevation of Th17 cytokines in aged mouse cells. Importantly, transfer of CD4⁺CD45Rb^{hi} cells from aged mice induced more severe colitis in RAG^{-/-} mice compared to cells from young mice. Taken together, these results suggest that Th17 immune responses are elevated in aging humans and mice and may contribute to the increased development of inflammatory disorders in the elderly.

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Keywords

Th17 cell; Aging; IL-17; IL-22; T cell; Dendritic cell

Introduction

Th17 cells, which secrete interleukin 17 (IL-17) and IL-22, comprise a recently identified subset of CD4⁺ T cells that is distinct from the Th1 and Th2 subsets (1–4). The IL-17 cytokine family consists of several members (IL-17A–F), though only IL-17A and IL-17F, which are most similar in structure and receptor usage, are routinely studied in inflammation. IL-17 has been shown to be a primary mediator in several autoimmune and inflammatory diseases, including rheumatoid arthritis, multiple sclerosis, and inflammatory bowel diseases (5).

The factors regulating Th17 differentiation and function have been intensively studied. Stimulation with IL-6 and TGFβ induces the differentiation from naïve mouse CD4⁺ T cells *in vitro* (6–7), while human Th17 cell differentiation requires TGFβ, IL-1β and IL-6 (8–11). The orphan nuclear receptor RORγt has been identified as the key transcription factor involved in Th17 cell differentiation (12). The differentiation of Th17 cells is also regulated by several positive and negative feedback loops, such as recently described IL-21, IL-23R, IL-10 and IL-27. New studies demonstrate that IL-21 is important for Th17 cell development in both human and mice (11,13–14). We have found that IL-10 negatively regulates Th17 cell differentiation (15) and other studies have shown that IL-27 also plays a negative role in Th17 cell generation (16–17). In addition to RORγt, several other transcription factors including RORα, STAT3 and IRF4 are also involved in the development of Th17 cells (5,18–19).

The incidence of cancer, infectious diseases, and autoimmune disorders increases with age. In addition, aging is accompanied by a number of changes in immune functions, including decreased lymphocyte proliferative responses to both mitogens and antigens, reduced delayed type hypersensitivity reactions, and decreased antibody responses to vaccination and infection (20–22). It has been postulated that these age-related diseases can be explained partially by an overall dysregulation in immune function. Although the most consistent and dramatic age-related changes have been demonstrated in T cells, the causes of age-associated alterations in immune function have not been established. Given the integral role that cytokines play in regulating immune system both in homeostasis and during an immune response, the impact of age on cytokine production may well be pivotal in determining age-associated changes in immunity. Murine models of aging, as well as human studies, have demonstrated that there is an age-associated dysregulation in Th1 and Th2 cytokine synthesis. In both murine and human studies IL-2 and IL-2R expression consistently decreases with age (23–25). However, the dysregulation of the Th1 cytokine IFN-γ and the Th2 cytokines IL-4 and IL-5 in aged humans is not as clear. Some reports showed that these cytokines were increased in aged humans, while others showed decreased cytokine expression by aged human T cells (26–28). Goetzl and colleagues showed that spleen CD4⁺ T cells from aged mice produced higher IL-17 than cells from young mice (29). However, it is unclear whether this is also true in aged humans and nor is clear the biological function of enhanced Th17 response in aged mice.

We demonstrate here that the induction of Th17 cytokines is significantly elevated in both aged humans and mice. In addition, we found that memory T cells are an important cell type for the induction of IL-17, and transfer of CD4⁺CD45Rb^{hi} cells from aged mice induced

more severe colitis in RAG^{-/-} mice compared to cells from young mice, Our study provides an in-depth understanding of the induction and progression of inflammation in aged humans.

