Aging Is Associated With Increased T-Cell Chemokine Expression in C57Bl/6 Mice

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To better understand the contribution of the chemokine system in immune senescence, we determined the aging effect on CD4+ and CD8+ T-cell chemokine expression by microarray screening and ribonuclease protection assays. Compared with young C57BL/6 mice, freshly isolated CD4+ cells from aged mice express increased level of interferon-γ-inducible protein 10 (IP-10), macrophage inflammatory protein (MIP)-1α, MIP-1β, regulated upon activation, normal T-cell expressed and secreted (RANTES), and lymphotactin (Ltn). T-cell receptor (TCR)/coreceptor stimulation up-regulates MIP-1α, MIP-1β, and Ltn, and down-regulates IP-10 and RANTES expression in CD4+ T cells. A similar increase in chemokine expression was demonstrated in the CD8+ T cell. Enzyme-linked immunosorbent assays confirmed increased T-cell chemokine protein production in old CD4+ and CD8+ T cells. Finally, supernatant of cultured T cells from old animals caused an enhanced leukocyte chemotaxis response compared with that from young animals, suggesting that the age-related difference in T-cell chemokine expression has an important functional consequence.

CHEMOKINES, or chemotactic cytokines, are small proteins (8–12 kd) with 4 conserved cysteines that form 2 essential disulfite bonds (1,2). Approximately 50 chemokines have been identified (3). Depending on whether the first 2 cysteines are adjacent or separated by 1 amino acid, most chemokines can be classified as belonging to either the CC or CXC chemokine subfamilies. In addition, 3 amino acids separate the 2 cysteines in the CX3C subfamily, and only the second and fourth cysteine residues are preserved in the C subfamily. The principal targets of chemokines are bone marrow-derived cells (3). Chemokines and their receptors are fundamental determinants of leukocyte localization during immune and inflammatory responses. In addition, they play an important role in the pathogenesis of many diseases prevalent in the elderly population including rheumatoid arthritis (4), coronary artery diseases (3,5,6), and human immunodeficiency virus (HIV)-1 infection (7–9). However, very little is known about the chemokine response in aging.

Despite investigations in the past few decades, there remains a poor correlation between the known age-related changes in protective immune response and the observed worse clinical outcome of many diseases that preferentially affect elderly people. Of the many factors studied, alterations in the T-cell compartment are believed to play a critical role in explaining immunosenescence (10). We recently reported the association between aging and changes in T-cell chemokine receptor expression (11). CD4+ T cells from aged mice were found to express a higher level of CCR1, 2, 4, 5, 6, 8, and CXCR2-5, and a lower level of CCR7 and 9 compared with cells from young mice. The changes are not strain specific and cannot be explained by the aging-associated differences in T-helper 1 (Th1)/Th2 or naïve/memory profile. Additionally, caloric restriction partially or completely restored the aging effects. In this article, we further examined the aging effect on the chemokine system by comparing T-cell chemokine expression in young and old C57Bl/6 mice.

METHODS

Mice
Young (3–4 months) and old (20–22 months) C57Bl/6 mice were obtained from the National Institute on Aging (NIA) Aged Rodent Colonies through Harlan Sprague Dawley, Inc. (Indianapolis, IN). All mice were maintained in a pathogen-free environment provided by the Unit for Laboratory Animal Medicine at the University of Michigan until they were used.

CD4 and CD8 Cell Isolation
CD4+ and CD8+ T cells were isolated as per previously published protocol (11). Careful inspection was done to exclude aged animals with cancer or lymphoma. CD4+ and CD8+ T cells were isolated by the magnetic cell separation (MACS) MicroBeads technology (Miltenyi Biotec, Bergisch-Gladbach, Germany) according to the manufacturer’s instructions. CD4+ cells were negatively selected using a combination of CD8a (Ly-2), CD11b (Mac-1), and CD19 Microbeads. CD8+ cells were selected using the same protocol except that CD4 microbeads were used. Alternately, CD4 and CD8 cells were positively selected using CD4
(L3T4) or CD8 Microbeads. Purity of the isolated cells was confirmed by staining with fluorescein isothiocyanate conjugated (FITC) anti-CD4, anti-CD8, and control IgG2a antibodies (all from BD PharMingen, CA) and was consistently between 94%–99%.

