Chronic resveratrol intake reverses pro-inflammatory cytokine profile and oxidative DNA damage in ageing hybrid mice

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Abstract Thymic involution and shrinkage of secondary lymphoid organs are leading causes of the deterioration of the T-cell compartment with age. Inflamm-aging, a sustained inflammatory status has been associated with chronic diseases and shortened longevity. This is the first study to investigate the effect of treating aging hybrid mice with long-term, low-dose resveratrol (RSV) in drinking water by assessing multiple immunological markers and profiles in the immune system. We found that hybrid mice exhibited marked age-related changes in the CD3+CD4+, C3+CD8+, CD4+CD25+, CD4M and CD8M surface markers. RSV reversed surface phenotypes of old mice to that of young mice by maintaining the CD4+ and CD8+ population in splenocytes as well as reducing CD8+CD44+

(CD8M) cells in the aged. RSV also enhanced the CD4+CD25+ population in old mice. Interestingly, pro-inflammatory status in young mice was transiently elevated by RSV but it consequently mitigated the age-dependent increased pro-inflammatory cytokine profile while preserving the anti-inflammatory cytokine condition in the old mice. Age-dependent increase in 8OHdG, an oxidative DNA damage marker was ameliorated by RSV. Immunological-focused microarray gene expression analysis showed that only the CD72 gene was significantly down-regulated in the 12-month RSV-treated mice compared to age-matched controls. Our study indicates that RSV even at low physiological relevant levels is able to affect the immune system without causing marked gene expression changes.

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Introduction

Recent studies indicate that nearly every component of the immune system undergoes dramatic age-associated reorganization, leading to diminished overall functions (Weiskopf et al. 2009). Immunosenescence is defined as the state of dysregulated immune function that contributes to the increased susceptibility of the elderly to infection and to autoimmune disease and cancer (Pawelec 1999). Indeed, it has been proposed that immunosenescence is part of gradual developmental processes, encompassing complex events such as compensatory mechanisms including qualitative alterations in function (Globerson and Effros 2000).

One of the most characterized changes during immunosenescence is age-associated thymic involution which is a progressive reduction in size of the thymus and replacement of lymphoid by fat tissue (Aw et al. 2008). This is associated with the loss of thymic epithelial cells and impairment in thymopoiesis (Aspinall and Andrew 2000; Aw et al. 2008). The decline in the output of newly developed T cells results in a diminished number of circulating naive T cells (CD45RA+; Pfister et al. 2006) and the accumulation of expanded clones of memory (CD45RO+) and effector T cells (Hannet et al. 1992; Saule et al. 2006).

Continuous antigenic challenge can result in a progressive pro-inflammatory status, which appears to be a major characteristic of the immune-aging process. This phenomenon has been termed, “inflamm-aging” (Franceschi et al. 2000a). Studies have shown that centenarians largely escaped major age-related diseases (Bonafe et al. 2001) that are characterized by a complex remodeling of immune responses (Franceschi et al. 2000b). Innate immunity is a major component of inflammation and a chronic, low-grade inflammatory status appears to be a key component of the most common age-related diseases, such as diabetes, osteoporosis and osteoarthritis, dementia, cardiovascular diseases, and cancer (Sansonetti et al. 2007).

Retardation or reversal of some of the changes associated with immunosenescence in rodents provides

the basis for the hypothesis that improvement of the immune function by diet intervention can contribute to better health and extended lifespan (Miller 1996; Walford 1982). So far, no long-term study has been conducted to examine the effects of resveratrol (RSV), a putative calorie restriction mimetic on the aging immune system. Caloric restriction studies done in rodents and primates were found to preserve and maintain several parameters of the immune function well into advanced age, at a level typically seen in healthy animals (Chen et al. 1998; Nikolic-Zugich and Messaoudi 2005; Pahlavani 2004).

The goals of this study were fourfold: (1) to determine whether splenocyte T cell subsets and cytokine levels in 12- (young), 18- (middle-aged), and 30-month (old) animals show clear age-dependent changes in genetically heterogeneous four-way cross F2 hybrid mice. To address this, we measured proportions of the major T-lymphocyte subsets including: CD4+ and CD8+ cells, memory T cells (CD4+CD44+ (CD4M) and CD8+CD44+ (CD8M)), CD4+CD25+ and CD4+69+ under basal and activated conditions, (2) to examine the effects of RSV on the T-lymphocyte surface marker phenotypes and cytokine profiles with respect to the age-related changes and finally, to explore possible mechanisms in the long-term low dose RSV intake in mice by (3) measuring the levels of oxidative DNA damage in T lymphocytes and (4) investigating gene expression changes using microarray.