Materials and Methods

Human subject

15 healthy young donors (age 20–30) and 15 healthy aged donors (age 70–80) were recruited at Tongji Hospital, Wuhan, China. The donors were sex matched and they have no history of chronic disease. Blood samples were obtained after informed consent and with approval of the Clinical Research Ethics Board of the Tongji Medical College. All subjects did not smoke cigarettes or use illicit drugs, or suffer from diabetes, hypertension or metabolic disease. Peripheral blood mononuclear cells (PBMC) were isolated by centrifugation over a Ficoll-Hypaque gradient. The PBMCs were used for the following experiments.

Mice

C57BL/6 mice were purchased from the Jackson Laboratories (Bar Harbor, ME), and aged mice were obtained from the National Institute of Aging. We obtained OT-II mice from Dr. Mayer's lab in Mount Sinai school of Medicine. The mice were maintained in the barrier facility at the Mount Sinai School of Medicine according to IACUC guidelines.

Human T cell culture in vitro

Human CD4⁺ T cells were isolated from PBMCs with the human CD4⁺ T Cell Isolation Kit II (Miltenyi Biotec, Auburn, CA), and cultured in the presence of anti-CD3 and CD28 antibodies (0.5 µg/ml each). 72 h later, the cells were activated with PMA and ionomycin for another 6 hrs and harvested for RNA isolation or flow cytometric analysis. In addition, CD3⁺CD4⁺CD25⁻ T cells were sorted from PBMCs (BD FACSAria™ cell sorter). The purity (>98%) was confirmed by flow cytometry. 2×10⁴ sorted cells then were stimulated in 96-well plate in the presence or absence of anti-CD3 and CD28 antibodies (0.5 µg/ml each), IL-2 (25 U/ml) and all-trans-retinoic acid (100 nM; Sigma-Aldrich). 6 days later, cells were harvested for Foxp3 analysis.

Murine T cell differentiation

Naive CD4⁺ T cells (CD62L⁺CD44^{lo}) were prepared by fluorescence-activated cell sorting from spleens and lymph nodes of young and old mice. The sorted cells were primed for 96 hrs with anti-CD3 (10 µg/ml; 145-2C11; BD Biosciences) and soluble anti-CD28 (2 µg/ml; 37.51; BD Biosciences). IL-6 (20 ng/ml), human TGFβ (3 ng/ml), and IL-23 (6 ng/ml) (all from R&D Systems, Minneapolis, MN) were added to the culture for 4 days to drive the differentiation of Th17 cells. No cytokines were added in contro cultures. Cells were then re-stimulated with plate-bound anti-CD3 with PMA and ionomycin for 6 hrs.

Preparation of murine bone marrow-derived dendritic cells

Mouse bone marrow-derived dendritic cells were generated from bone marrow stem cells obtained from the femurs of the mice as described previously (30). After lysis of the red blood cells 1 × 10⁶ bone marrow cells were cultured in each well of 24 well plates with complete DMEM in the presence of GM-CSF (10 ng/ml) for 7 days.

ELISA

Cytokine secretion in the cell free supernatants was analyzed using ELISA kits from eBioscience and R&D systems.