**T-Cell Culture and Stimulation With Monoclonal Antibodies**

All the monoclonal antibodies (mAbs) used were from BD PharMingen (San Diego, CA) unless otherwise stated. Combined anti-CD3 and anti-CD28 mAbs were used to provide maximum T-cell receptor (TCR)/costimulation to the CD4 and CD8 T cells as before (11). Briefly, anti-CD3e (2.5 μg/ml final concentration) was diluted in PBS (phosphate-buffered saline) and immobilized to the individual wells of 6-well flat bottom tissue culture plates (Corning Glass Works, Corning, NY) in a final volume of 6 ml. RNAs from the splenic CD4+ and CD8+ lymphocytes of 15 animals were used for the experiment. Total RNA was isolated using Trizol reagent (GIBCO-BRL), followed by an oligo(dT)24 primer containing a T7 RNA polymerase promoter site added 3’ of the indicated time period. RNAs from unstimulated and anti-CD3/anti-CD28-stimulated cells were isolated by TRIzol LS reagent (Life Technologies, Grand Island, NY), and a second cleanup step was performed by using the Qiagen RNeasy Total RNA isolation kit (Qiagen, Valencia, CA). Intracellular proteins were isolated from the phenol–ethanol supernate with isopropyl alcohol after precipitation with ethanol, as per standard protocol.

**RNA Expression by GeneChip Microarrays**

Chemokine gene expression of young and old, unstimulated, and mAbs-stimulated CD4 and CD8 cells was initially screened using the AffyMetrix GeneChip Gene microarray gene expression system (AffyMetrix, Inc., Santa Clara, CA) as before (11). To minimize individual variability, pooled RNAs from the splenic CD4+ and CD8+ lymphocytes of 15 animals were used for the experiment. Total RNA was isolated using Trizol reagent (GIBCO-BRL), followed by clean-up on a RNeasy spin column (Qiagen), then used to generate cRNA probes. Preparation of cRNA, hybridization and scanning of the mouse genome U74A arrays were performed according to the manufacturer’s protocol (AffyMetrix). Briefly, 5 μg of total RNA was converted into double-stranded cDNA by reverse transcription using a cDNA synthesis kit (Superscript Choice System, GIBCO-BRL) with an oligo(dT)24 primer containing a T7 RNA polymerase promoter site added 3’ of the poly T (Genset, La Jolla, CA). Following second-strand synthesis, labeled cDNA was generated from the DNA sample by an in vitro transcription reaction supplemented with biotin-11-CTP and biotin-16-UTP (Enzo, Farmingdale, NY). The labeled cRNA was purified by using RNeasy spin columns (Qiagen). Next, 15 μg of each cRNA was fragmented at 94°C for 35 minutes in fragmentation buffer (40 mM Tris-acetate (pH 8.1), 100 mM potassium acetate, 30 mM magnesium acetate) and then used to prepare 300 μl of hybridization cocktail (100 mM MES, 1 M NaCl, 20 mM EDTA, 0.01% Tween 20) containing 0.1 mg/ml of herring sperm DNA (Promega, Madison, WI), 500 μg/ml acetylated BSA (GIBCO-BRL), and a mixture of control cRNAs for comparison of hybridization efficiency between arrays and for relative quantitation of measured transcript levels. Prior to hybridization, the cocktails were heated to 94°C for 5 minutes, equilibrated at 45°C for 5 minutes, then clarified by centrifugation (16,000 × g) at room temperature for 5 minutes. Aliquots of each sample (10 μg of fragmented cRNA in 200 μl of hybridization cocktail) were hybridized to the mouse genome U74A arrays at 45°C for 16 hours in a rotisserie oven set at 60 rpm. The arrays were then washed with nonstringent wash buffer (6X SSPE) at 25°C, followed by stringent wash buffer (100 mM MES [pH 6.7], 0.1 M NaCl, 0.01% Tween 20) at 50°C, stained with streptavidin-phycoerythrin (Molecular Probes), washed again with 6X SSPE, stained with biotinylated antistreptavidin IgG, followed by a second staining with streptavidin-phycoerythrin, and a third washing with 6X SSPE. The arrays were scanned using the GeneArray scanner (AffyMetrix). Data analysis was performed using GeneChip 4.0 software. The U74A chips contain approximately 12,000 probe sets, with each probe set representing a transcript. Each probe set typically consists of 20 perfectly complementary 25 base long probes as well as 20 mismatch probes that are identical except for an altered central base. We subtract the mismatch probe values from the perfect match values and average the middle 50% of these differences as the expression measure for that probe set. A quantile normalization procedure was used to adjust for differences in the probe intensity distribution across different chips. We then applied a mono-tone linear spline to each chip that mapped quantiles 0.02 up to 0.98 (in increments of 0.02) exactly to the corresponding median quantiles for all the samples. Then, the transform log(100 + max[ X + 100; 0]) was applied to the data from each chip. The chemokine results were then calculated as changes relative to the expression levels of unstimulated young CD4+ and CD8+ lymphocytes.

