Decreased c-rel activation contributes to aberrant interleukin-2 expression in CD4+T cells of aged rats

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A B S T R A C T
Studies indicated significantly decreased expression of interleukin-2 (IL-2) with age. This decrease could be a major contributory factor to the increased frequency of morbidity and mortality among the elderly. C-rel is a key coregulator of IL-2 expression. However, it is unknown whether aging inhibits normal c-rel activation, thereby decreasing production of IL-2. We analyzed the dynamics of IL-2 expression in CD4+T cells from different aged rats (young group: around 6 months (n=6), aged group: around 24 months (n=6)). The expression of the CD3 receptor and CD28 receptor in the CD4+T cells was assessed by flow cytometry. Translocation of c-rel and its protein level in the cytoplasm and nucleus at different time points were detected by confocal microscopy and Western blotting. Chromatin immunoprecipitation (ChiP) was used to analyze the status of c-rel binding to the IL-2 promoter region in the different aged rats. Our results showed the CD4+T cells from young rats and aged rats showed different expression kinetics of IL-2 after stimulation. The expression level of IL-2 was higher in young rats compared with aged rats at 24 h and 48 h. Data showed lower CD3 receptor expression on CD4+T cells from aged rats compared with young rats. Although the CD28 receptors declined on the aged CD4+T cells, the difference was not significant. After stimulation for 0.5 h, more c-rel was translocated into nucleus markedly compared with that in the aged group. ChiP showed that in aged CD4+T cells, c-rel DNA binding was inhibited compared with that in young cells. Therefore, reduced IL-2 production in activated CD4+T cells from aged rats is associated with concomitant impairments in the activation of c-rel.

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1. Introduction

Significant increases in human longevity raise the crucial issue of healthy aging. IL-2 is a hallmark in maintaining T cell homeostasis (Malek, 2008) and plays a key role in the proliferation and differentia-
tion of T cells, in the activation of Th1 and Th2 cells (Karanfilov et al., 1999) and in immunological responses. Clinical studies (Larbi et al., 2004) and veterinary animal models (Adolfsson et al., 2001) have demonstrated that activated T cells exhibit decreased IL-2 production in response to mitogenic and antigenic stimuli with aging. Dysregulation of IL-2 bioavailability during aging has profound consequences, including influenza infection (Thompson et al., 2003), neoplasms (Provinciali et al., 2000) and lethal autoimmunity (Zheng et al., 2007). Activated peripheral CD4+T cells mainly produce IL-2; however, regulation of its production is complex. One important cause of the decline in IL-2 production during aging may be related to underlying disturbances in transcriptional regulatory proteins needed for the normal expression of IL-2.

The promoter region of the IL-2 gene mediates transcriptional regulation of IL-2 mRNA and contains DNA recognition sequences that bind several transcription factors, including transcription factor nuclear factor (NF)-kappa B (Park et al., 2009; Weaver et al., 2007). After stimulation, the free NF-κB heterodimer is translocated into the nucleus where the NF-κB subunits engage cognate κB enhancer elements and modulate the transcription of IL-2 or the CD25/IL-2 receptor alpha chain (Oh et al., 2010; Algarte et al., 1995). However, reports of impaired IL-2 production by rat CD4+T cells during aging being linked to aberrant activation of NF-κB are conflicting. Whisler et al. (1996) indicated that the age-related
alterations in the basal and stimulated levels of NF-κB p50 did not correlate with the levels of IL-2 produced by stimulated T cells from elderly human using electrophoretic mobility shift assays (EMSA). Another report from Haynes showed that age-related defect in CD4+ T cell response also occurs in vivo and that it is correlated with reduced NF-κB activation in mice, also by EMSA (Haynes et al., 2004). NF-κB consists of a variety of canonical Rel-domain family dimers. Of these, only p50-p65 and p50-c-rel play an activating role in IL-2 gene regulation (Rothenberg and Ward, 1996). However, c-rel is required for a correctly altered chromatin state across the IL-2 proximal promoter in CD3/CD28-activated primary T cells in preference to other NF-κB proteins (McKarns and Schwartz, 2008; Rao et al., 2003).