Real-time RT-PCR

Cells were lysed in TRIzol reagent (Invitrogen) and total RNA was extracted according to the manufacturer's instructions. For real time RT-PCR assays, the cDNA sequences of all detected genes were retrieved from the NCBI database. Primers were designed using Oligo Primer Analysis 4.0 software and the sequences were blasted (<http://www.ncbi.nlm.nih.gov/BLAST/>). The primer sequences were as follows: human IL-17A, sense 5'-GAAGGCAGGAATCACAATCC-3', antisense 5'-TCAGGTTGACCATCACAGTC-3'; human IL-17F, sense 5'-GCTTGCCTTTCTGAGTGAG-3', antisense 5'-CTTCATACTACCTCCTGGC-3'; human IL-22, sense 5'-GAGAACTGTTCCACGGAG-3', antisense 5'-TGCTTAGCCTGTTGCTGAG-3'; human RORC2, sense 5'-CAAGGCTCAGTCATGA GAAC-3', antisense 5'-CCTCACAGGTGATAACCC-3'; human GAPDH, sense 5'-CCCTTCATTGACCTCAACTAC-3', antisense 5'-GGTGATGGGATTTCCATTG-3'; mouse IL-17, sense 5'-CTCCAGAAGGCCCTCAGACTAC-3', anti-sense, 5'-AGCTTTCCCTC-CGCATTGACACAG-3'; mouse IL-22, sense 5'-CATGCAGGAGGTGGTACCTT-3', anti-sense, 5'-CAGACGCAAGCATTCTCAG-3'; mouse ROR γ t, sense 5'-CCGCTGAGAGGGCTTCA-3', anti-sense, TGCAGGAGTAGGCCACATTACAC-3'; mouse ubiquitin, sense 5'-GTGGAGATTGTTGCACAACG-3', antisense 5'-CAGTG GATGCAGGGATGATGTTCTG-3'. 100 ng of total RNA was used for reverse transcription using Superscript II RNase H reverse transcriptase (Invitrogen) in a volume of 25 μ l. Then 2 μ l of cDNA was amplified with SYBR Green Universal PCR Mastermix (Bio-Rad, Richmond, CA) in duplicate. For sample analysis, the threshold was set based on the exponential phase of products, and a CT value for samples was determined. The resulting data were analyzed with the comparative CT method for relative gene expression quantification against the house keeping gene ubiquitin.

Intracellular staining

Brefeldin A was added into the cultured cells for an additional six hours prior to intracellular staining. Cells were fixed with IC Fixation Buffer (eBioscience), incubated with permeabilization buffer, and stained with FITC-conjugated anti-human IL-17 or FOXP3 antibody, or PE-anti-mouse IL-17, FITC-anti-mouse IFN- γ , and Cy5-anti-mouse CD4 antibodies. All fluorophore-conjugated antibodies and the corresponding isotypes were purchased from eBioscience. The stained cells were used for flow cytometric analysis.

T cell transfer colitis studies

For adoptive transfer study, purified CD4⁺CD45RB^{hi} T cells from young and old mice were injected intraperitoneally into *Rag1*^{-/-} recipients (5×10^5 cells per mouse in 200 μ l sterile PBS per injection). Mice were weighed every week throughout the course of the experiments. Mice were sacrificed and colon tissues were excised. Entire colon was fixed in 10% buffered formalin and stained with hematoxylin and eosin. The severity of colitis was evaluated by an experienced pathologist without prior knowledge of the type of the treatment. The degree of inflammation in the epithelium, submucosa, and muscularis propria was scored separately.

Statistical analysis

Statistical analysis was performed using Student's t-Test. *P* values <0.05 were considered significant.

Results

Th17 cytokines are elevated in aged humans

To analyze the effect of the aging process on the Th17 immune response, we purified CD4⁺ T cells from the peripheral blood of healthy aged humans (between 70 and 80 years old) and healthy young humans (between 20 and 30 years old). After stimulation with PMA and ionomycin, Th17 cell-associated cytokines IL-17A, IL-17F and IL-22 were significantly increased at the mRNA levels in the aged CD4⁺ T cell population (Figure 1A). We then quantified by FACS intracellular IL-17A in CD4⁺ T cells activated as above and found that the population of IL-17-producing cells was significantly increased in aged individuals (Figure 1B). ROR γ t is a transcription factor that is critical for the differentiation of mouse Th17 cells. RORC2 is the human homologue of mouse ROR γ t. We found that the increase in IL-17 mRNA and IL-17 expressing CD4⁺ T cells in aged individuals was accompanied by an increase in the mRNA for RORC2 (Figure 1C). Thus, there is intrinsic bias towards a Th17 immune response in aging process.