**RNA Protection Assays**

Changes in T-cell chemokine expression were confirmed by ribonuclease protection assays (PRAs). Pooled RNAs from equal number of purified CD4 and CD8 T cells from young and old mice in groups of 4–6 animals were used to minimize individual variability. The probes were synthesized by modification of the manufacturer’s protocol. Briefly, GACU nucleotide pool and [α-32P]UTP, RNasin, T7 RNA polymerase were added to the multiprobe template set mCK-5 (Ltn, RANTES [regulated upon activation, normal T-cell expressed and secreted], Eotaxin, MIP-1β; [macrophage inflammatory protein], MIP-1α, MIP-2, IP-10 [interferon-γ-inducible protein 10], MCP-1 [monocyte chemoattractant protein], TCA-3 [T-cell activation gene-3]) (BD PharMingen, San Diego, CA) and placed on a heat block at 37°C for 1 hour. Although changes in other chemokines were found in the microarray experiments, the 9 chemokines were chosen because their probes were available commercially. The reaction was terminated by adding DNase, and the samples incubated at 37°C on a heat block for 30 minutes. Appropriate volumes of EDTA, Tris-saturated phenol, chloroform:isoamyl alcohol (CIAA)
(50:1), and yeast tRNA were then added to the mixture, as suggested by the manufacturer. The aqueous layer was extracted by CIAA, then pelleted by adding a 1:5, 4 M ammonium acetate and ice-cold 100% ethanol mixture. Next, 5 μg of total RNA from each T-cell sample was used for hybridization. The protected probes were then allowed to be resolved by electrophoresis using a 5% acrylamide gel, exposed to a phosphor screen, and quantified by a Phosphor-imager using Image Quant software (Molecular Dynamics).

Chemokine Enzyme-Linked Immunosorbent Assays
Changes in RNA expression may not correlate to alteration of protein level due to aging-associated differences in post-transcriptional regulation. Quantitation of MIP-1α and MIP-1β and RANTES protein levels in the supernatant of cultured (at 24 and 48 hours) young and old CD4 and CD8 T cells were therefore measured using commercially available enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN), according to the manufacturer’s instructions.

In Vitro Chemotaxis Assay
Dual-chamber chemotactic assays were performed to compare the transmigration response of splenic lymphocytes from 8-week-old C57BL/6 mice to supernatants from young and old T cells. Briefly, freshly isolated 4 × 10^5 splenocytes in 100 μl RPMI 1640 medium supplemented with 0.5% BSA were placed in Transwell Clear culture inserts with 5 μm pores (Corning-Costar, Cambridge, MA). The inserts were then placed in 24-well tissue culture plate (Corning-Costar) containing 600 μl of supernatants from young and old CD4+ T cells for 5 hours in a humidified incubator at 37°C. Cells from the top and bottom chambers were then harvested and counted with a Beckman Coulter counter as before (11).

Statistical Analysis
The difference between means was tested using Student’s two-tailed t test whenever applicable.