In this study we examined the activation of c-rel in aged rat CD4+ T cells and the correlation with impaired of IL-2 expression.

2. Materials and methods

2.1. Animals

Young (4 mo) and aged (24 mo) male Sprague Dawley (SD) rats, purchased from the Animal Center of the Chinese Academy of Sciences, were housed in filtered cages maintained at a constant temperature (23 °C) with a 12-h light–dark cycle and fed autoclaved forage and water ad libitum. The Animal Care and Use Committee of Shanghai University of Traditional Chinese Medicine, in accordance with the National Research Council’s Guide for the care and use of laboratory animals, approved all animal handling and conditions. Rats exhibiting tumors, splenomegaly, grossly visible skin lesions, or significant pathology were excluded from the study.

2.2. Flow cytometry assay of a subset of T cells

Lymphocytes were isolated and the different subsets of T cells were counted from whole blood. Lymphocyte surface expressions of CD3+CD4+ and CD28+CD4+ were determined using three-color direct immunofluorescence assays. Peripheral blood mononuclear cells (PBMCs) were stained for 30 min at 4 °C with APC-anti-CD3 and FITC-anti-CD4 or PE-anti-CD28 and FITC-anti-CD4 monoclonal antibodies or related isotype controls (Ebioscience, Mountain View, CA, USA). The concentration of each antibody was 0.5 μg/106 cells. After washing three times with PBS containing 0.1% bovine serum albumin (Sigma–Aldrich, St. Louis, MO, USA), lymphocytes were re-suspended in 0.5 ml PBS–BSA and the cytometric analysis was performed using a flow cytometer (FACScalibur, BD Biosciences, San Jose, CA, USA).

Forward scatter against FITC fluorescence was used to identify and gate the CD4 positive and CD4 negative lymphocytes. The surface expression of CD3 or CD28 was determined on the CD4 positive gate. Data from 10,000 gated mononuclear lymphocyte events were acquired for analysis. Total numbers of lymphocytes expressing the measured cell-surface antigens were determined by the percentage values obtained from the corresponding total lymphocyte and lymphocyte subset counts.

2.3. Sorting of CD4+ T cells by fluorescence activated cell sorting (FACS)

Spleens were collected aseptically and minced. Single-cell suspensions were obtained in phosphate-buffered saline (PBS) by passage through 100 μm pore size Nitex nylon fabric. Lymphocytes were isolated on a Ficoll gradient and then stained with an anti-CD4 antibody directly conjugated with fluorescein isothiocyanate (FITC) (Ebioscience, Mountain View, CA, USA) for 30 min on ice in PBS with 5% fetal bovine serum (FBS), followed by washing with PBS/bovine serum albumin (BSA). After washing, the cells were filtered through a 100 μm pore size Nitex nylon fabric and resuspended in PBS with 1% FBS. FACS analysis was performed using a BD FACSARIA™ III instrument (BD Biosciences). The purity of the isolated CD4+ T cells was assessed in every experiment and ranged from 96% to 99%.

2.4. Cell culture

Purified CD4+ T cells were cultured in sterile, endotoxin-free cRPMI 1640 media (Gibco, Grand Island, NY, USA) supplemented with 10% heat-inactivated FBS, 2 mM glutamine, antibiotics (all from Life Technologies, Grand Island, NY, USA) at 37 °C in 5% CO2. For stimulations via the T cell receptor (TCR), all the cells were stimulated with 5 μg/ml plate-bound anti-CD3e antibody (Ebioscience) and 2 μg/ml soluble anti-CD28 antibody (Ebioscience) at a density of 2 × 106 cells/ml in 24-well flat-bottom plates (Corning, Corning, NY, USA). Cultures were harvested at the indicated time points.

