

Chronic lung inflammation in aging mice

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Received 22 June 2007; accepted 28 June 2007

Available online 5 July 2007

Edited by Barry Halliwell

Abstract To determine whether aging is associated with a pro-inflammatory shift in the lung, inflammation and inflammation-related gene expression in the lungs of 12-week-old and 24-month-old Balb/c mice were studied. cDNA microarray and quantitative reverse transcription-polymerase chain reaction analyses showed that eight inflammation-related genes, including CD20, Burkitt lymphoma receptor 1, CXCR-3, provirus integration site for Moloney murine leukemia virus-2, CD72, IL-8RB, C-Fgr, and CD8 β , were upregulated in the aged mice. Immunohistochemistry showed that the lungs of the aged mice contained increased numbers of CD4 cells, CD8 cells, B cells and macrophages. These results suggest that a pro-inflammatory shift occurs in the lungs of mice with aging.

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Keywords: Aging; Inflammation; Lung; Lymphocytes; Macrophages; Cytokine

1. Introduction

Aging is associated with an increased risk of chronic inflammatory lung diseases; in fact, aging per se may be associated with changes in immune function that predispose to these diseases [1]. We hypothesized that normal aging is associated with a pro-inflammatory shift in the profile of lung gene expression. The mouse is a good model of human aging, since murine physiology is very similar to human physiology, and the gene expression profile of mouse overlaps considerably with that of humans [2]. In the present study, a cDNA array technique was used to screen the expression profiles of the genes encoding cytokines, chemokines, their receptors, and other inflammation-related molecules in the lungs of young and aged mice. The results were confirmed using quantitative reverse transcription-polymerase chain reaction (RT-PCR) analyses. We also counted the numbers of inflammatory cells infiltrating the lungs of the mice using a histological technique.

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Abbreviations: RT-PCR, Reverse transcription-polymerase chain reaction; HRP, Horseradish peroxidase; B1r1, Burkitt lymphoma receptor 1; Pim-2, provirus integration site for Moloney murine leukemia virus-2; IGF-BP3, Insulin-like growth factor binding protein 3; BMP-5, bone morphogenetic protein-5; GKLF, gut-enriched Kruppel-like factor; MMP-2, matrix metalloproteinase; Glut4, glucose transporter type 4 isoform; COPD, chronic obstructive pulmonary disease

2. Materials and methods

2.1. Experimental animals

The protocol of the animal experiments was approved by the Institutional Animal Care and Use Committee at the animal facility of Tokyo Women's Medical University. Eight-week-old female Balb/c mice were maintained under specific pathogen-free conditions, with a dark period from 8 PM to 8 AM; they were given free access to water and food. They were housed in accordance with the guideline for rodents used in aging studies on the basis of operational criteria, such as ambient air temperature, relative humidity, lighting levels, sound level, cage size, and ventilation rate [3]. Mice aged 12 weeks and 24 months were sacrificed by intraperitoneal injection of an overdose of pentobarbital, and thoracotomy was performed. The right lung was reserved for RNA analysis and frozen in liquid nitrogen, and the left lung was inflated by manual instillation with 50% Optimal Cutting Temperature compound, quickly frozen, sectioned (3 μ m), acetone-fixed, and processed for immunohistochemistry.

2.2. RNA isolation and cDNA array hybridization

Total RNAs were prepared from the lung tissue using the RNeasy RNA isolation kit (Qiagen K.K., Tokyo, Japan); the mRNAs were isolated by incubation with oligo-dT-magnetic beads (Toyobo Co., Tokyo, Japan). cDNA array hybridization was performed using the GeneNavigator cDNA Array System – Mouse Immunology selected – (Toyobo Co.) according to the manufacturer's instructions [4]. The genes spotted on the filter consist of 538 immunology-related mouse genes plus nine housekeeping mouse genes and two nonmammalian genes as negative controls. A list of the set of genes on the filter is shown at the following web site: (<http://www.toyobo.co.jp/seihin/xr/product/genenavi/genenavigator.html>). Biotin-labeled probes were generated by incorporating biotin-16-deoxyuracil triphosphate during cDNA synthesis. The cDNA reaction was achieved using reverse transcriptase (ReverTraAce- α [®], Toyobo Co). Specific signals on the filters were detected with the Imaging High[®] chemiluminescence detection kit (Toyobo Co.) and a chemiluminescence image of the filter was acquired with the Lumino Imaging Analyzer (FAS-1000, Toyobo Co). Gene expression images were quantified by measuring the intensity of the signals with the Array-Pro[®] Analyzer (Toyobo Co). The intensity of the signals was converted into relative numbers based on the median intensity of the total genes on each filter. To minimize artifacts arising from low expression values, only genes with raw intensity values of greater than the intensity of luciferase and pUC were chosen for the differential analysis.