RESULTS
Screening of Chemokine Expression by Microarray
Chemokine gene expression of CD4 and CD8 T cells from young and old animals was initially screened by using microarray. Significant difference is arbitrarily set as changes greater than or equal to twofold as before (11). The results (Figure 1A) showed significant increased expression of selected chemokines including Ltn, RANTES, and MIP-1β, and a smaller increased in MIP-1α in freshly isolated old compared with young CD4 T cells. Old CD8 cells also have a greater than or equal to twofold increased expression of MIP-1α, MIP-1β, and MIP-2 and a smaller increase in Ltn, RANTES, IP-10, and TCA-3 (Figure 1B). SDF-2 expression was similar in young and old T cells. Relative expression of selected chemokines of old and young (= 1) CD4 (Figure 1C) and CD8 (Figure 1D) cells are also shown. Although a number of other chemokines were screened (MCP-1, MCP-2, MCP-3, TCA4, SDF-1, SDF-4, fractalkine) their low level of expression (below the background fluorescence) was considered to be beyond the sensitivity of the assay. Anti-CD3/anti-CD28 stimulation resulted in significant (≥ twofold) increased expression of MIP-1α, MIP-1β, and TCA-3, and decreased expression of RANTES, IP-10, and MIP-2 in both CD4 and CD8 cells. Anti-CD3/anti-CD28 stimulation causes increased Ltn expression in both young (9.4-fold) and old (12.9-fold) CD8 cells but has little effect on CD4 cells.

Chemokine ELISAs
We next determined if the change in chemokine gene expression resulted in altered protein production. RANTES, MIP-1α, and MIP-1β protein levels were assayed in the culture supernatants generated from the anti-CD3/CD28-stimulated (24 and 48 hr) CD4+ and CD8+ young and old T cells. The results confirmed the gene expression results that showed increased RANTES, MIP-1α, and β gene expression by CD4+ and CD8+ T cells from aged compared with young animals (Figure 4).

Chemotactic Assay
Dual-chamber chemotactic assays were done using freshly isolated, unstimulated splenocytes from 8-week-old C57BL/6 mice and supernatant from anti-CD3/anti-CD28-stimulated (24 and 48 hr) CD4 young and old T cells to determine if the observed aged-associated increase in chemokine expression had functional consequence. The results showed that supernatants from aged mice had a significantly greater chemotactic response than that elicited from young mice (Figure 5), confirming that the increased chemokine protein production in aged T cells have an important functional consequence.

DISCUSSION
The basis for the aging-associated increase in disease susceptibility is incompletely understood. Immunosenesence has been put forward as a possible explanation for the increased incidence and poor disease outcome in elderly persons. In particular, changes in the T-cell compartment...
have been studied in great detail and are believed to play a crucial role in explaining the alteration in immune function with age (10). The shift from naïve to memory T-cell phenotype, presumably due to chronic antigen stimulation over time, may account for many functional changes in T cells. In contrast, inconsistent reports of small decline in the number of T-cell subsets are unlikely to have important clinical relevance. Furthermore, attempts to directly correlate currently known age-related changes in immunity with disease susceptibility or outcome in elderly people have been largely unsuccessful.

Despite the growing understanding of the importance of the chemokine system in immunobiology, there remains relatively little information on the consequence of aging in this system. Selected murine studies have provided evidence that aging may affect chemokine expression levels. For example, GRO/CINC-1 (interleukin-8 [IL-8]-like chemokine) gene expression and production are increased in the nasal mucosa of old (~18 months) compared with young rats (12). A similar increase in selected chemokine expression has been demonstrated in the brain of aged rodents (13). However, the effect of aging on murine
immune cell chemokine expression and function is unknown.