2.5. RNA purification and real-time quantitative RT-PCR

Total RNA was extracted using Trizol (Life Technologies) in accordance with the manufacturer’s instructions and treated with RNase-free DNase to remove residual genomic DNA. First-strand cDNA synthesis reactions were conducted using KOD plus following standard protocols (Toyobo Co., Ltd., Osaka, Japan). SYBR® Green Realtime PCR analysis was conducted using the Applied Biosystems PRISM 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA). PCR was performed in a 25 μl reaction mix containing: 30 ng of cDNA with PCR grade water, 10 μl 2 x Quanti Tect SYBR Green PCR master mix (TOYOBO), 0.4 μl Forward primer and 0.4 μl Reverse primer (designed by Sangon Biotech, Shanghai, China). The reaction conditions comprised: an activation step at 95 °C for 15 min; 10 gradient cycles of 95 °C for 0.5 min, 60 °C for 0.5 min and 72 °C for 0.5 min (−0.5 °C per 0.5 min), 40 cycles of 95 °C for 0.5 min, 55 °C for 0.5 min, 72 °C for 0.5 min and 60 °C for 0.75 min, were performed, with a final extension step at 72 °C for 10 min and a dissociation step at 95 °C for 0.25 min, 60 °C for 1 min and 95 °C for 0.25 min.

All primer sets used gave no signal in the control reactions lacking template. Dissociation-curve analysis showed that each primer set generated single products with the expected Tm values. To normalize the samples, control β-actin (ACTB) Cβ values were subtracted from the IL-2 Cβ values for each sample (ΔCβ). Then, ΔCβ of the non-stimulated sample was subtracted from ∆Cβ of the stimulated sample (Δ∆Cβ). The relative levels of IL2 mRNA were calculated as 2−ΔΔCβ.

The primers used were as follows:

<table>
<thead>
<tr>
<th>IL2</th>
<th>Forward (F):</th>
<th>Reverse (R):</th>
</tr>
</thead>
<tbody>
<tr>
<td>II-2</td>
<td>F: aagcttggtgaggttgactc</td>
<td>R: atagtctgttagctaggtcta</td>
</tr>
<tr>
<td>β-Actin</td>
<td>F: cacccgaggatcccacctt</td>
<td>R: cccatatcaccacccatcacc</td>
</tr>
</tbody>
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2.6. Western blotting analysis

CD4+ T cells nuclear and cytoplasmic proteins were extracted using an NE-PER kit (Pierce Biotechnology, Inc., Rockford, IL, USA). Nuclear and cytoplasmic proteins were analyzed by Western blotting for NF-κB p65 nuclear translocation. Protein content was determined by using the BCA assay (Pierce Biotechnology). Sample buffer (0.1 M Tris–HCl, 20% glycerol, 4% SDS, and 0.01% bromophenol blue) was added to the supernatant fractions from the lysates. Samples were boiled for 5 min. Aliquots of 80 μg protein were subjected to 10% SDS-PAGE. The separated proteins were transferred to a PVDF membrane (Roche Diagnostics Corporation, Indianapolis, IN, USA) for 1 h at 350 mA. The membrane was pre-blocked with 5% nonfat milk or 5% (w/v) BSA in TBS containing 0.1% Tween 20,
incubated with anti-c-rel (Cell Signaling Technology, Danvers, MA, USA) at 4 °C with gentle shaking, overnight. Each membrane was washed three times for 5 min and incubated with the secondary horseradish peroxidase-linked antibody (Cell Signaling Technology). Quantitation of detected bands was performed with the Scion Image analysis software (Scion Corp., Frederick, MD, USA). Each density was normalized by the intensity of the corresponding β-actin protein band (40 μg sample) or H2AX (40 μg sample) protein band as internal controls. The density of the control group for relative comparison was standardized as 1.0 to compare across groups.

2.7. Confocal laser scanning microscopy

CD4+ T cells were stimulated for 1–3 h with plate-bound anti-CD3 and soluble anti-CD28. After being fixed in 4% paraformaldehyde for 15 min, the fixed cells were resuspended in Permeabilization Wash Buffer (Biologend, San Diego, CA, USA) and centrifuged at 350 × g for 5 min. Cells were then stained intracellularly using 1:1000 anti-c-rel (Cell Signaling Technology) on ice in the dark for 30 min. After washing, the slides were incubated for 30 min with 1:2000 Alexa Fluor 555 donkey anti-rabbit (H+L) (Life Technologies). For a negative control, we used mouse IgG (BD Biosciences) in place of anti-c-rel monoclonal antibodies. DAPI (Life Technologies) was used as a nuclear stain. Imaging was performed using confocal microscopy at 60× magnification (Confocal Fluorescence Imaging Microscope, Leica TCS-SPS) and in the fluorescent mode (Leica, Heidelberg, Germany).