2.3. Real-time quantitative RT-PCR

Quantitative RT-PCR was performed by monitoring the increase in fluorescence of the SYBR green dye in real time with a Light Cycler system (Roche Diagnostics, Tokyo, Japan). Total RNA was reverse-transcribed with ReverTraAce- α [®] (Toyobo Co). Thermocycling was performed in a 20- μ l volume containing SYBR Green Realtime PCR Master Mix, Primers A and B, and cDNA. The sequences of the PCR primers are shown in Table 1. PCR products were quantified by using lightcycler analysis software, checked by melting point analysis, and normalized to β -actin mRNA expression.

2.4. Immunohistochemistry

Rat monoclonal anti-mouse CD4 (BD Pharmingen, Tokyo, Japan), rat monoclonal anti-mouse CD8 (BD Pharmingen), rat monoclonal

Table 1
Gene-specific primers for real-time quantitative RT-PCR

Gene	Accession no.	Forward primer	Reverse primer
CD20	M62541	5'-CCTCTCTGGGGAGGCATTAT-3'	3'-AATGGCAGCAAAGAGGCTTA-5'
CD8β	X07698	5'-CGATACGGCAAAGATGTCCT-3'	3'-CACTGTCCTCTGGCTTACA-5'
CXCR-3	AF045146	5'-AGCCCTCACCTGCATAGTTG-3'	3'-GCCACTAGCTGCAGTACACG-5'
CD72	J04170	5'-ATAAGTCAGCTGGGGCAGGA-3'	3'-CAGGTTCTCCTTGGTCTCG-5'
Blr1	X71788	5'-CCTCTCCATCCACATCACCT-3'	3'-ACGTAACACCATCCCATCAC-5'
Pim-2	L41495	5'-ACTCAGTCACCTGCCCACTT-3'	3'-GCCACGACTTGGGTAAAGAA-5'
IL-8RB	L23637	5'-GCCCTGCCATCTTAATTCT-3'	3'-CAGTGTGAACCCGTAGCAGA-5'
NF-κB	AF15573	5'-ACCTTTGCTGGAACACACC-3'	3'-GTATCCCTCTCAGGCCCTTC-5'
iNOS	M84373	5'-GACGAGACGGATAGGCAGAG-3'	3'-GCACATGCAAGGAAGGGAAAC-5'
β-Actin		5'-AAATCGTGCGTGACATCAAAG-3'	3'-AAGGAAGGCTGGAAAAGAGC-5'

anti-CD45R/B220 (B-cell marker, BD Pharmingen), rat monoclonal anti-mouse macrophage (BD Pharmingen), goat anti-neutrophil elastase (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), rabbit polyclonal anti-Brl1 (Santa Cruz), and rabbit polyclonal anti-CXCR-3 (Santa Cruz) were used as the primary antibodies. The primary antibodies were reacted either with biotinylated goat anti-rat IgG and streptavidin-Horseradish peroxidase (HRP), with HRP-conjugated donkey anti-goat IgG, or with HRP-conjugated goat anti-rabbit IgG. Immunoreactants were visualized with 3,3'-diaminobenzidine and were counterstained with a hematoxylin solution.

The numbers of CD4⁺ T-lymphocytes, CD8⁺ T-lymphocytes, B-lymphocytes, and macrophages located in the lung parenchyma, airway walls, and artery walls were estimated using an image analysis system (Win Roof Version 3.5; Mitani Corporation, Fukui, Japan). The length of the alveolar wall in 15 randomly selected fields of the lung parenchyma was digitized and the number of positive staining cells in the alveolar wall, or in close contact with it, was counted. A minimum of 10 mm of the alveolar wall was examined per slide, and the data are expressed as cell number/mm of alveolar wall. A minimum of five medium-sized bronchovascular bundles (airway diameter ~400 μm) was selected, and the numbers of positively stained cells in the airway wall and in the artery wall were counted. A minimum of 3000 μm² of the airway wall and a minimum of 8000 μm² of artery wall were studied per slide; the data are expressed as cell number/mm².