Th17 cytokines are enhanced in aged mice

To facilitate studies of the Th17 immune response in aging, murine models were used in our further studies. Naïve CD4⁺ T cells from aged mice (20 to 22 months old) and young mice (6 to 8 weeks old) were incubated *in vitro* with IL-23, IL-6, and TGF- β to promote Th17 differentiation. We found that CD4⁺ T cells from aged mice expressed significantly higher levels of IL-17 and IL-22 mRNA (Figure 2A) and secreted more IL-17 protein as determined by ELISA (Figure 2B), which was comparable to our finding in human cells (Figure 1). We then quantified intracellular IL-17 in CD4⁺ T cells differentiated under Th0 or Th17 conditions from aged and young mice. As expected, there were very limited IL-17-producing cells when cells were differentiated under Th0 conditions. However, under Th17-polarizing conditions, 31.9% of CD4⁺ T cells from aged mice were IL-17 positive compared to 12.6% from young mice (Figure 2C). Since CD8⁺ T cells also express IL-17 (31–32), we analyzed IL-17-producing CD8⁺ T cells in aged mice. Similarly, the population of IL-17 positive CD8⁺ T cells was significantly increased (Figure 2D). In agreement with results with human PBMC, qRT-PCR analysis showed that ROR γ t mRNA expression was also significantly increased by cells from aged mice (Figure 2E). Lamina propria lymphocytes (LPL) from aged mice differentiated under Th17 polarizing conditions also expressed significantly higher levels of IL-17 and IL-22 mRNA than LPL from young mice (Figure 2F). In summary, in aged mice the expression of IL-17 and IL-22 in CD4⁺ T cells cultured under Th17 differentiation conditions were both significantly enhanced.

Aging directly affects IL-17-producing CD4⁺ T cells

To determine if the effect of aging on the Th17 immune response is a result of an intrinsic property of aged T cells or the influence of antigen presenting cells, we co-cultured dendritic cells from aged and young mice with naïve CD4⁺ T cells derived from OTII mice (specific for OVA323-339), and activated with OVA for 72 hrs in the presence or absence of Th17 polarizing conditions. We found the percentage of IL-17-producing CD4⁺ T cells to be similar between the two co-cultures (Figure 3A). In addition, there was no significant difference between young or aged groups in the expression of IL-17, IL-22, and ROR γ t mRNA by real time RT-PCR (data not shown). These data suggest that the enhanced Th17 cells in aged mice are not due to antigen presenting cells. We also activated dendritic cells from both aged and young mice with LPS (1 μ g/ml) for 6 hrs and quantified cytokines by real time RT-PCR. The profile of cytokine expression including IL-6, TNF α , IL-12/23 p40, TGF- β , IL-27 p28, and EBI3 by dendritic cells from aged and young mice was similar (Figure 3B). However, the expression of IL-6 and TGF- β mRNA expression was

significantly enhanced in CD4⁺ T cells of aged mice. The results suggest that the aging process directly affects the differentiation of CD4⁺ T cells towards Th17 cells.

Memory T cells are an important cell type for IL-17 production

Phenotypical naïve T cells are present in the peripheral blood of elderly individuals, but their numbers dwindle and few of these cells express cell surface markers of recent thymic emigrants. Thus, we wondered whether such variation of naïve and memory T cells affected IL-17-producing cells during the aging process. For this purpose, spleen cells from both young and aged mice were analyzed by FACS and, as expected, we found that both CD62L⁺ naïve CD4⁺ and CD8⁺ T cells were significantly reduced in aged mice compared to young mice (Figure 4A, B). Conversely, CD44⁺ CD4⁺ and CD8⁺ memory T cells were greatly increased in aged mice (Figure 4A, B). These data are consistent with previous reports, suggesting that memory T cells are dominant in the T cell population of aged mice. Next we incubated spleen cells from both young and aged mice with IL-6, IL-23, and TGF- β for 4 days to force Th17 differentiation. We then quantified by FACS intracellular IL-17 in CD4⁺ T cells differentiated under Th0 or Th17 conditions from aged and young mice. We found that 7.2% of CD44⁺ CD4⁺ T cells were IL-17 positive compared to 3.2% of young mice (Figure 4C). Thus, the results suggest that memory T cells are an important cell source for IL-17 production.