Materials and methods

Animals

F2 hybrid four-way cross mice (CB6F1 x C3D2F1 (C3H x DBA/2)) of three age groups (Fig. 1) were obtained from the National Institute of Aging (Bethesda, MD, USA). Upon arrival, animals were kept in an AAALC-accredited facility at the Biopolis Resource Centre, Singapore on a daily cycle of alternating 12-h periods of light and dark under specific pathogen-free conditions. All mice received food and drinking water ad libitum. All mice were given the standard rodent chow diet (Teklad 2018 Global Rodent, Harlan Madison, WI, USA). RSV-treated mice received RSV in their daily drinking water equivalent to an average daily intake of 1.50–

Fig. 1 Age cohorts of the hybrid mice and the study durations. All mice were given food and water ad libitum. The RSV mice were given RSV in their daily drinking water from the starting point until the end point of the study

2.27 mg/kg body wt. This water was prepared fresh every 2–3 days. RSV concentration and stability in the solution was monitored for 5 days and we found no reduction in resveratrol concentration over a period of 3 days under our study conditions (20–23°C, protected against UV light in animal drinking bottles). Concentration of RSV in drinking water during the study was determined by gas chromatography with mass spectrometer and was found to be 14.09 ± 3.35 mg/L and was stable over a 3-day period (mean $\pm$ SD; $n=10$, different study days; Wong et al. 2009). All procedures were performed in compliance with relevant regulations approved by the Institutional Animal Care and Use Committee of the Biological Resource Centre, Singapore.

Tissue collection

Animal sacrifices were performed approximately at the same time in the mornings to minimize any day-to-day variations in the levels of oxidative damage biomarkers. Following intraperitoneal anesthesia with a mixture of ketamine (150 mg/kg) and xylazine (10 mg/kg), blood was withdrawn via cardiac puncture and spleen was aseptically removed. A small section of the spleen was rinsed with ice cold $1 \times$ phosphate buffer saline, blotted dry and flash frozen in liquid nitrogen followed by storage at -80°C until further analysis. The remaining spleen was stored in

ice-cold complete RPMI-1640 medium and splenocytes were isolated within 2 h of dissection.

Splenocyte and T cell isolation

Spleens were kept in sterile ice-cold RPMI-1640 medium upon harvest and used immediately.

Splenic lymphocytes were isolated under sterile conditions by a procedure described previously (Ansar Ahmed et al. 1989). Briefly, spleen tissues were gently teased on a $40 \mu\text{m}$ mesh cell strainer (BD Bioscience, CA, USA). Cells were washed twice in RPMI-1640 media and the cell pellet was suspended in 3 ml of $1 \times$ PharmLyse and incubated at room temperature for 5 min. After incubation, the cells were washed two times (400 g, 5 min, 4°C) in RPMI-1640 medium. The splenocytes were resuspended in complete RPMI-1640 medium containing 10% FBS (Sigma), 2 mM L-glutamine (ICN, Costa Mesa, CA, USA), 50 IU/ml penicillin (ICN), and 50 $\mu\text{g}/\text{ml}$ of streptomycin (ICN) and the numbers and viability were assessed by the trypan blue exclusion method. Half of the splenocytes were used for further T cell purification process while the remaining splenocytes were used for T cell surface marker phenotyping and cytokine profiling assays. Splenocytes were maintained on ice during the entire isolation procedure with the exception of the erythrocyte-lysis step.