2.5. Statistical analysis

To assess the signal intensity of each gene in the young mice ($n = 4$) and the aged mice ($n = 4$) on the cDNA array analysis, the Mann-Whitney U test was used; genes with significantly different intensities

($P < 0.05$), either with an average fold difference of 2.5 or more or an average fold difference of 0.4 or less, were designated as being differentially expressed. In the quantitative RT-PCR analysis, the data are reported as the average differential expression. In the analysis of immunohistochemical data, the number of infiltrating cells was compared between young mice ($n = 5$) and aged mice ($n = 6$) using Welch's t -test.

3. Results and discussion

In the cDNA microarray analysis, 15 genes were differentially expressed in the aged mice compared to the young mice (Table 2 and Table 1E in the online supplement). Nine genes were upregulated (≥ 2.5 -fold) in the aged mice. Eight of them encoded pro-inflammatory molecules, such as B-cell signaling molecules (CD20 and CD72), a T-cell signaling molecule (CD8β), chemokine receptors (Burkitt lymphoma receptor 1 (Blr1), CXCR-3, IL-8RB), a chemokine signaling molecule (C-Fgr), and a lymphocyte pro-survival kinase (provirus integration site for Moloney murine leukemia virus-2, Pim-2), and one of the upregulated genes encoded the anti-inflammatory transcription factor IκB-ε. Seven out of the nine upregulated genes were chosen for quantitative RT-PCR verification, which corroborated the cDNA microarray data

Table 2
Differences in gene expression in the lungs of aged mice compared to young mice

Gene	Accession no.	Function	Differences (-fold)	
			Microarray	qRT-PCR
<i>Upregulated</i>				
CD20	M62541	B cell signaling	10.5	4.5
Blr1	X71788	Chemokine receptor	7.6	5.6
CXCR-3	AF045146	Chemokine receptor	4.3	4.1
Pim-2	L41495	Lymphocyte pro-survival kinase	4.0	2.5
CD72	J04170	B cell signaling	3.9	7.0
IL-8RB	L23637	Chemokine receptor	3.8	2.8
C-Fgr	X16440	Chemokine signaling regulator	3.0	N.D.
IκB-ε	AF03089	Regulatory transcriptional factor	2.7	N.D.
CD8β	X07698	T-cell signaling	2.6	2.2
<i>Down-regulated</i>				
IGF-BP3	X81581	Cell growth regulator	0.27	N.D.
BMP-5	L41145	Cell growth regulator	0.36	N.D.
GKLF	U20344	Cell growth regulator	0.38	N.D.
MMP2	M84324	Matrix metalloprotease	0.39	N.D.
Glut4	M23383	Glucose metabolism	0.40	N.D.
GRB14	AF155647	Cell growth regulator	0.40	N.D.

Gene expression patterns of aged mice ($n = 4$) were compared to those of young mice ($n = 4$) by the mouse immunology GeneNavigator cDNA Array and real-time quantitative RT-PCR (see Section 2.5). Results are expressed as average fold difference for each gene in aged compared to young mice. N.D., not done.

(Table 2). Genes whose expression was down-regulated (≤ 0.4 -fold) in the aged mice were genes coding cell growth/metabolism regulators (insulin-like growth factor binding protein 3 (IGF-BP3), bone morphogenetic protein-5 (BMP-5), gut-enriched Kruppel-like factor (GKLF), matrix metalloproteinase (MMP-2), glucose transporter type 4 isoform (Glut4), and GRB14). These results suggest that expression of pro-inflammatory genes in the lung is upregulated during normal aging.

A quantitative analysis of immunostained lung tissue sections showed that compared with the lungs of the young mice, the lungs of the aged mice contained increased numbers of CD4⁺ T-lymphocytes, CD8⁺ T-lymphocytes, B-lymphocytes, and macrophages in their alveoli, airway wall, or artery wall (Fig. 1 and Table 3). The number of neutrophils was not counted, since because very few neutrophils were present in the lungs of the mice examined in this study. Immunohistochemistry of lung tissue section also showed that the lungs of aged mice contained increased numbers of cells immunoposi-

tive for Blr or CXCR-3, corroborating the results of cDNA microarray and quantitative RT-PCR (Fig. 1E and Table 2E in the online supplement).