Treg cells are significantly increased in aged humans and mice

Although there was an enhanced Th17 immune response in aged individuals, no clear signs of inflammatory disorders were observed in the clinic. It is possible that there may be a suppressive mechanism in aged population, perhaps due to an enhanced population of Treg cells. For this purpose, we sorted CD4⁺CD25⁻ T cells from PBMC isolated from 7 healthy young and 7 aged individuals. The CD4⁺CD25⁻ T cells were then incubated with anti-CD3 antibody, anti-CD28 antibody, IL-2, and all-trans-retinoic acid for 6 days to drive differentiation of Tregs. We found that by quantitative RT-PCR analysis that Foxp3 mRNA was clearly increased in both groups of cells after activation but that the expression of Foxp3 mRNA was significantly higher in cells from aged people than that of young people (Figure 5A). By FACS analysis we found that 37.6% of cells from aged people were Foxp3 positive compared with 20% from young people (Figure 5B). In addition, we also examined CD4⁺CD25⁺ cells in young and aged mice and found that CD4⁺CD25⁺ cells were increased in aged mice even without stimulation (Figure 5C). The mRNA expression of Foxp3 was similarly increased in TGF- β activated spleen cells from aged mice (Figure 5D). Taken together, these data suggest that Treg cells were comparably increased with age and that these might counteract the enhanced Th17 immune response.

Transfer of naïve T cells from aged mice induced more severe colitis in RAG^{-/-} mice

More and more evidence suggests that Th17 cells are critical pathogenic effector T cells involved in autoimmune and inflammatory diseases including inflammatory bowel disease (IBD) (33–35). To further assess the function of aging on Th17 cell differentiation *in vivo*, we performed adoptive transfer experiments using CD4⁺CD62L⁺CD45RB^{hi} cells from young mice and aged mice to induce colitis in recombination-activating gene 1 deficient (Rag1^{-/-}) mice. Rag1^{-/-} mice reconstituted with T cells from aged mice lost more weight than the control group. These results were associated with a more severe inflammatory cell infiltration in Rag1^{-/-} mice reconstituted with T cells from aged mice and significantly higher pathologic scores relative to the control group (6A, B, C, and data not shown). In addition, mice reconstituted with T cells from aged mice had a significantly higher expression of Th17 signature cytokines in colons than control mice. Thus, the results suggest that aging promotes an environment where it is easy to generate a pathologic Th17 immune response under the appropriate conditions/microenvironments.

Discussion

We report in this study that the Th17 immune response is elevated in both aged humans and mice. The percentage of IL-17-producing cells in the population of CD4⁺ T cells activated with PMA and ionomycin was significantly increased in aged healthy humans. In agreement with these results, the mRNAs encoding IL-17A, IL-17F, IL-22, and RORC2 were also significantly increased in aged humans. Similar to humans, Th17 cells were dramatically increased in aged mice, as were mRNAs encoding IL-17, IL-22, and ROR γ t. In addition, IL-17-producing CD8⁺ T cells were also significantly increased in aged mice. To investigate the contribution of antigen processing cells to the enhanced Th17 response in aging, we co-cultured naïve T cells from OT-II mice with dendritic cells from young or aged mice in the presence of OVA under Th17 differentiation conditions. We found that dendritic cells from both young and aged mice have a similar ability to induce a Th17 profile in naïve T cells, suggesting dendritic cells do not play a role in the elevated Th17 cells in aged mouse T cell cultures. Importantly, transfer of CD4⁺CD45Rb^{hi} cells from aged mice induced more severe colitis in RAG^{-/-} mice compared to cells from young mice. Taken together, these results suggest that a T cell intrinsic predisposition to develop a Th17 immune response in aged humans and mice and the enhanced Th17 immune response can contribute to the development of inflammatory disorders.