2.8. Chromatin immunoprecipitation (ChIP) assay

The status of c-rel binding on the IL-2 promoter region was detected using an EZ-Magna ChIP™ A/G Chromatin Immunoprecipitation Kit (Millipore, Billerica, MA, USA), following the protocol provided by the manufacturer. For crosslinking, 2 × 10^6 cells were resuspended in warm medium containing 1% formaldehyde for 15 min at 37 °C. Cells were lysed in lysis buffer containing a proteinase inhibitor cocktail and sonicated 12 times for 10 s each to produce a chromatin size of 100–500 bp. ChIP antibodies were directed against c-rel (Cell Signaling Technology). Real-time PCR analysis of ChIP samples was performed using the Applied Biosystems PRISM 7900HT Sequence Detection System. PCR was performed in 25 μl reactions with SYBR Green (TOYOBO). All the primer sets used produced no signal in control reactions lacking template. Only primer sets yielding slopes close to the theoretical values of 1.9- to 2-fold amplifications per cycle were used. The amount of each sequence in the input and precipitated DNA was calculated from the Ct for each primer set. The relative units recovered for each primer set were determined by dividing the calculated amount of precipitated sequence by the amount of that sequence in the input DNA. The results are shown as the means ± SD of three independent experiments.

2.9. Statistical analysis

Comparisons of data from experimental groups and the control group were analyzed with a two-tailed Student’s t-test using SPSS 18.0. A p-value of 0.05 was considered statistically significant.

**Fig. 1.** IL2 mRNA expression was determined by quantitative real-time PCR (qRT-PCR) at the indicated time points after anti-CD3e/CD28 stimulation in different groups (A: young group; B: aged group; PTX: PTX group). The results shown are the means ± SD of three independent experiments. qRT-PCR indicated lower expression of IL-2 mRNA in both the young and aged group T cells after stimulation for 0.5 h, which markedly increased after 4 h, peaking at 24 h. The quantity of IL2 mRNA at 24 h and 48 h was lower in the aged group compared with the young group (**p < 0.01 vs. young group; *p > 0.05 vs. young group; n=6). After treatment with 50 μmol/L PTX, CD4+ T cells expressed less IL-2 at every time point. Data showed the IL2 expression ability of young CD4+ T cells were higher than aged CD4+ T when costimulated with anti-CD3ε and anti-CD28, and the classical c-rel pathway was involved in the expression of IL2.

**Fig. 2.** The percentage of peripheral blood CD4+ T cells expressing CD3 and CD8 in the young and aged rats as analyzed by flow cytometry. Statistically significant differences are indicated by *p < 0.05 between the young and aged subjects. There were significance differences in the percentage of CD4+ T cells counts between young and aged rats. The subset of CD3 cells on the CD4 positive gate declined in the aged rats compared with the young rats (p < 0.05). CD28 receptor expression also declined in the aged CD4+ T cells, but with no statistical difference between the younger and older subjects (p > 0.05).
3. Results

3.1. IL-2 expression declined in rat CD4+T cells of various ages

The primary CD4+T cells purified from splenocytes were costimulated with both anti-CD3ε and anti-CD28 antibodies. Total RNA and supernatant were purified from stimulated and non-stimulated cells. Fig. 1 shows that costimulation of CD4+T cells induced IL-2 mRNA transcription markedly in both age groups after 4 h, peaking at 24 h. The quantity of IL-2 mRNA at and after 24 h was lower in the aged group than in the young group. To reveal whether NF-κB was involved in the TCR-induced activation of CD4+ cells, we treated the CD4+T cells from young rats with 50 μmol/L pentoxyfylline (PTX group) 30 min before costimulation. We found that the expression of IL-2 decreased, indicating that the classical c-rel pathway is involved in the expression of IL-2. These results were confirmed in two other independent experiments.