In the present study, we provide the first comprehensive determination of the effect of aging on chemokine gene expression in CD4 and CD8 T cells of C57Bl/6 mice using microarray gene scanning and ribonuclease protection assays. C57Bl/6 mice were chosen because this mouse strain has already been used extensively as an animal model to study human aging. We demonstrated that aging is associated with the increased mRNA expression of eotaxin, MIP-2 (macrophage inflammatory protein-2), MCP-1 (monocyte chemotactic protein-1), and T-cell activation gene-3 (TCA-3) are considered too low for reliable quantitation and are therefore excluded from analysis. Lanes 1 are unstimulated young T cells. Lanes 2 are old unstimulated T cells. Lanes 3 are anti-CD3/anti-CD28-stimulated (72 hr) young T cells. Lanes 4 are anti-CD3/anti-CD28-stimulated (72 hr) old T cells. The results represent mean ± SEM (standard error of mean) of 4 independent experiments, and each experiment has 4–6 mice in each age group. Gel-loading is corrected with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and L32 expression. *p < .05; the difference between means of young and old was compared using Student’s t test. RANTES = regulated upon activation, normal T cell expressed and secreted; IP = interferon-γ-inducible protein.

Figure 2. Effect of aging on CD4+ T-cell chemokine expression as measured by ribonuclease protection assays (RPAs). Chemokine gene expression of pooled CD4+ cells from unstimulated (0 hr) and anti-CD3/CD28 stimulated (24, 48, and 72 hr) young (3–4 months), middle-aged (12–14 months), and old (20–22 months) C57Bl/6 mice were measured using the mCK-5 probe set. An autoradiograph of representative RPAs and histograms shows the effect of aging and anti-CD3/anti-CD28 stimulation on CD4+ T cells. The expression levels of eotaxin, MIP-2 (macrophage inflammatory protein-2), MCP-1 (monocyte chemotactic protein-1), and T-cell activation gene-3 (TCA-3) are considered too low for reliable quantitation and are therefore excluded from analysis. Lanes 1 are unstimulated young T cells. Lanes 2 are old unstimulated T cells. Lanes 3 are anti-CD3/anti-CD28-stimulated (72 hr) young T cells. Lanes 4 are anti-CD3/anti-CD28-stimulated (72 hr) old T cells. The results represent mean ± SEM (standard error of mean) of 4 independent experiments, and each experiment has 4–6 mice in each age group. Gel-loading is corrected with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and L32 expression. *p < .05; the difference between means of young and old was compared using Student’s t test. RANTES = regulated upon activation, normal T cell expressed and secreted; IP = interferon-γ-inducible protein.

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The mechanism for the observed enhanced chemokine response in aging is unclear. Th1 and Th2 cells are known to preferentially express specific chemokines (1–3). However, whether aging is associated with a shift from Th1 to Th2 cytokine profile is controversial (10). Parallel increases in inflammatory cytokine responses in both human and murine aging have been documented by our group and others (11,24–28). In earlier studies (11) using the same T cells used in the microarray experiment, we showed that old CD4$^+$ T cells had selected increased proinflammatory cytokines including interferon gamma at both the RNA and protein levels. Changes in cytokine response in aging are accompanied by the accumulation of a memory T-cell subset that exhibits aberrant early signaling events (29–31). This has led to the suggestion that the accumulation of dysfunctional memory T cells may play an important role in the altered cytokine production in aging (32–34). Whether similar mechanisms underlie the current observation of increased CD4 and CD8 T-cell chemokine responses in aging is unknown. A recent study examined chemokine production by T cells from C57Bl/6 mice and showed that RANTES, MIP-1$\beta$, and Ltn mRNAs were primarily expressed in memory T cells (35). This was consistent with other earlier reports (36–38). Thus, our observed differences in T-cell chemokine expression may at least be partly related to the accumulation of memory T cells in aging. Freshly isolated memory T cells contain high levels of RANTES mRNA. However, unlike other β-chemokines (e.g., MIP-1α and MIP-1$\beta$), RANTES protein secretion is regulated at the post-transcriptional level independent of mRNA transcription, most likely through inhibition of the translation of cytoplasmic RANTES mRNA (35). Engagement of TCR releases the RANTES mRNA from this translational silencing. This may provide an explanation for our results.