Th17 cells have been implicated in the pathogenesis of autoimmune and inflammatory diseases in both mice and humans. In patients with multiple sclerosis, *in situ* hybridization showed that significantly higher numbers of IL-17 mRNA-expressing cells were found in the cerebrospinal fluid (36). IL-17 has been detected in the sera and colonic biopsies of inflammatory bowel disease patients (37–38). In addition, Th17 cytokines are present in the sera and diseased tissues of patients with rheumatoid arthritis, scleroderma, and systemic lupus erythematosus (39–41). Murine models of human inflammatory diseases including experimental autoimmune encephalitis, collagen-induced arthritis, and colitis have clearly demonstrated a role for Th17 cells in disease pathology (5). Taken together, these results suggest that the Th17 cells are directly involved in the pathogenesis of autoimmune and chronic inflammatory diseases. Thus, an intricate understanding of Th17 cell development and function is critical for the development of effective therapies for these diseases.

It has been reported that the number and intrinsic helper function of naïve CD4⁺ T cells decrease with age and that CD4⁺ T cells from 22–24 month old mice are less effective at forming immune synapses with antigen presenting cells than cells from young mice (42). The increase in memory CD4⁺ T cells and concomitant decrease of naïve T cells in aged mice is in part due to the lymphoid environment as well as to reduced generation of naïve T cells in the thymus. In agreement with published results, we found that the numbers of CD62L⁺ CD4⁺ and CD8⁺ T cells were significantly decreased in aged mice. However, the population of CD44⁺ T cells was dramatically enlarged. Interestingly we found that naïve CD4⁺ T cells from aged mice under Th17 conditions generated significantly larger numbers of IL-17 producing cells compared to young mice, although the increase in CD44^{hi} IL-17-producing CD4⁺ T cells was only moderately increased in aged mice. Our results suggest that naïve CD4⁺ T cells in aged mice are biased towards Th17 effector T cells under appropriate conditions although the number of naïve T cells is decreasing with aging.

In addition to CD4⁺ T cells, other cell types including CD8⁺ T cells, $\gamma\delta$ T cells, neutrophils, and macrophages also express IL-17 (5,15,31). However, the functional significance of IL-17 expression by these types is not clear. Neutrophils and $\gamma\delta$ T cells may produce IL-17 in the early phase of immune response against infection and thereby regulate innate inflammatory responses such as macrophage or neutrophil infiltration into infected tissues. Murine CD8⁺ T cells produce IL-17 in response to IL-1 and IL-23 and human memory

CD8⁺ T cells have been reported to secrete IL-17 following stimulation of PBMC with PMA and ionomycin (43–44). In the present study, we have shown that IL-17-producing CD8⁺ T cells were significantly increased in aged mice, suggesting aging process may also affect CD8⁺ T cells function. Future studies need to address the function of IL-17-producing CD8⁺ T cells in aging process.

Most recent attention on IL-17-producing cells has been focused on CD4⁺ T cells. It is well established that naïve CD4⁺ T cells in the presence of IL-6, TGF β , and IL-23 will differentiate into IL-17-producing cells. Recently, IL-23, which induces IL-17 expression by mouse memory T cells, has emerged as a crucial regulator of IL-17 expression. However, we found that the expression of IL-12/IL-23 p40, IL-6, TGF- β in LPS-induced dendritic cells from aged mice was actually comparable with that seen in DCs from young mice, suggesting that the increased expression of IL-17 by CD4⁺ T cells during the aging process may not be dependent upon APC derived factors such as IL-6, TGF- β or IL-23.

