

Impaired GATA3-Dependent Chromatin Remodeling and Th2 Cell Differentiation Leading to Attenuated Allergic Airway Inflammation in Aging Mice¹

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Age-related changes in lymphocytes are most prominent in the T cell compartment. There have been substantial numbers of reports on T cell function in aged mice and humans, such as on the production of Th1 and Th2 cytokines, but the results show considerable variation and contradictions. In the present study, we used 8- to 12-mo-old aging mice and a well-established in vitro Th1/Th2 cell differentiation culture system to identify molecular defects in Th1/Th2 cell differentiation that can be detected in the relatively early stages of aging. The capability to differentiate into Th2 cells is reduced in aging mouse CD4⁺ T cells. Decreased activation of the ERK MAPK cascade upon TCR stimulation, but normal intracellular-free calcium ion concentration mobilization and normal IL-4-induced STAT6 activation were observed in aging mouse CD4⁺ T cells. In addition, reduced expression of GATA3 was detected in developing Th2 cells. Chromatin remodeling of the Th2 cytokine gene locus was found to be impaired. Th2-dependent allergic airway inflammation was milder in aging mice compared with in young adult mice. These results suggest that the levels of Th2 cell differentiation and resulting Th2-dependent immune responses, including allergic airway inflammation, decline during aging through defects in the activation of the ERK MAPK cascade, expression of GATA3 protein and GATA3-dependent chromatin remodeling of the Th2 cytokine gene locus. In the present study, we provide the first evidence indicating that a chromatin-remodeling event in T cells is impaired by aging. *The Journal of Immunology*, 2006, 176: 2546–2554.

The CD4⁺ T cells consist of two distinct Th cell subpopulations, Th1 and Th2 cells (1). Th1 cells produce IFN- γ and are involved in cell-mediated immunity against intracellular pathogens. Th2 cells produce IL-4, IL-5, and IL-13 and control humoral immunity and allergic reactions. Naive CD4⁺ T cells differentiate into Th1 cells following recognition of Ags in the presence of IL-12, whereas IL-4 drives differentiation into Th2 cells (2–4). In addition to the cytokines mentioned above, TCR stimulation by Ags is also indispensable for both Th1 and Th2 cell differentiation. We reported that the efficient TCR-mediated activation of p56^{lck}, calcineurin, and the Ras-ERK MAPK signaling cascade is required for Th2 cell differentiation (5–7). Several transcription factors that control Th1/Th2 cell differentiation have been identified (8, 9). Among them, GATA3 appears to be a master

transcription factor for Th2 cell differentiation (10–13) and Th2 cell maintenance (14, 15). Recently, we reported that the activation of the ERK MAPK cascade inhibits the ubiquitin-dependent degradation of GATA3 in developing Th2 cells and facilitates GATA3-dependent chromatin remodeling of the Th2 cytokine gene locus (16).

In the elderly, there is an increase in the frequency and severity of infectious diseases (17–19). Age-related changes in the immune system occur mainly in the T cell compartment (20–22). There may be related to a decrease in the ability of T cells to proliferate, and are associated with a reduction in IL-2 production (23) and reduced IL-2R expression (24–26). Various alterations in signaling have been described in comparison with young T cells. CD4⁺ T cells from old mice show defects in TCR signal transduction that include diminished TCR- ζ phosphorylation, decreased elevation of intracellular Ca²⁺, and diminished activation of the MEK/ERK pathway (20, 27, 28). In contrast, aging does not affect Zap70-TCR- ζ association (29).

T cells in the elderly are often characterized by a shift from naive to memory phenotypes (30, 31). The production of the type 1 cytokine IFN- γ has been reported to be increased (32–34) or decreased (35–37) in aged mice and humans. Also, the production of type 2 cytokines such as IL-4 and IL-5 has been reported to be increased (38, 39) or decreased (33, 35) in vitro. These controversial observations on cytokine production may be a result of variations among the species or strains used in experiments, housing conditions or experimental culture systems. At present, age-related molecular defects in developing Th1/Th2 cells that control Th1/Th2 cytokine gene chromatin remodeling have not been formally investigated. In addition, it is not well clarified whether the severity of Th2-dependent allergic responses, such as allergic asthma, is modulated by aging.

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In the present study, we demonstrate that Th2 cell differentiation and Th2-dependent immune responses *in vivo*, including OVA-induced airway inflammation, are attenuated in aging mice. We detected several molecular defects in aging mouse CD4⁺ T cells that may account for the attenuated Th2 responses, i.e., 1) reduced activation of the ERK MAPK cascade upon TCR stimulation, 2) decreased GATA3 expression in developing Th2 cells, and 3) impaired chromatin remodeling of the Th2 cytokine gene locus.

Materials and Methods

Mice

C57BL/6 and BALB/c were purchased from Charles River Laboratories. All young adult (5–6 wk) and aging (8–12 mo) mice including OVA-specific TCR $\alpha\beta$ transgenic (DO.11.10 Tg) mice (40) used in this study were maintained under specific-pathogen-free conditions. Animal care was in accordance with the guidelines of Chiba University.

Immunofluorescent staining and flow cytometric analysis

In general, 1 million cells were incubated on ice for 30 min with the appropriate staining reagents, according to a standard method (41). The reagents used in this study, anti-CD4-PE (RM4-1-PE), anti-CD4-FITC (RM4-1-FITC), anti-CD44-PE, anti-IL-4R α Ab, anti-CD25-FITC, and anti-CD69-FITC, were purchased from BD Pharmingen. Anti-rat Ig-FITC was purchased from CAPPEL. Anti-TCR β -FITC (H57-FITC), anti-TCR β -biotin, and anti-CD3-FITC (2C11-FITC) were prepared in our laboratory. Flow cytometric analysis was performed on a FACSCalibur (BD Biosciences), and the results were analyzed with CellQuest software (BD Biosciences). Intracellular staining of IL-4 and IFN- γ was performed as described previously (6). FITC- or allophycocyanin-conjugated anti-IFN- γ Ab (XMG1.2; BD Pharmingen) and PE- or allophycocyanin-conjugated anti-IL-4 Ab (11B11; BD Pharmingen) were used for detection.

Cell purification

Splenic CD4⁺ T cells were stained with anti-CD4-FITC and then purified using anti-FITC magnetic beads (Miltenyi Biotec) and an AutoMACS sorter (Miltenyi Biotec), yielding a purity of >98%. In some experiments, spleen cells were stained with anti-CD4 and anti-CD44, and naive CD4⁺CD44^{low} T cells were sorted by a FACS Vantage (BD Biosciences) and used as responder T cells as described previously (42).