Surface marker phenotyping and intracellular cytokine staining for lymphocytes

Splenocytes were divided into six aliquots with the following conditions applied in duplicates: (1) cells activated with phorbol 12-myristate-13-acetate (PMA, Sigma; 1 μ l of 200 μ g/mL; 20 ng/mL), ionomycin (Sigma; 1 μ l of 1 mM; 0.1 μ M) and Brefeldin A (Pharmingen; 10 μ l of 1.0 mg/ml; 1 μ g/mL). These cells were used for surface marker phenotyping and intracellular cytokine assays; (2) cells activated with PMA (1 μ l of 200 μ g/mL) and ionomycin (1 μ l of 1 mM). These cells were used for surface marker phenotyping and extracellular cytokine profiling assays and (3) unstimulated cells, containing only complete RPMI-1640 medium as controls. This was used for basal surface marker phenotyping assay. All cells mentioned are considered as unstimulated unless otherwise stated. All cells were incubated at 37°C in a humidified 5% CO₂ incubator for 6 h and immediately harvested for further surface marker and intracellular cytokine staining analysis. After centrifugation, culture medium from conditions (2) and (3) were aliquoted and kept at –80°C until further analysis for extracellular cytokine profiling assay using the Luminex-Bioplex multiplexing bead assay. Cells were collected and stained for intracellular cytokines as well as for surface marker subsets, according to Collins et al. (1998) with minor modifications. Surface marker phenotyping and intracellular cytokine staining were performed according to the following conditions: cells from (1) were divided into four aliquots containing either unstained cells, anti-CD69-PE-Cy7/anti-CD4-APC-Cy7/anti-IL-2-APC/IL-4-PE, anti-CD69-PE-Cy7/anti-CD4-APC/anti-IL-5-APC/IL-6-PE or CD69-PE-Cy7/CD4-APC-Cy7/IL-10-APC/IFN- γ -PE fluorescent-conjugated monoclonal antibodies (Pharmingen, Becton–Dickinson, CA, USA). Cells from (2) and (3) were divided into 4 aliquots respectively. Each aliquot contained either unstained cells, CD3-PE/CD4-APC-Cy7/CD8-PE-Cy7, CD4-APC-Cy7/CD25-PE-Cy7/CD44-APC/CD69-PE or CD8-APC-Cy7/CD25-PE-Cy7/CD44-APC/CD69-PE fluorescent-conjugated monoclonal antibodies (Pharmingen, Becton–Dickinson, CA, USA).

For surface marker staining, cells were incubated in BD staining buffer (Pharmingen, Becton–Dickinson, CA, USA) containing the above fluorochrome-

conjugated antibodies or the respective isotype controls (Pharmingen, Becton–Dickinson, CA, USA) for 30 min at 4°C. After staining, cells from (2) and (3) were washed with BD staining buffer and resuspended in 1% paraformaldehyde for the final flow cytometry analyses. Cells from (1) used for intracellular cytokine staining were incubated in BD Cytofix/Permeabilising Solution (Pharmingen, Becton–Dickinson, CA, USA) for 20 min at 4°C after staining for surface markers. The cells were then washed with 1 $\times$ BD permeabilising wash buffer and incubated with 4% of paraformaldehyde solution for 15 min at 4°C. Cells were resuspended in BD staining buffer and kept overnight at 4°C. On the following day, the cells were incubated in 1 $\times$ BD Permeabilising Wash Buffer (Pharmingen, Becton–Dickinson, CA, USA) for 15 min at 4°C. After the removal of 1 $\times$ BD Permeabilising Wash Buffer, anti-cytokine mAbs or the matching blocking control antibodies (Pharmingen, Becton–Dickinson, CA, USA) were added to the different aliquots. Cells were further incubated for 30 min at 4°C, washed with 1 $\times$ BD permeabilisation wash buffer and fixed in 1% paraformaldehyde. To examine cell surface molecules and intracellular cytokines, three- and four-color analyses were performed using the BD FACSArray software (Becton–Dickinson) and lymphocytes were gated according to the forward and side scattering properties. Quadrants and gates were set on the basis of mouse isotype/blocking control antibodies.

Extracellular cytokine profiling assay

The culture supernatant from the cells in (2) and (3) were assayed for IL-2, IL-4, IL-5, IL-6, IL-10, IFN- γ , and tumour necrosis factor- α (TNF- α) using the Bio-Plex multiplexing suspension array technique according to the manufacturer's instructions (BioRad Ltd, USA).

8-hydroxy-2'-deoxyguanosine

DNA was extracted and purified in a method previously described (Gedik and Collins 2005) with some modifications. Briefly, 100 mg of frozen spleen was pulverized in liquid nitrogen. The tissue was resuspended in ice-cold buffer A (10 mM Tris, 0.32 M sucrose, 5 mM MgCl₂, 0.1 mM deferoxamine mesylate (DEF), 0.1 mM diethylenetriamine penta-acetic acid (DTPA), and 1% Triton X-100; pH 7.5).