Inflammatory bowel diseases (IBDs), including Crohn's and ulcerative colitis, involve an interplay between host genetics and environmental factors. Animal models of IBD have demonstrated that chronic inflammation can result from overproduction of inflammatory responses or deficiencies in key negative regulatory pathways. Recent studies have shown that Th17 cells play a pathogenic role in the development of colitis (5,45). It has been generally held that there is a bimodal distribution in the age of onset of IBD. The first mode occurs reliably in the 20s and the second peak occurs more variably between the ages of 50 and 80 (46). In the present study we have found that Th17 cells were significantly increased in both aged humans and mice, suggesting aging may affect pathological Th17 immune response. In addition, transfer of naïve CD4⁺ T cells from aged mice induced more severe colitis in RAG^{-/-} mice. These results may suggest that aging promotes an intrinsic predisposition towards the pathological Th17 immune response, which may explain the second peak in the incidence of IBD in humans.

Our current studies are consistent with the studies of Goetzl and colleagues (29), demonstrating that splenic CD4⁺ T cells from aged mice produce more IL-17 than those from young mice. However, it has remained unclear as to whether T cells from aged humans produce more IL-17 and other Th17 cytokines. Here, we demonstrated that human T cells from healthy aged individuals expressed significant higher IL-17, IL-22 and RORC2. In addition, we have also found a parallel increase of CD4⁺CD25⁺Foxp3⁺ Treg cells from aged human and mice. A number of studies have evaluated Foxp3 expression in aged mice and human. Generally these studies have found increased numbers of Foxp3 in aged hosts, which correlated to the increased proportion of memory cells (47–51). Furthermore, these cells were found to be functional; their suppressive capacity has been described as being at least equivalent to Tregs derived from young hosts, and in some cases as being enhanced compared to cells from young hosts (48–49,51). It has been suggested that this increased number of Foxp3⁺ Tregs may actually contribute to disease in older host, where they may contribute to the proliferation of cancer cells or the reactivation of latent chronic diseases by suppressing protective host immune responses (47–49,51). Significantly, more studies will be required to address the apparent paradox of increased Tregs and increased IL-17 in aged individuals, and how this contributes to disease development.

Taken together, we demonstrate that the number of Th17 cells was significantly increased in aged humans and mice, with a concurrent increase in the expression of Th17 cytokines and ROR γ t. In addition, antigen presenting cells do not contribute to the enhancement of Th17 immune response in aging. Furthermore, transfer of naïve T cells from aged mice induced more severe colitis in RAG^{-/-} mice. The results suggest that aging process may play an

important role in the generation of pathological Th17 immune response, contributing to the development of inflammatory diseases including IBD.

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Fig. 1. IL-17 and IL-22 expression is elevated in healthy aged individuals

(A) CD4⁺ T cells from peripheral blood of healthy aged and young people were incubated with anti-CD3 and anti-CD28 antibodies for 72 hrs and then activated with PMA/ionomycin for 6 hrs. Total RNA was extracted and analyzed for IL-17A, IL-17F and IL-22. *, $p < 0.01$ vs young individuals. (B) The above stimulated cells were harvested and stained for intracellular IL-17. (C) Total RNA was extracted from cells as in A and analyzed by real time RT-PCR for RORC2 mRNA. *, $p < 0.01$ vs young individuals.

Figure 2D

Fig. 2. Th17 cytokines are enhanced in aged mice

(A) Naïve T cells from both young and aged mice were differentiated under Th17 conditions for 4 days. Cells were then activated with PMA/inomycin for another 6 hrs. Total RNA was extracted and analyzed for IL-17 and IL-22 mRNA by real time PCR, *, $p < 0.01$ vs young mice. (B) Supernatants were collected from the cell culture as in A and IL-17 protein was measured by ELISA, *, $p < 0.01$ vs young mice. (C) Splenocytes from both young and aged mice were differentiated under Th17 conditions for 4 days. Cells were then harvested and stained for intracellular IL-17 and CD4. (D) Naïve T cells from both young and aged mice were differentiated under Th17 conditions for 4 days. Cells were then harvested and stained for intracellular IL-17 and CD8. (E) Total RNA was extracted from cells as in A and

analyzed by real time PCR for ROR γ t mRNA. (F) Lamina propria lymphocytes (LPL) from both young and aged mice were differentiated under Th17 conditions for 4 days. Total RNA was extracted and analyzed for IL-17 mRNA and the supernatants were analyzed for IL-17. *, p<0.01 vs young mice. The data are representative of three similar experiments.