Proliferation assay

Splenic CD4⁺ T cells (2×10^5) prepared by the AutoMACS sorter were stimulated in 200- μ l cultures for 40 h with immobilized anti-TCR β mAb (H57-597). [³H]Thymidine (37 kBq/well) was added to the stimulation culture for the last 16 h, and the incorporated radioactivity was measured on a beta plate (6).

Analysis of the efficiency of cell division

Splenic CD4⁺ T cells purified by the AutoMACS sorter were labeled with CFSE (Molecular Probes) as described previously (42).

Measurement of intracellular-free calcium ion concentration ([Ca²⁺]_i)³

Splenic CD4⁺ T cells purified by the AutoMACS sorter were loaded with Indo-1 (Indo-1 AM; Molecular Probes) in the presence of F127 (41). After washing, the cells were incubated with anti-CD4-FITC and anti-TCR β -biotin on ice. The stained cells were washed and subjected to Ca analysis on a FACS Vantage (BD Biosciences). TCR was cross-linked with avidin, the [Ca²⁺]_i was monitored for 512 s, and the results were analyzed with CellQuest software (BD Biosciences).

In vitro Th1/Th2 cell differentiation cultures

DO11.10 Tg CD44^{low}CD4⁺ T cells (1.5×10^4) purified by cell sorting were stimulated with antigenic OVA peptide (OVA; 323–339, 10 μ M) and irradiated (3000 rad) BALB/c APCs (1×10^5) in the presence of exogenous IL-4 or IL-12 as described previously (6).

ELISA for the measurement of cytokine concentration

The productions of IL-2, IL-4, IL-5, IL-13, and IFN- γ were measured by ELISA as described previously (43).

RT-PCR analysis

The quantitative RT-PCR analysis of GATA3 expression was performed as described previously (44).

Retroviral vectors and infection

cDNA for human GATA3 was inserted into a multicloning site of pMX-IRES-GFP (16). The methods for the generation of the virus supernatant and infection were described previously (16).

OVA immunization and ELISA for the measurement of serum Ig concentration

Young (6 wk old) and old (10 mo old) BALB/c mice were immunized i.p. with 100 μ g of OVA emulsified in CFA (Difco) on days 0 and 7. Blood was collected from the tail vein on day 14. The concentrations of IgE in the serum were measured with a mouse IgE ELISA kit (BD Biosciences). The concentrations of OVA-specific Igs (IgG1 and IgG2a) in the serum were determined by ELISA as described previously (5).

Immunoblotting

Immunoblotting was performed as described previously (6). For ERK1 and ERK2 phosphorylation, naive CD4⁺ T cells from C57BL/6 mice were purified with anti-CD4 mAb (RM4-5) and magnetic beads sorting (MACS sorting), and then the cells were incubated with anti-TCR mAb (H57-597) on ice. After incubation, the cells were stimulated with anti-hamster Igs (which cross-reacts with both H57-597) for 3, 10, or 30 min at 37°C, and then total cell lysates were subjected to phospho-ERK immunoblotting (Cell Signaling Technology). For STAT6 phosphorylation, naive CD4⁺ T cells from C57BL/6 mice were activated with immobilized anti-TCR mAb and IL-4 (100 U/ml) for 2 days (induction culture). To assess IL-4-induced tyrosine phosphorylation, stimulated cells were washed, cultured for 8 h without cytokines, and stimulated with IL-4 (100 U/ml) for 3, 10, 30, or 60 min at 37°C. For IL-4 titration, 10–100 U/ml IL-4 was used. Anti-phosphotyrosine (RC20; BD Transduction Laboratories) or antiserum reactive with STAT6 (R&D Systems) was used for detection. For the detection of GATA3 or JunB, nuclear extracts were prepared with NE-PER Nuclear and Cytoplasmic Extraction Reagent (Pierce) according to the manufacturer's protocol. Immunoblotting was performed with anti-GATA3 mAb or anti-JunB mAb (Santa Cruz Biotechnology). HRP-conjugated anti-mouse Ig Ab (Amersham Biosciences) was used for GATA3 or JunB visualization (45).

Chromatin immunoprecipitation (ChIP) assay

Acetylation status of histone H3-K9/K4 was assessed using histone H3 (K9/14) ChIP assay kits (no. 17-245; Upstate Biotechnology) as described previously (46). The ChIP assay for di-methylated histone H3-K4 was performed using anti-histone H3 dimethyl K4 antiserum (no. 07-030; Upstate Biotechnology) (47). Semiquantitative PCR was performed with DNA samples from 3×10^4 or 1×10^4 cells at 28 cycles. PCR products were resolved in an agarose gel and visualized and quantified using an ATTO L&S analyzer (ATTO). The primers used were described previously (46).

Sensitization and inhalation with OVA

Young (6 wk old) and old (12 mo old) BALB/c mice were immunized i.p. with 250 μ g of OVA (chicken egg albumin purchased from Sigma-Aldrich) in 4 mg of aluminum hydroxide gel (alum) on days 0 and 7. Mice were made to inhale aerosolized OVA in saline (10 mg/ml) for 30 min, using a supersonic nebulizer (model NE-U07; Omron) on days 14 and 16 to assess eosinophilic inflammation as described previously (48).

Collection of bronchioalveolar lavage (BAL) fluid and lung histology

Two days after the last OVA inhalation on day 16, BAL was performed as described previously (49). Total BAL fluid was collected and the cells in 100- μ l aliquots were counted. One hundred thousand viable BAL cells were cytocentrifuged onto slides using a Cytospin3 (Thermo Shandon) and stained with May-Grünwald-Giemsa solution (Merck) as described previously (50). Two hundred leukocytes were counted on each slide. Cell types were identified using morphological criteria. The percentages of each cell type were calculated.

³ Abbreviations used in this paper: [Ca²⁺]_i, free calcium ion concentration; ChIP, chromatin immunoprecipitation; BAL, bronchioalveolar lavage.

For lung histology, mice were sacrificed by CO₂ asphyxiation 48 h after the last OVA inhalation on day 16, and the lungs were infused with 10% (v/v) Formalin in PBS for fixation. The lung samples were sectioned, stained with H&E reagents, and examined for pathological changes under a light microscope at $\times 200$. Numbers of infiltrated mononuclear cells in the perivascular and peribronchiolar regions were enumerated by direct counting of four different fields per slide as described previously (51).