The nuclei were pelleted by centrifugation at 1,500×*g* for 5 min (4°C). After removing RNA and proteins using RNase and Proteinase-K, respectively, the mixture was cooled to 4°C and DNA was precipitated using cold NaI solution (40 mM Tris, 20 mM Na₂EDTA, 7.6 M NaI, 0.3 mM DEF, 0.3 mM DTPA; pH 8.0) and 2-propanol. The DNA pellet was washed with ice-cold 40% 2-propanol and 70% ethanol (−20°C). The ethanol was removed and the DNA was left to dry in the tube for 5 min. The DNA was suspended in 0.1 mM DEF, immediately frozen, and stored at −80°C. For hydrolysis, the enzymatic digestion was done as previously reported (Wong et al. 2009). Briefly, 30 µg of DNA was digested using DNase I, nuclease P1, alkaline phosphatase, and phosphodiesterase I at 37°C. Samples were kept in the 4°C autosampler of the HPLC system until injection. Samples were analyzed using the HPLC system (Model 2695, Waters Corp., USA) coupled with a UV-photodiode array (PDA-UV/VIS, Model 2996, Waters Corp., USA) and an electrochemical detector (Model 2465, Waters Corp., USA). Data from both the UV and EC detectors were acquired using the Waters Empower Software (Waters Corp., version 1.6). The degree of DNA damage was expressed as the ratio of 8-hydroxy-2'-deoxyguanosine (8OHdG) nmol to 10⁶ nmol of 2-deoxyguanosine.

cDNA microarray analyses on purified T splenocytes

Mouse-specific topic-defined PIQOR™ Immunology Microarrays (Miltenyi Biotec, Cologne, Germany) consisting of 1,076 selected cDNA fragments were used to compare gene expression profiles of middle-aged hybrid mice: array 1 comprises T cells isolated from splenocytes of five LT RSV mice and array 2 comprises T cells isolated from splenocytes of control mice. To obtain purified T cells, single-cell suspension of the splenocytes were processed according to the MACS Pan T Cell Isolation Kit manual (Miltenyi Biotec GmbH). Finally, T cells were adjusted to 1×10⁶ cells/ml in MACS PrepProtect Stabilization Buffer (Miltenyi Biotec GmbH) until further cDNA microarray analysis. RNA was isolated using standard RNA extraction protocols (NucleoSpin RNA II, Macherey-Nagel). Hybridization and post-hybridization washes were carried out according to the PIQOR User manual. Subsequently, fluorescent-labeled samples were hybridized overnight to the topic defined

PIQOR Immunology Microarrays Human Antisense using the a-Hyb Hybridization Station. Slides were scanned by Miltenyi Biotec on a ScanArray 4000 Lite scanner (Perkin-Elmer, Wellesley, MA, USA). ImaGene software version 4.1 (BioDiscovery, El Segundo, CA, USA) was used for signal quantification and analysis as described. Normalized ratios are shown as Cy5 signal intensity divided by Cy3 signal intensity of the respective gene.

Gene expression analysis

To better understand the biological meaning of our cDNA microarray results, the gene expression data was analyzed using GeneSpring GX 10.0 and potentially important genes were identified by sorting quantitative and statistical results by *P* value.

Statistical analyses

All data were analyzed using SPSS for Windows version 13.0. All data are presented as means ± SEM unless otherwise specified. Variables were continuous and normality was ascertained using Kolmogorov–Smirnov test. All data are found to be normally distributed except data from the multiplexing extracellular cytokine assay. For normally distributed data which met the equal variances criteria, the Student's *t* test was used to determine significant difference between the different aged groups and between control and RSV-treated mice. Similarly, non-parametric Mann–Whitney *U* test was applied for the multiplexing extracellular cytokine data. *P*<0.05 was considered as statistical significant.

Results

T cell subsets within the spleen across the age spectrum

In control mice, we examined different T cell subsets in splenocytes taken from young, middle-aged, and old hybrid mice (Fig. 2). In unstimulated cells (in the absence of *in vitro* stimulation with PMA-ionomycin and CD69 expression), we observed a significant age-dependent decrease in the number of CD4 T-lymphocytes (old vs. young, *P*<0.01) and a concomitant increase in CD8 T-lymphocytes with age (old vs. young, *P*<0.05).