Figure 3A

Figure 3B

Figure 3C

Fig. 3. Dendritic cells do not contribute to the enhanced expression of Th17 cytokines in aged mice

(A) Bone marrow-derived dendritic cells from young and aged mice were co-cultured with naïve CD4⁺ T cells from OT-II mice. The cells were either activated with OVA peptide (323–339) for 72 hrs or polarized under Th17 conditions for 4 days. Cells were harvested and stained for intracellular IL-17. The data are representative of three similar experiments.

(B) Bone marrow-derived dendritic cells were activated with LPS (1 µg/ml) for 6 hrs and total RNA was extracted. Real time RT-PCR was performed for the analysis of mRNA expression of IL-12/IL-23 p40, IL-6, EBI3, TNFα, TGF-β, and IL-27 p28. (C) CD4⁺ T cells were purified from spleens and lymph nodes of young and old mice. Total RNA was extracted and real time RT-PCR was performed for the analysis of IL-6 and TGF-β mRNA expression. *, p<0.01 vs young mice. The data are the representative of two similar experiments.

Figure 4A

Figure 4B

Figure 4C

Fig. 4. Memory T cells are an important cell type for IL-17 production

(A) Naive from young and aged mice were prepared and analyzed for CD62L and CD44 expression of CD4⁺ T cells by FACS. (B) Splenocytes from young and aged mice were prepared and analyzed for CD62L and CD44 expression of CD8⁺ T cells by FACS. (C) Splenocytes from both young and aged mice were differentiated under Th17 conditions for 4 days. Cells were harvested and stained for intracellular IL-17 by FACS. The cells were gated on CD4. The data are representative of three similar experiments.

Figure 5B

Fig. 5. Treg cells are increased in healthy aged individuals

(A) Peripheral blood mononuclear cells (PBMCs) were prepared from seven healthy young and aged individuals and CD4⁺CD25⁻ cells were sorted out and incubated with anti-CD3, anti-CD28 antibodies (0.5 µg/ml), IL-2 (25 U/ml), and all-trans-retinoic acid (100 nM) for 6 days. Total RNA was extracted and analyzed for Foxp3 mRNA by RT-PCR (upper panel) and real time RT-PCR (lower panel). (B) The above cells were harvested for FOXP3 analysis by FACS. (C) Naïve T cells from both young and aged mice were prepared and

analyzed for the population of CD4⁺CD25⁺ cells by FACS. (D) Naïve T cells from both young and aged mice were incubated with TGF- β for 4 days. Total RNA was extracted and analyzed for Foxp3 mRNA expression by real time PCR.

Fig. 6. Transfer of naïve T cells from aged mice induced more severe colitis in $RAG^{-/-}$ mice (A) Changes in body weight of $Rag1^{-/-}$ mice (n=4–5 mice per group) after intraperitoneal transfer of $CD4^{+}CD45RB^{hi}$ T cells from young and aged mice. Data are presented as the mean $\pm$ sd of the percentage of initial body weight and are representative of two similar experiments. *, $p<0.01$ vs cells from young mice. (B) Disease scores of $Rag1^{-/-}$ mice (n=4–5 mice in each group) after intraperitoneal transfer of $CD4^{+}CD45RB^{hi}$ T cells from young and aged mice. (C) Sections of colons obtained from $Rag1^{-/-}$ mice with colitis 9 weeks after T cell transfer described in (A). The data are representative of three similar experiments.