Results

Phenotypic and functional characterization of aging mouse CD4⁺ T cells

We initiated the analysis of T cells of aging C57BL/6 mice (8–12 mo) maintained under specific pathogen-free conditions by examining the expression of cell surface molecules on splenic CD4⁺ T cells. Representative CD4/CD8 profiles for young and aging mice are shown in Fig. 1A. The yields of spleen cells were $112 \pm 24 \times 10^6$ ($n = 8$) for young mice and $134 \pm 34 \times 10^6$ ($n = 8$) for aging mice. The percentages of CD4⁺ and CD8⁺ T cells in the spleen were similar between young and aging mice, and the cell surface expression of TCR $\alpha\beta$ and CD3 on the splenic CD4⁺ T cells was also similar (Fig. 1B). However, the numbers of CD25⁺ cells, CD69⁺ cells, and memory type (CD44^{high}) CD4⁺ T cells in aging mice were higher than in young mice. The expression level of IL-4R α on CD4⁺ T cells was slightly higher in aging mice. As for T cell function, the anti-TCR β mAb-induced proliferative responses of CD4⁺ T cells were lower in aging mice (Fig. 1C). We examined the anti-TCR-induced cell division of CD4⁺ T cells in Th1- or Th2-skewed differentiation cultures and found that CFSE-labeled naive CD4⁺ T cells were stimulated by the anti-TCR β mAb in the presence of IL-2 and IL-12 (Th1 culture conditions) or IL-2 and IL-4 (Th2 culture conditions). After 72 h of culture, the cells had divided three to four times in the case of both young and aging mouse T cells. The rate of cell division of aging mouse CD4⁺ T cells was slightly impaired under either Th1- or Th2-skewed conditions (Fig. 1D). Similar results were obtained using CD4⁺ T cells from BALB/c mice (data not shown). These results suggest that CD4⁺ T cells from aging mice have a moderately decreased proliferative response to anti-TCR stimulation, a finding consistent with previous reports (17, 18).

In vitro Th1/Th2 cell differentiation of naive CD4⁺ T cells from aging mice

Th1/Th2 cell differentiation of naive CD4⁺ T cells from young and aging mice was examined using a well-established in vitro culture system (5). Naive CD4⁺ T cells from aging OVA-specific TCR $\alpha\beta$ Tg (DO11.10 Tg) mice were sorted and stimulated with antigenic OVA peptide in the presence of young BALB/c APCs for 5 days. CD4⁺ T cells from young DO11.10 Tg mice preferentially differentiated into IL-4-producing Th2 cells in an Ag dose-dependent fashion (Fig. 2, left upper panels). However, for aging mice, the generation of Th2 cells was decreased, and a significant increase in the number of IFN- γ -producing Th1 T cells was observed (Fig. 2, left panels). The levels of Th2 cell differentiation induced by a minimal dose of antigenic peptide (0.1 μ M), and exogenous IL-4 was also decreased in old DO11.10 Tg T cell cultures (Fig. 2, middle panels). In contrast, the IL-12-dependent induction of Th1 cell differentiation remained intact (Fig. 2, right panels). These results suggest that the efficiency of Th2 cell differentiation is reduced in aging mice.

Anti-TCR-induced cytokine production profiles of splenic CD4⁺ T cells from aging mice

Purified BALB/c CD4⁺ T cells from young and aging mice were stimulated in vitro with immobilized anti-TCR β mAb, and the level of cytokines in the culture supernatant was determined by

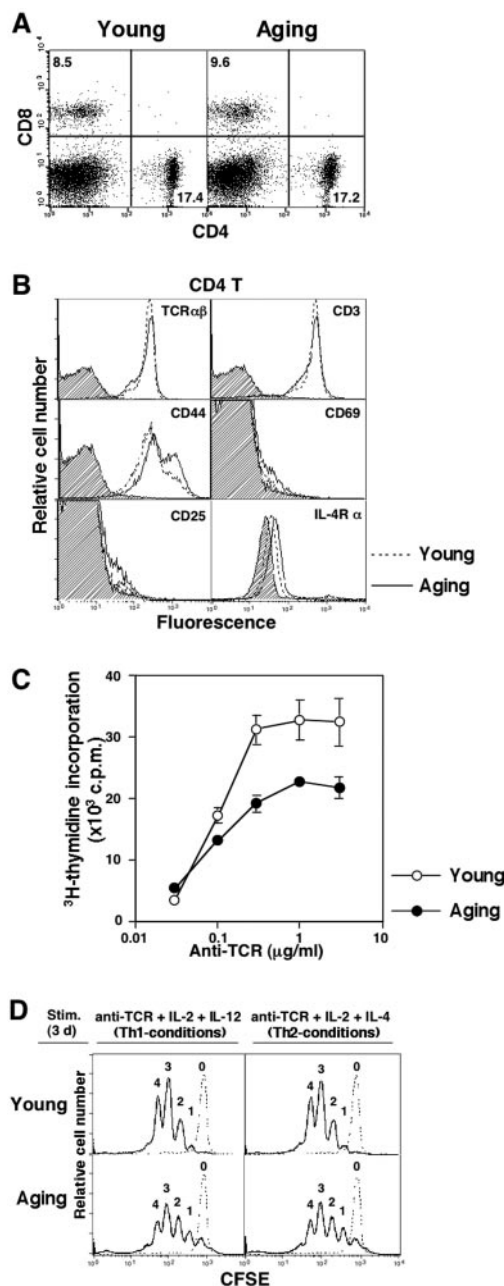


FIGURE 1. Phenotypic and functional characterization of aging mouse T cells. *A*, Representative CD4/CD8 profiles of splenocytes of young (6 wk old) and aging (9 mo old) C57BL/6 mice are shown. Percentages of cells present in each area are also shown. *B*, Each histogram depicts the expression of the indicated marker Ags on electronically gated splenic CD4⁺ T cells from young (dotted lines) and aging (solid lines) mice. Background staining is shown as hatched areas. *C*, Splenic CD4⁺ T cells from young (○) and aging (●) mice were stimulated with immobilized anti-TCR β mAb. The mean [³H]thymidine incorporation of each group is shown with SDs. *D*, Naive CD4⁺ T cells were labeled with CFSE and stimulated with immobilized anti-TCR β mAb under Th1- or Th2-skewed conditions. After 3 days of culture, the number of cell divisions (0 to 4) was assessed by flow cytometry.