Figure 3. Effect of aging on CD8$^+$ T-cell chemokine expression as measured by ribonuclease protection assays. Chemokine gene expression of pooled CD8$^+$ cells from unstimulated (0 hr) and anti-CD3/CD28-stimulated (24, 48, and 72 hr) young (3–4 months), middle-aged (12–14 months), and old (20–22 months) C57Bl/6 mice were measured using the mCK-5 probe set. Lanes 1 are unstimulated young T cells. Lanes 2 are old unstimulated T cells. Lanes 3 are anti-CD3/anti-CD28-stimulated (72 hr) young T cells. Lanes 4 are anti-CD3/anti-CD28-stimulated (72 hr) old T cells. The results represent mean ± SEM (standard error of measurement) of 4 independent experiments, and each experiment has 4–6 mice in each age group. Gel-loading is corrected with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and L32 expression. *p < .05; the difference between means of young and old was compared using Student's $t$ test. RANTES = regulated upon activation, normal T cell expressed and secreted; MIP = macrophage inflammatory protein; IP = interferon-γ-inducible protein; TCA = T-cell activation gene-3; MCP = monocyte chemotactic protein.
that showed decreased RANTES mRNA expression but increased RANTES protein secretion following TCR stimulation in both young and old T cells.

Consistent with our data, a number of studies have documented increased serum and T-cell production of selected chemokines in human aging. Serum levels of IL-8 (36) and MCP-1 (37) are increased in elderly humans. Others have shown age-related increased production of unfraccionated peripheral blood cell IL-8, MCP-1, MIP-1α, and RANTES with or without stimulation with anti-CD3 mAb and lipopolysaccharide (LPS) (38). Mariani and colleagues (39) recently examined the RANTES and MIP-1α response in nonagenarians and found increased T-cell and monocyte RANTES and MIP-1α production. Others also demonstrated increased serum MCP-1 in healthy elderly people and RANTES in centenarians (40). "Inflammatory chemokines" such as MIP-1α, MIP-1β, and RANTES are produced by T cells typically in response to infection and to recruit effector cells to sites where pathogens are present (41). The increased chemokine
response seen in murine and human T cells in aging suggest that changes in the chemokine system are not responsible for the worse infection outcome in elderly people. However, it is also clear that excessive proinflammatory chemokine responses may be harmful to the hosts, such as during overwhelming infection and septic shock (42–44). Furthermore, an aging-associated increased chemokine response may also contribute to increased incidence or severity of other T-cell chemokine-dependent diseases in elderly persons. For example, T cells accumulate early in atheroma formation and persist at sites of lesion growth and rupture. T-cell-activating chemokines have been shown to be produced by atheroma-associated endothelial cells, smooth muscle cells, and macrophages, and are believed to play an important role in both early and late plaque formation in both human and murine genetic models of atherosclerosis (45–48). Another example is rheumatoid arthritis. The prevalence of this disease increases with age, and is most common in the most elderly group studied (49,50). The incidence of rheumatoid arthritis also increases with age, with the peak incidence occurring in the seventh and eighth decades (51). Interestingly, older-onset rheumatoid arthritis is also a distinctly different disorder from younger-onset rheumatoid arthritis, with a more abrupt onset of disease (“infectious-like”) (52). The effect of age on experimental arthritis has not been examined extensively, with reports of increased (53) and reduced (54) joint inflammation in aged rodents. Currently known age-related “decline” in immune or hormonal function cannot account for the observed aging-related increased incidence and immune/inflammatory response in this disease. A central role of leukocyte chemokine response in the recruitment and retention of leukocytes in the rheumatoid joint has recently been established (55,56). Taken together, it is tempting to postulate that the age-associated increase in chemokine response may contribute to the increased susceptibility and altered clinical course of these diseases in elderly people.

Summary
We have provided the first comprehensive look at the effect of aging on T-cell chemokine receptor expression in a murine model of human aging. We propose that an increased chemokine response may play a role in T-cell chemokine-dependent diseases in aging.

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Figure 5. Dual-chamber chemotactic assay. The chemotactic response of C57Bl/6 mice splenocytes to supernatants from 24-hour and 48-hour cultures of CD4 T cells of young and old C57Bl/6 mice was compared. Increased chemotaxis is seen at 24 and 48 hours. Results are expressed as mean ± SD (standard deviation) of duplicate determinations. *p < .05.
CHEMOKINES, OR CHEMOTACTIC CYTOKINES, ARE SMALL PROTEINS