3.2. CD3 expression declined in aged rat CD4+T cells

PBMCs were isolated from the blood of different rat groups (young group and aged group). After staining with FITC-anti-CD4, APC-anti-CD3, and PE-anti-CD28, the percentage values obtained from the lymphocyte subset counts were divided by corresponding total lymphocytes. Fig. 2 shows that CD3 receptor expression declined significantly in the CD4+T cells of aged rats, compared with those of young rats.

**Fig. 3.** C-rel translocation analyzed by confocal microscopy and Western blotting (A: young group; B: aged group; PTX: PTX group). (a) Confocal microscopy showing the merged results. C-rel is shown in red and the nucleus in blue, with the merged results in purple. C-rel is clustered in the cytoplasm of the non-stimulated cells in both groups, showing strong red staining in the cytoplasm around the blue nucleus. After stimulation with anti-CD3ε/anti-CD28 for 30 min, strong purple staining in the nuclear region, indicating that stimulation induced translocation of c-rel. However, after treatment for 1.5 h, c-rel translocated back to the cytoplasm. The cells previous treated with 50 μmol/L PTX translocated weakly. Original magnification: ×60. The results demonstrated that costimulation with anti-CD3ε/anti-CD28 could induce c-rel translocation quickly and transiently. (b) Western blotting analysis of c-rel protein levels in the cytoplasm and nucleus. Before stimulation, the c-rel protein is highly expressed in the cytoplasm and only slightly in the nucleus. After costimulation for 30 min, c-rel expression increased in the nucleus significantly. In the aged group, there was a significant difference in translocation behavior compared with the young cells after stimulation (**p<0.01 vs. young group; *p<0.05 vs. young group; n=6). Western blot analysis suggested that aging markedly attenuated the translocation activity of NF-κB induced by anti-CD3ε/anti-CD28 (c). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)
The percentage of CD4+T-cells expressing CD28 for the aged rats also declined in aged rats, but not statistically significantly (p > 0.05).

3.3. C-rel nuclear translocation declined in the stimulated aged rat CD4+T cells

C-rel translocation represents its activation from the cytosol to the nucleus. Cells were analyzed by confocal microscopy (Fig. 3a). C-rel was highly clustered in the cytoplasm of the primarily cells in both age groups. Stimulation with anti-CD3e/anti-CD28 for 0.5 h induced c-rel translocation; however, there were no detectable levels of IL-2 production in either age group at that time point. After treatment for 1.5 h, c-rel translocated back to the cytoplasm. In addition, the cells previous treated with 50 μmol/L pentoxifylline showed weak translocation. We then investigated c-rel protein levels in the cytoplasm and nucleus at the same time (Fig. 3b). In the primarily cells both from young and aged rats, the c-rel protein was expressed highly in the cytoplasm and slightly in the nucleus. After costimulation for 30 min, c-rel expression decreased in the cytoplasm and increased in the nucleus significantly (Fig. 3c). Furthermore, in the aged group, there was a significant difference in translocation behavior compared with the young model under stimulation (p < 0.05, n = 6). The Western blot analysis suggested that aging markedly attenuated the translocation of NF-κB induced by anti-CD3e/anti-CD28.

3.4. C-rel binding on the IL-2 promoter region declined in the aged rat CD4+T cells

Nuclear extracts were generated before or after costimulation with anti-CD3/anti-CD28 and examined by ChIP. DNA-binding activity was undetectable before stimulation, but increased markedly after costimulation for 30 min. There was no difference in the basal levels of c-rel DNA binding activity on the IL-2 promoter between young and aged CD4+T cells (Fig. 4b). In stimulated aged CD4+T cells, c-rel DNA binding was blocked in comparison with young cells. These results indicated that part of the classical NF-κB pathway that regulates IL-2 gene expression in CD4+T cells is impaired in aged rats. The defective NF-κB DNA binding could be responsible for the inability of the aged cells to produce IL-2.