ELISA (Fig. 3). The levels of IL-2 were not obviously decreased on day 2 but decreased on day 3. The production of IFN- γ in aging mouse CD4⁺ T cell cultures was substantially increased on day 5 and day 7. In contrast, the levels of all Th2 cytokines, IL-4, IL-5, and IL-13, in aging mouse T cell cultures were decreased on days

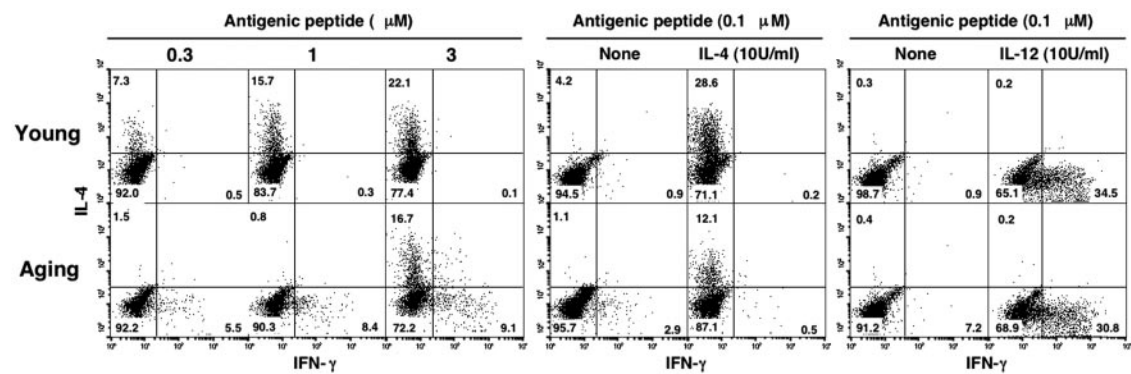


FIGURE 2. In vitro Th1/Th2 cell differentiation of naive CD4⁺ T cells from aging mice. Naive (CD44^{low}) CD4⁺ T cells purified by cell sorting from the spleens of aging OVA-specific TCRαβ Tg (DO11.10 Tg) mice were stimulated with the indicated doses of antigenic peptide (OVA; 323–339) and irradiated young BALB/c APCs (*left panel*), a minimal dose of peptide (0.1 μM) and APCs in the presence of exogenous IL-4 (*middle panel*) or IL-12 and anti-IL-4 mAb (*right panel*) for 5 days. Intracellular staining profiles of IFN-γ and IL-4 are shown with percentages of cells in each area. The results are representative of five experiments.

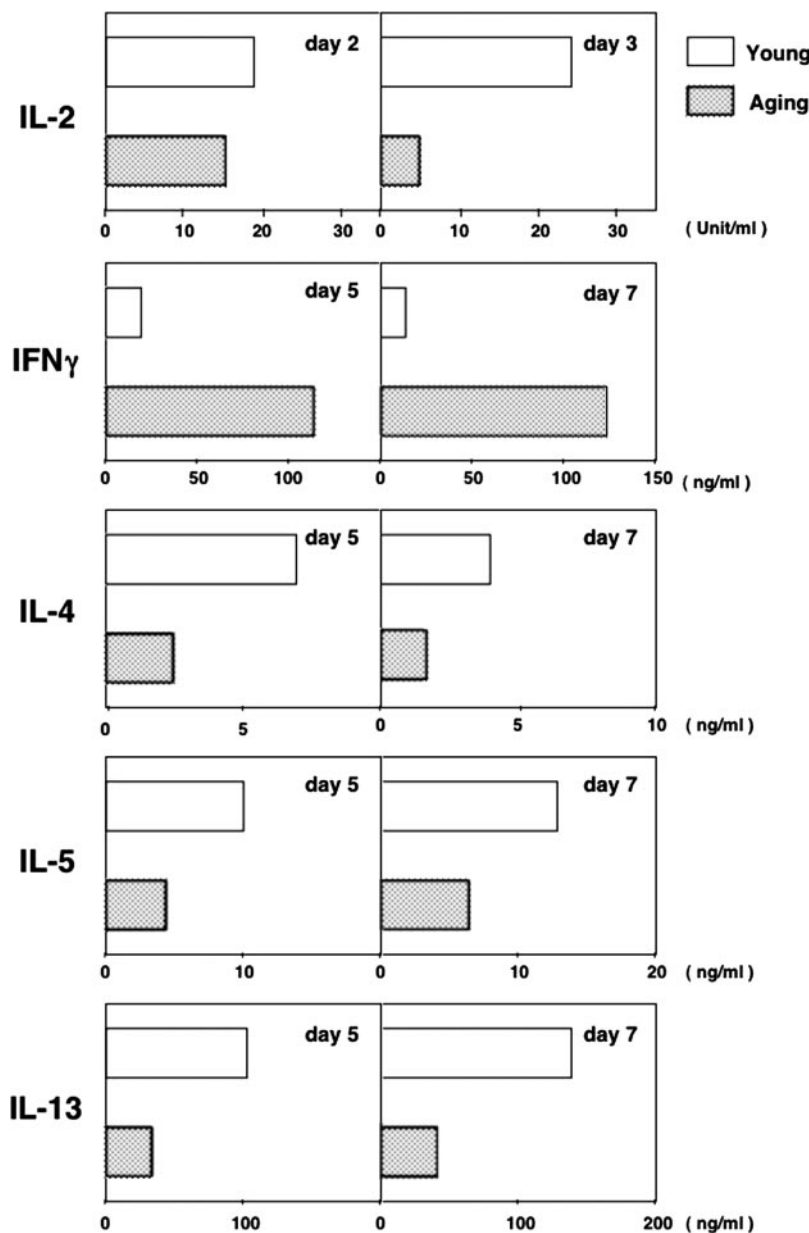


FIGURE 3. Decreased Th2 cytokine production in aging mouse CD4⁺ T cells. Purified BALB/c splenic CD4⁺ T cells were stimulated with immobilized anti-TCRβ mAb for 2 and 3 days (IL-2), or 5 and 7 days (IL-4, IL-5, IL-13, and IFN-γ). The concentration of cytokines in the culture supernatant was determined by ELISA. Three independent experiments were performed with similar results.

5 and 7. Similar cytokine production profiles were obtained in the anti-CD3 stimulation cultures (data not shown).

Th1/Th2-dependent Ab production in aging mice

Th2 cells play an important role in the stimulation of B cells to produce high levels of Ag-specific IgG1 and IgE in vivo, whereas the IgG2a isotype is a consequence of the generation of Th1 cells. Young and aging BALB/c mice were immunized with OVA-CFA, and the serum concentrations of total IgE, OVA-specific IgG1 and IgG2a were measured. As expected, the serum concentration of total IgE was significantly decreased in aging mice (Fig. 4, *left panel*). The serum concentration of IgG1 was significantly lower in aging mice (Fig. 4, *middle panel*), while Th1-dependent OVA-specific IgG2a levels were not decreased (Fig. 4, *right panel*). The production of Ag-specific IgE was not detected (data not shown). These results suggest that Th2-dependent Ab responses in vivo are decreased in aging mice preserving Th1 responses intact.