These results demonstrate that a pro-inflammatory shift develops in the lungs with aging. This may reflect not only the aging process but also the lung's response to airborne pollutants encountered over the mice's life span. For example, chronic exposure to dust from bedding or pathogens other than for those tested may have exacerbated lung inflammation. Aging-associated inflammation is not peculiar to the lung; it appears to be present in many organs. In fact, the results of our study corroborate those of previous studies showing that pro-inflammatory genes are upregulated in the brain, heart, liver, and blood vessel walls in aged rodents [5–7]. Studies in humans have also shown that the plasma levels of the pro-inflammatory cytokines IL-6 and TNF α increase with age, and that the increases are correlated with morbidity and mortality [8,9]. These findings, together with our own, suggest that

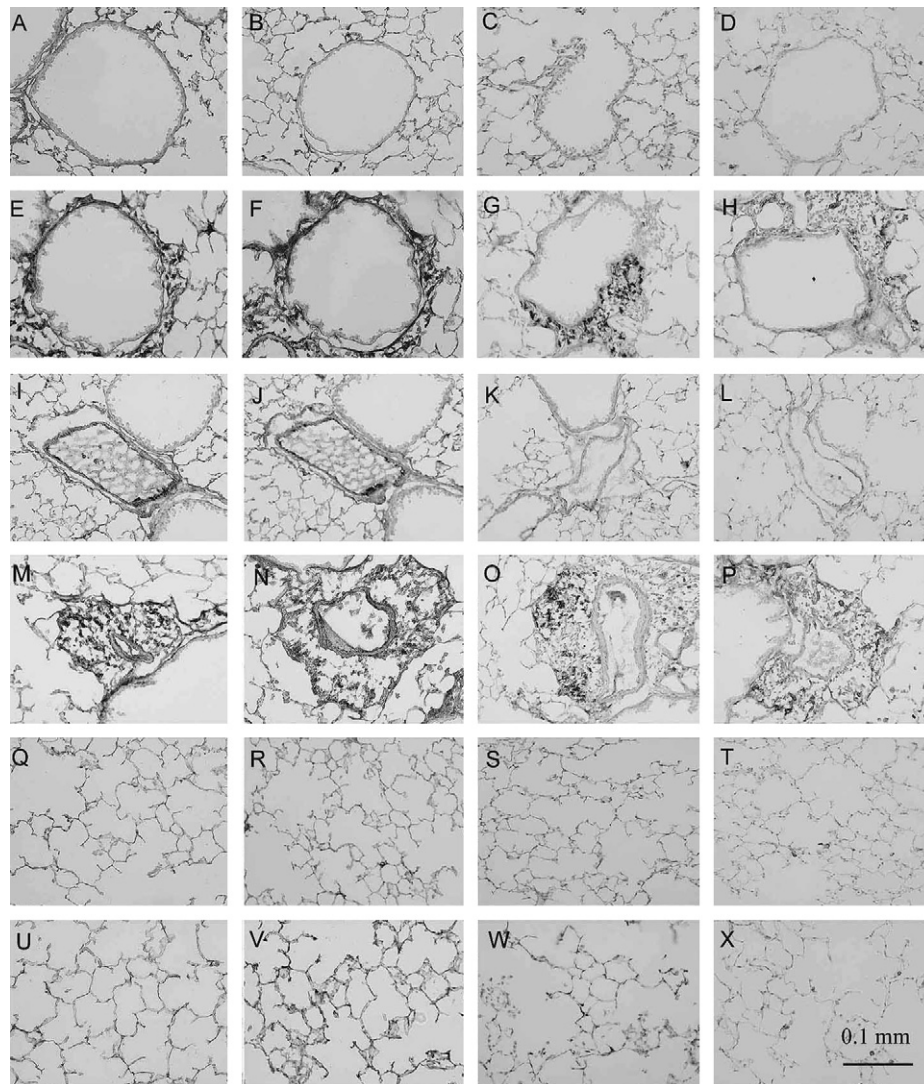


Fig. 1. Representative photomicrographs of lung tissue sections (original magnification $\times 200$) immunostained for CD4⁺ T-lymphocytes (A, E, I, M, Q, and U), CD8⁺ T-lymphocytes (B, F, J, N, R, and V), B-lymphocytes (C, G, K, O, S, and W), and macrophages (D, H, L, P, T, and X) in young (A–D, I–L, and Q–T) and aged (E–H, M–P, and U–X) mice. (A–H) Airways, (I–P) pulmonary arteries, (Q–X) lung parenchyma. In the original photomicrographs, the immunopositive cells are stained brown. These photomicrographs are representative of the results from six aged mice and five young mice.