4. Discussion

Aging is associated with impairment of T-cell functions, in particular the decline in the ability of T lymphocytes to produce IL-2 on mitogenic stimulation. It is considered a major contributory factor to the increased frequency of morbidity and mortality among the elderly. IL-2 gene regulation is subject to dual control, its expression depends acutely on signaling by particular combinations of pathways. NF-κB is an important transcriptional coregulators that binds immediately upstream of the transcriptional site of IL-2 gene after activation. However, there are conflicting results about whether aberrant NF-κB activation contributes to impaired IL-2 production by T-cells with aging, although there have been some reports on the contribution of inflammation factors to IL-2R expression (Whisler et al., 1996; Haynes et al., 2004). In the NF-κB family, p50 homodimers, p50–p65 heterodimers, and p50–c-rel heterodimers are most often found in T-cells. Of these, only p50–p65 and p50–c-rel play an activating role in IL-2 gene regulation. C-rel is required for a correctly altered chromatin state across the IL-2 proximal promoter in CD3/CD28-activated primary T cells. Other NF-κB proteins, including p65 (Rel A), are not sufficient to generate an accessible chromatin configuration across the promoter, being dispensable for IL-2 transcription (Rao et al., 2003). Thus, the correlation between aberrant activation of NF-κB with aging impairment is dimers-specific.

The aim of this study was to evaluate the possibility that impairments in IL-2 production by aged CD4+T cells is linked to underlying defects in the activation of NF-κB. The results showed that the IL-2 mRNA expression displayed a different kinetics in CD4+T cells from young and old mice: CD4+T cells from old rats produce less IL-2 upon costimulation with anti-CD3/anti-CD28 as compared to CD4+T cells from young rats at various stages.

However, in a number of systems, both naive and memory T cells from aged animals exhibit decreased responses compared with the same populations from young mice. Reports showed that both naive and memory cells from aged mice are hyporesponsive to costimulation by anti-CD28 mAB compared with T cells from young rats (Engwerda et al., 1994). In primary T cells, we showed that there was no IL-2 expressed. Simultaneous stimulation of the T-cell receptor (TCR)/CD3 complexes and costimulatory receptors such as CD28 induced the activation of cytosolic tyrosine kinases, such as Lck, ZAP70 and Syk (Weiss and Littman, 1994). These protein kinases and phosphatases initiate signal transduction cascades leading to the optimal activation of NF-κB, which ultimately controls the transcription of cytokines such as IL-2, and T-cell proliferation (Wang et al., 2004). In our experiment, CD3 receptor expression declined significantly and CD3/CD28 costimulation initiated signal transduction cascades leading to activation of IκB kinase (IKK), which perhaps represents one explanation for the
abnormal NF-κB activation in the aged rat T cells. IKK then phosphor- lates I-κBs, triggering rapid phosphorylation, dissociation and subsequent degradation of I-κB (Wang et al., 2004; Schaecher et al., 2004). Activated NF-κB complexes rapidly translocate into the nucleus, where they bind DNA at NF-κB--binding motifs, modulating the transcription of numerous genes involved in immune and inflammatory responses. At 0.5 h of stimulation, there were very weak or no detectable levels of IL-2 production in both age groups. At that time, significant amounts of c-rel had translocated from the cytoplasm to the nucleus indicating that activation of c-rel occurs before IL-2 expression. Pentoxifylline (PTX), a xanthine derivative, is a selective suppressor of c-rel (but not the other NF-κB family members) translocation to the nucleus (Wang et al., 1997). Here, PTX inhibited the translocation of c-rel in vitro and was negatively correlated with IL-2 mRNA expression, indicating the inhibition of c-rel might contribute to age-related impairments in IL-2 expression, as has described for some other inflammatory mediators or costimulatory responses (Goodman and Stein, 1994; Engwerda et al., 1994) in which NF-κB appears to be involved.

Our results showed that the DNA-binding activities of c-rel play non-redundant roles in the transcriptional activity of the IL-2 gene. Reduced IL-2 production by activated CD4⁺T cells from aged rats was associated with concomitant impairments in the activation of c-rel.

Conflict of interest

The authors declare no conflict of interest.

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