Signal transduction downstream of IL-4R in CD4⁺ T cells from aging mice

To assess the activation of the IL-4R signaling pathway, freshly prepared CD4⁺ T cells from young and aging mice were stimulated with IL-4, and then the tyrosine phosphorylation of STAT6 was examined. No significant differences in the magnitude or time course of phosphorylation of STAT6 were observed (Fig. 5A). Protein expression of STAT6 was comparable between young and old mice. Moreover, STAT6 phosphorylation induced by various doses of IL-4 was also comparable (Fig. 5B). Thus, the IL-4R signaling cascade appears to be intact in CD4⁺ T cells from aging mice.

Signal transduction through TCR in CD4⁺ T cells from aging mice

Next, we assessed the levels of signaling activation downstream of TCR. First, [Ca²⁺]_i mobilization in CD4⁺ T cells was assessed after TCR cross-linking, and a slightly higher percentages of responding cells and slightly higher magnitude of the response were observed (Fig. 5C). Next, naive CD4⁺ T cells from young and aging mice were stimulated with anti-TCR mAb, and the tyrosine phosphorylation of ERK1 and ERK2, reflecting MAPKK activation, was examined (Fig. 5D). Although the expression levels of ERK1 and ERK2 protein were comparable, the levels of phosphorylation of both ERK1 and ERK2 were reduced substantially in CD4⁺ T cells from aging mice. The background phosphorylation was also slightly reduced (see time 0). These results suggest that the activation of the Ras-ERK MAPK cascade is impaired in aging mouse CD4⁺ T cells.

Decreased GATA3 induction in CD4⁺ T cells differentiated under Th2 culture conditions

Because the levels of GATA3 and JunB expression are reported to be critical for Th2 cell differentiation (11), we assessed the protein and mRNA expression levels of GATA3 and JunB in developing Th2 cells. Naive CD4⁺ T cells from young and aging BALB/c mice were stimulated with anti-TCR β mAb in the presence of IL-4 and anti-IL-12 mAb for 5 days, and the protein expression levels of GATA3, JunB, and tubulin- α (Fig. 6A), and the mRNA expression levels of GATA3 (Fig. 6B) were assessed. The levels of GATA3 protein were decreased substantially in aging mouse CD4⁺ T cells, while the levels of JunB were unchanged. Regarding the GATA3 mRNA levels, the decrease was significant but less dramatic in aging mouse T cells. These results suggest that GATA3 induction is significantly impaired in developing Th2 cells of aging mice.

In an attempt to rescue the inefficient Th2 cell differentiation in aging mouse T cells, we introduced GATA3 by retrovirus infection into aging mouse developing Th2 cells on day 2 of Th1/Th2 cell differentiation culture, and the generation of Th1/Th2 cells was assessed on day 5 (Fig. 6C). Although not complete, a substantial rescue of Th2 cell generation was observed. These results suggest that the inefficient Th2 cell differentiation in aging mouse T cells is due, at least in part, to the decreased expression of GATA3.

Chromatin remodeling of the Th2 cytokine gene locus in CD4⁺ T cells from aging mice

We reported that Th2 responses are highly dependent on the extent of activation of the Ras-ERK MAPK cascade (6, 48). The hyperacetylation of histones associated with the Th2 cytokine gene locus is dependent on the expression of GATA3 (43, 46). Activation of the ERK-MAPK cascade is required for GATA3-dependent histone H3 hyperacetylation of the Th2 cytokine gene locus (16). Consequently, we wished to examine the chromatin remodeling of the Th2 cytokine gene locus in CD4⁺ T cells from aging mice. The acetylation levels of histones associated with the Th2 cytokine gene locus (IL-4 promoter, IL-5 promoter, and IL-13 promoter) were reduced significantly in Th2 cells from aging mice (Fig. 7, A and B). The acetylation of the CNS1 region was reduced slightly in aging mouse Th2 cells. Similarly, the methylation levels of histones associated with the Th2 cytokine gene locus (IL-4 promoter, IL-5 promoter, and IL-13 promoter) and CNS1 region were significantly reduced in Th2 cells from aging mice (Fig. 7, A and C). These results indicate that histone H3 hyperacetylation and methylation of the Th2 cytokine gene locus are significantly decreased in developing Th2 cells of aging mice.

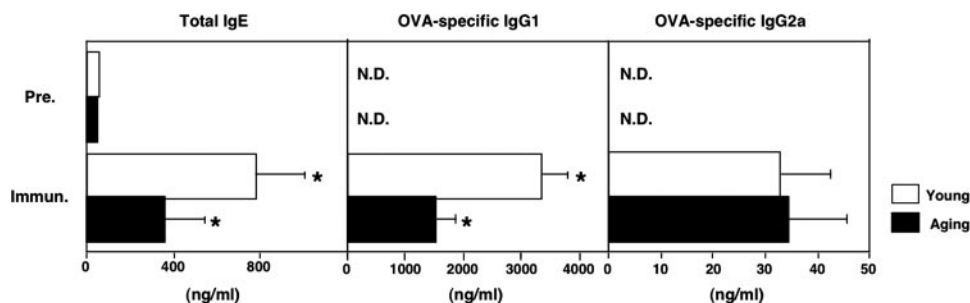


FIGURE 4. Th1/Th2-dependent immune responses in aging mice. Young (6 wk old) and aging (9 mo old) BALB/c mice (five mice per group) were immunized with OVA with CFA. Two weeks later, mean concentrations of total IgE and OVA-specific IgG1 and IgG2a Abs in the serum were determined. Data are shown with SEM. *, $p < 0.05$ by Student's t test. N.D., not detected.