Table 3
Numbers of inflammatory cells in the lungs of young and aged mice

	Lung parenchyma (mm of alveolar wall)		Airway (mm ² of airway wall)		Pulmonary artery (mm ² of artery wall)	
	Young	Aged	Young	Aged	Young	Aged
CD4	0.96 ± 0.03 vs. 1.60 ± 0.31 (1.7-fold)		85.0 ± 3.6 vs. 306.6 ± 101.7 (3.6-fold)		64.9 ± 22.7 vs. 988.4 ± 295.7* (15.2-fold)	
CD8	0.81 ± 0.05 vs. 1.71 ± 0.2** (2.1-fold)		93.7 ± 19.8 vs. 198.6 ± 58.3 (2.1-fold)		106.2 ± 29.9 vs. 543.1 ± 82.4** (5.1-fold)	
CD4/CD8 ratio	1.21 ± 0.1 vs. 0.99 ± 0.24		1.10 ± 0.25 vs. 1.52 ± 0.23		0.83 ± 0.27 vs. 1.65 ± 0.35	
B cells	0.41 ± 0.04 vs. 1.15 ± 0.10** (2.9-fold)		50.9 ± 6.3 vs. 307.7 ± 74.4* (6.0-fold)		77.3 ± 13.3 vs. 1237.9 ± 301.5* (16.0-fold)	
Macrophages	0.66 ± 0.11 vs. 1.01 ± 0.16 (1.5-fold)		36.6 ± 7.3 vs. 105.5 ± 14.6** (2.9-fold)		42.6 ± 6.9 vs. 138.6 ± 59.9 (3.3-fold)	

Data are expressed as means ± S.E.M. Values in parenthesis are the mean differences in aged mice ($n = 6$) compared to young mice ($n = 5$).

* $P < 0.05$.

** $P < 0.01$.

normal aging is associated with a chronic inflammatory state that develops both locally and systemically. The results of our study of healthy aged mice may have clinical relevance to the aging process of humans, since the BAL fluid of seemingly healthy, elderly, never-smokers contains a higher number of neutrophils, lymphocytes, and activated T-cells, and a higher concentration of immunoglobulin and IL-6 than that of young, healthy, never smokers [1,10].

The pro-inflammatory genes that are upregulated in the lungs of aged mice encode signaling molecules that are known to activate inflammatory cells to migrate, proliferate, and survive. For example, CD20 and CD72 are expressed by B-lymphocytes and have been shown to transmit signals that stimulate the proliferation of B-lymphocytes [11,12]. B1r1 (CXCR-5), CXCR3, and IL-8R (CXCR-2) are chemokine receptors, and after binding to their ligands, they have been shown to elicit signals leading to migration of B-lymphocytes, T-lymphocytes, and phagocytes, respectively. Similarly, C-Fgr, a member of the Src gene family kinases, has been shown to stimulate chemotaxis by phagocytes [13]. Moreover, Pim-2 is a serine/threonine kinase that phosphorylates different signaling molecules, most of which have been reported to promote the proliferation and survival of lymphocytes and phagocytes [14].

Recent studies have suggested that the mechanisms of age-related inflammatory responses involve activation of NF- κ B and the expression of inducible NO synthase (iNOS) [15]. However, the present study's results of the cDNA microarray analysis of gene expression in the lung did not reveal any age-related increases in the transcriptional levels of NF- κ B family members (NF- κ B p52: 1.5-fold, $P = 0.9$; RelB: 0.9-fold, $P = 1.0$) or iNOS (0.4-fold, $P = 0.2$). The cDNA microarray data were corroborated by the results of the quantitative RT-PCR, which did not show any significant increases in the transcription levels of NF- κ B p100 (1.6-fold, $P = 0.16$) or iNOS (1.7-fold, $P = 0.3$). Alternatively, the pro-inflammatory shift during aging may be the results of altered gene expression in senescent cells that accumulate in aging tissue [16]. In fact, senescent cells have recently been shown to exhibit a pro-inflammatory phenotype that contributes to the etiology of tissue inflammation [17,18].

The pro-inflammatory state of aged lungs may be beneficial because it prevents microbial invasion. On the other hand, it can also be harmful, because it may predispose to the develop-

ment of chronic inflammatory lung diseases, such as chronic obstructive pulmonary disease (COPD), interstitial pneumonia, and persistent lower respiratory tract infection, whose prevalence increases with age.

Acknowledgement: This work was partly supported by a Grant to the Respiratory Failure Research Group from the Ministry of Health, Labour and Welfare, Japan.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.febslet.2007.06.075](https://doi.org/10.1016/j.febslet.2007.06.075).

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