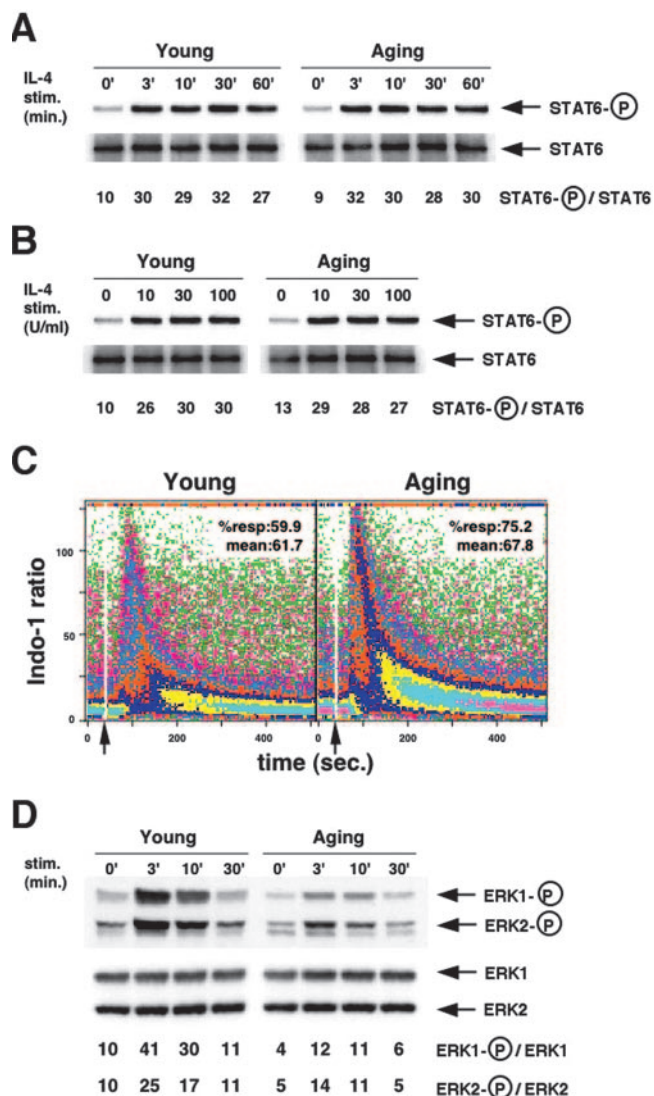


FIGURE 5. Signal transduction through TCR or IL-4R in CD4⁺ T cells from young and aging mice. *A* and *B*, Naive CD4⁺ T cells from young and aging mice were cultured for 2 days with immobilized anti-TCR mAb and IL-4, cultured without IL-4 for 8 h, and then the IL-4-induced phosphorylation on STAT6 (3–60 min with 100 U/ml IL-4 in *A*; 10 min with 10–100 U/ml IL-4 in *B*) was assessed by immunoprecipitation and immunoblotting with anti-phosphotyrosine mAb. The amount of STAT6 protein was also determined by reblotting the same membrane with specific mAbs. Arbitrary densitometric ratios (phospho-STAT6/STAT6) are shown under each band. Four independent experiments were done with similar results. *C*, Intracellular-free calcium ion levels after TCR-cross-linking (arrows) were measured by flow cytometric analysis of Indo-1-labeled naive CD4⁺ T cells from young and aging mice. The mean ratio of violet to blue fluorescence of Indo-1 is plotted vs time following stimulation. Shown are data obtained by gating electronically on CD4⁺ T cells. The percentages of responding cells and the mean response (ratio) of the responding cells are also shown. *D*, TCR-induced MAPKK activation in splenic CD4⁺ T cells from aging mice. The phosphorylation status of ERK1 and ERK2 in splenic CD4⁺ T cells was assessed 3–30 min after TCR cross-linking. After stimulation, the cells were lysed, and the lysates were subjected to immunoblotting with anti-phospho-ERK or anti-ERK Abs. Densitometric measurements of the phosphorylated bands (p44 for ERK1 and p42 for ERK2) are shown under each band in arbitrary units. Three independent experiments were done with similar results.

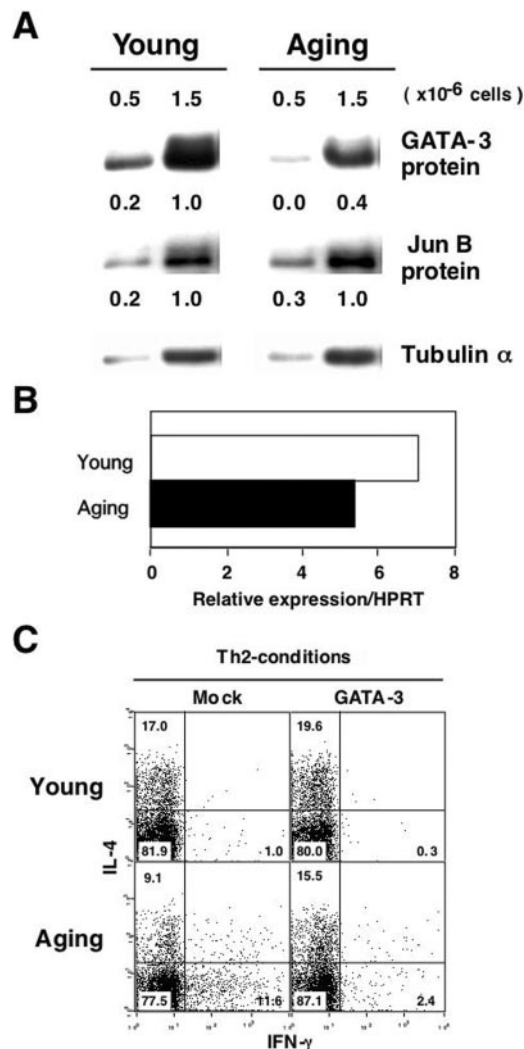
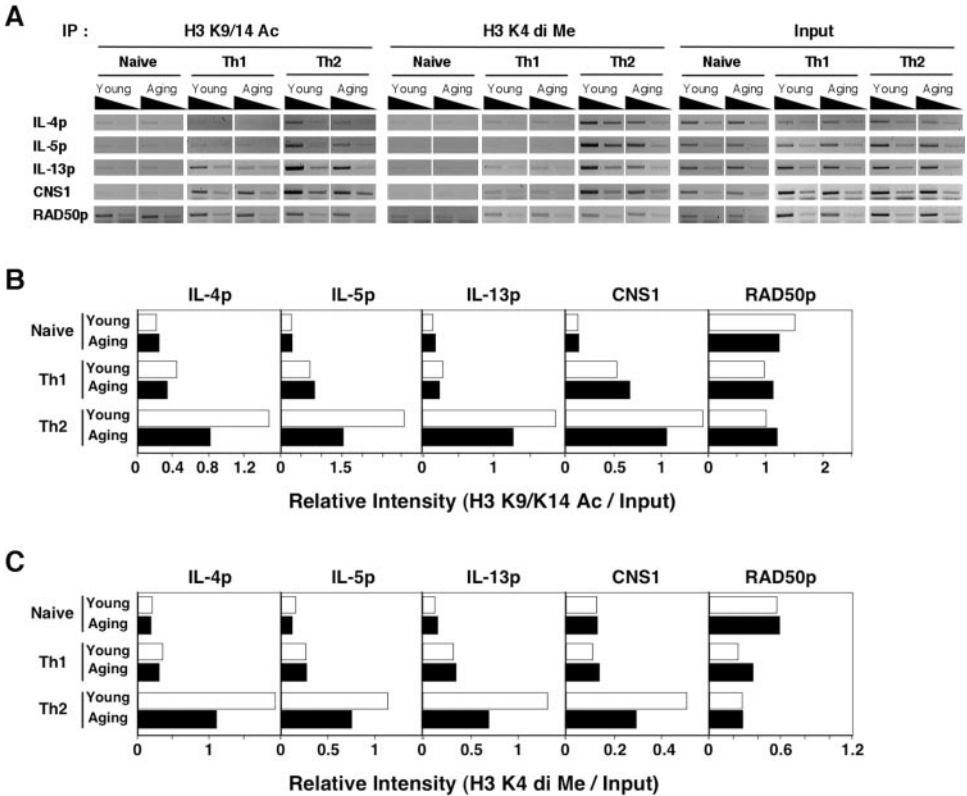


FIGURE 6. GATA3 expression and the effect of overexpression of GATA3 in developing Th2 cells from aging mice. *A*, GATA3 and JunB protein expression in developing Th2 cells. Naive CD4⁺ T cells from BALB/c mice were stimulated with anti-TCRβ mAb in the presence of IL-4 and anti-IL-12 mAb for 5 days, and total nuclear extracts were analyzed by immunoblotting with anti-GATA3 mAb, anti-JunB mAb, or anti-tubulin-α mAb. Three independent experiments were performed; representative results are shown. Arbitrary densitometric units normalized to tubulin-α are shown under each band. *B*, GATA3 mRNA expression in young and aging developing Th2 cells. Th2 cells prepared as in *A* were subjected to quantitative RT-PCR analysis. *C*, Naive CD4⁺ T cells from BALB/c mice were stimulated with anti-TCRβ mAb in the presence of IL-4 and anti-IL-12 mAb for 2 days and then infected with a mock or GATA3-containing retrovirus vector. Th1/Th2 cell differentiation was assessed on day 5.

Decreased OVA-induced eosinophilic infiltration in BAL fluid and airway inflammation in aging mice

Next, we examined the effect of aging on Th2-dependent immune responses in vivo. Young and aging BALB/c mice were immunized twice with OVA-alum, and 2 wk later, exposed to inhaled OVA as described in *Materials and Methods*. BAL fluid was harvested and examined for infiltrating leukocytes (Fig. 8, *A* and *B*). Eosinophils, lymphocytes, neutrophils, and macrophages were determined based on morphological criteria, and the absolute numbers and percentages of each cell type were determined. A substantial decrease in the absolute numbers (Fig. 8*A*) and percentages

FIGURE 7. Histone modification of the Th2-cytokine locus in developing Th2 cells from aging mice. *A*, Acetylation and methylation status of histones associated with the Th2 cytokine gene locus. Freshly prepared naive splenic CD4⁺ T cells were stimulated under Th1 or Th2 conditions for 5 days. The acetylation status and methylation status of histone H3 in nucleosomes associated with the indicated regions was assessed by ChIP assay. An anti-acetylated histone H3 (K9 and K14) Ab and an anti-histone H3 di-methyl K4 antiserum were used. *B*, The relative intensity of histone hyperacetylation (Ac-H3/input) in each group is shown in *A*. *C*, The relative intensity of histone dimethylation (diMe-H3/input) in each group is shown in *A*. Representative results of three independent experiments are shown.



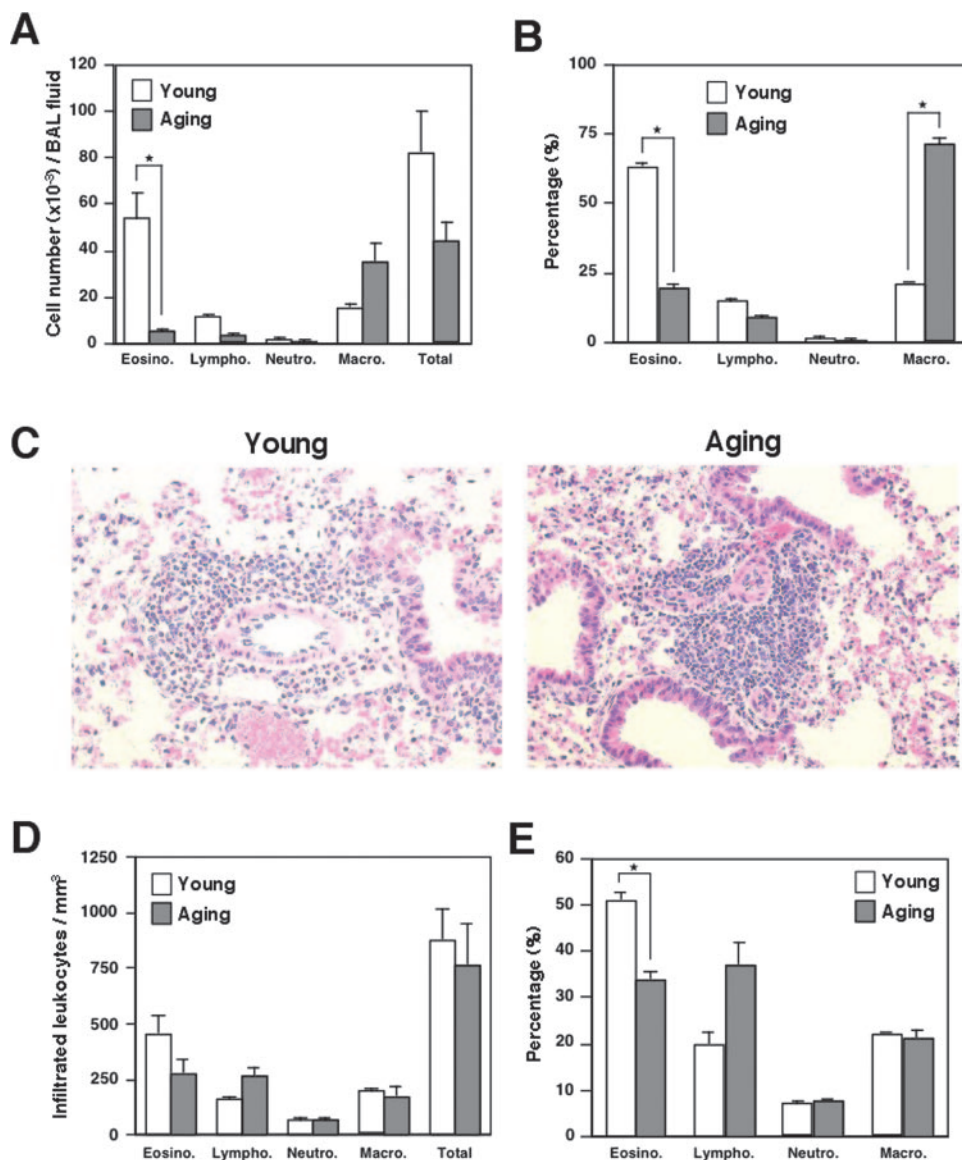
(Fig. 8*B*) of eosinophils was observed in aging mice. The infiltration of macrophages was increased in aging mice, and the number of total infiltrating cells was decreased in aging mice. These results indicate that OVA-induced eosinophilic infiltration in the BAL fluid is decreased in aging mice. Concurrently, histological changes in the lungs of young and aging mice were evaluated by HE staining (Fig. 8*C*). The absolute numbers and percentages of each cell type are shown (Fig. 8, *D* and *E*). The numbers of total infiltrating cells were indistinguishable between young and aging mice, although a slight decrease in the absolute number, and a slight, but significant, decrease in the percentage of eosinophils were observed. No apparent difference in the numbers of lymphocytes, neutrophils or macrophages was observed.

Discussion

In the present study, we used young adult (4–6 wk old) and aging (8–12 mo old) mice, to demonstrate that the levels of Th2 cell differentiation and Th2-dependent allergic airway inflammation are attenuated in aging mice. In addition, we found several molecular defects in aging mouse CD4⁺ T cells, i.e., limited activation of the ERK/MAPK cascade, decreased expression of GATA3 and impaired chromatin remodeling of the Th2 cytokine gene locus. Because 8- to 12-wk-old mice can be considered to be in the early stages of aging, the processes affected would be the most sensitive among various age-related alterations in T cells. Impaired Th2 cell differentiation in aging mouse CD4⁺ T cell cultures was not restored by the addition of excess amounts of exogenous IL-4 (10 U/ml; Fig. 2, *middle panel*). In addition, our in vitro cultures contained sufficient amounts of exogenous IL-2 (30 U/ml). The production of all Th2 cytokines (IL-4, IL-5, and IL-13) was decreased in aging mouse CD4⁺ T cells (Fig. 3). Thus, the defect in Th2 cell differentiation appears to be due to intrinsic alterations and not simply a secondary consequence of the imbal-

ance in the production of IL-2, IL-4, or IFN- γ by aging mouse CD4⁺ T cells in culture. Our observations are consistent with the results reported by H. al-Rayes et al. (52) that IL-4 production is diminished in aged human PBL. In our previous reports, we showed that Th2 cell differentiation and Th2-dependent airway inflammation are highly dependent on the TCR-mediated activation of the Ras-ERK MAPK cascade (6, 48). In the present study, we detected significantly reduced activation of the Ras/MAPK cascade in aging mouse CD4⁺ T cells (Fig. 5*D*). Thus, the impaired Th2 cell differentiation in old mice appears to be due to the decreased TCR-mediated activation of the Ras/MAPK signaling pathway. As for Th1 cell differentiation, we observed no detectable decrease in IL-12-induced Th1 cell generation in vitro (Fig. 2, *right panels*), suggesting that the efficiency in Th1 cell differentiation is less affected by aging. We detected significant numbers of IFN- γ producing cells in aging naive CD4⁺ T cell cultures when exogenous IL-4 was not included in the culture (Fig. 2, *left panels*). In addition, we detected increased production of IFN- γ by ELISA (Fig. 3). These results suggest that some shift from Th2 to Th1 cell differentiation occurs in aging mouse CD4⁺ T cell cultures. We previously reported a dramatic shift from Th2 to Th1 cell differentiation in dominant negative Ras Tg mice (6). Therefore, these results also support the notion that the impaired Th2 cell differentiation in aging mice is due to the decreased TCR-mediated activation of the Ras/MAPK signaling pathway. The expression levels of the GATA3 protein are critical for chromatin remodeling (46) and the maintenance of remodeled chromatin at the Th2 cytokine gene locus (14). In addition, we have recently demonstrated that the activation of the Ras-ERK MAPK cascade controls the stability of the GATA3 protein through the inhibition of ubiquitin-dependent degradation (16). The results presented in this report suggest that GATA3-dependent

FIGURE 8. OVA-induced eosinophilic infiltration in BAL fluid and airway inflammation in old mice. The absolute cell number (A) and percentage (B) of eosinophils (Eosino.), lymphocytes (Lympho.), neutrophils (Neutro.), and macrophages (Macro.) in BAL fluid are shown with SDs. Three young (6 wk-old) and three aging (9 mo-old) BALB/c mice were used in this experiment. Data include absolute cell number, percentage of cells, total cell number per milliliter, and the volume of BAL fluid recovered. *, $p < 0.01$ and other p values > 0.05 by Student's t test. C, OVA immunization and inhalation of OVA aerosol were done as in A. The lungs were fixed and stained with H&E, $\times 200$. Sections shown are representative of 10 lung sections/mouse from three mice in each group. The cell number (D) and percentage (E) of infiltrated eosinophils (Eosino.), lymphocytes (Lympho.), neutrophils (Neutro.), and macrophages (Macro.) in the perivascular and peribroncholar regions are shown with SDs. Three young (6 wk old) and three aging (9 mo old) BALB/c mice were used in this experiment. *, $p < 0.01$ and other p values > 0.05 by Student's t test.



chromatin remodeling of the Th2 cytokine gene locus is significantly reduced in aging mouse CD4⁺ T cells (Fig. 7). The expression levels of JunB (Fig. 6A) and the activation of NF- κ B were comparable between young and aging mouse CD4⁺ T cells (A. Hasegawa and T. Nakayama, unpublished observation). From these results, we conclude that the impaired chromatin remodeling of the Th2 cytokine gene locus in aging mice is due, at least in part, to the decreased expression of the GATA3 protein in developing Th2 cells.

Although it remains unclear why the activation of the Ras-ERK MAPK cascade is affected selectively during aging, this pathway may determine the characters of various T cell responses. In anergic CD4 T cells impaired activation of the Ras-ERK MAPK cascade has been reported (53, 54).

How allergic inflammation, such as allergic asthma is modulated by aging has not been reported. We show in the present study that the severity of Th2-dependent allergic airway inflammation is decreased in aging mice (Fig. 7). This appears to be due to the decreased Th2 cell differentiation in aging mice. Although there is as yet no clear evidence in humans, the data would suggest that the first onset of allergic asthma should occur less frequently in aged human beings. The confirmation of this hypothesis must await a comprehensive investigation in humans.

In summary, this study provides the first evidence that a chromatin-remodeling event in T cells, i.e., chromatin remodeling of the Th2 cytokine gene locus in developing Th2 cells, is compromised during aging. Moreover, we demonstrate attenuated Th2-dependent allergic airway inflammation in aging mice, which may reflect the nature of allergic diseases in aged humans.

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Disclosures

The authors have no financial conflict of interest.

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