RSV treatment did not produce significant effects in these cell subsets for young and middle-aged mice. By contrast, in old mice, RSV treatment resulted in a restoration in the number of CD4 T-lymphocytes to a level similar to that of young control mice (cell population percentage of 18.1% and 18.0% in old RSV and young control mice, respectively). The elevated population of CD8 T-lymphocytes in old mice was significantly reduced by the RSV treatment to a level found in middle-aged mice (cell population percentage of 17.1% in old RSV and 16.8% in middle-aged control mice, respectively). Unlike unstimulated cells, we observed no age-related changes in activated CD4 T-lymphocyte and CD8 T-lymphocyte populations (with co-expression of CD69). RSV treatment increased the basal levels of CD8 T-lymphocytes by 32.8% ($P < 0.01$) in old mice but had no effect in young and middle-aged mice.

While there was no age-related trend in unstimulated CD4+CD25+ cells, we detected a significant increase in activated CD4+CD25+ cells in old mice when compared to the young mice ($P < 0.05$). RSV treatment increased the populations of these cells in old mice compared to the aged-match controls ($P < 0.05$).

There was an elevated population of unstimulated CD4+CD69+ cells with age (old vs. young, $P < 0.01$). RSV treatment resulted in an enhanced level in young mice ($P < 0.05$) when compared to age-matched controls, but had no effect in middle-aged or old mice. However, there was an age-dependent reduction in the number of activated CD4+CD69+ cells (old control vs. young control, $P < 0.05$). Interestingly, this age-dependent decrease was ameliorated in middle-aged 12-months RSV-treated mice (cell population percentage of 9.7% in control and 13.4% in RSV mice, respectively, $P < 0.05$).

Fig. 2 T cell surface marker phenotyping for non-activated (a, b) and activated (c, d) T-helper and T-cytotoxic lymphocytes as assessed by CD3+CD4+, CD3+CD8+, CD4+CD25+, CD4+CD69+, CD4+CD44+, and CD8+CD44+ markers, respectively, measured as percentage of gated lymphocytes. Values indicate

mean $\pm$ SEM. Significant difference: a, b, c $P < 0.05$ and d, e, f $P < 0.01$ between the control mice: young and middle-aged, middle-aged and old, old and young; * $P < 0.05$, ** $P < 0.01$ between RSV and age-matched control mice

There were significant increases in the proportion of unstimulated CD4M (CD4+CD44+; old vs. young, $P<0.05$) and CD8M (CD8+CD44+; old vs. young, $P<0.01$) cells with age. While young RSV-treated mice exhibited an increase in CD4M cells ($P<0.05$, age-matched controls), this was not seen in the older groups. On the other hand, the RSV treatment resulted in reduced levels of CD8M in LT RSV and old RSV mice ($P<0.05$, $P<0.05$), compared to age-matched controls. Activated CD4M and CD8M cells did not project significant age-related or RSV effects. Overall, RSV helped maintain the juvenile status of old mice splenocytes in terms of combating the age-dependent changes in CD4 T-lymphocytes and CD8 T-lymphocytes.

Intracellular cytokines: ageing trends and the effects of chronic RSV intake

The three pro-inflammatory cytokines examined here were IL-2, IL-6, and IFN- γ , with the latter an important Th1 signatory cytokine. Th2 anti-inflammatory cytokines, IL-4, IL-5, and IL-10 were also investigated. Figure 3 dot plots show representative profiles of the percentage of IFN-gamma and IL-4 cells gated on the CD4+ population across the different age spectrum. The aging trends and the effects of the RSV treatment on the intracellular cytokine profiles in the hybrid mice are depicted in Fig. 4a and b, respectively.

The intracellular cytokine staining for IL-2 in the CD4+ cells did not reveal any significant age-dependent trend in the hybrid mice. However, surprisingly, young mice treated with RSV exhibited a marked elevation of IL-2 level (32.7% increase, $P<0.01$) compared to age-matched controls.

Similar to IL-2 CD4+ cells, there was no age-related trend across all mice cohorts for IL-6 positive CD4+ cells. RSV treatment reduced the intracellular IL-6 cytokine levels consistently in all age groups when compared to age-matched controls (percentage of reduction): old RSV mice (82.6%, $P<0.01$)>LT RSV (73.8%, $P<0.05$)>middle-aged RSV mice (46.2%, $P<0.05$)>young RSV mice (34.6%, $P<0.05$).

An age-dependent elevation of IFN- γ positive CD4+ cells was apparent in control mice: between young and old mice (73% increase, $P<0.01$) and between middle-aged and old mice (79.8% increase, $P<0.05$). RSV treatment enhanced intracellular IFN- γ level significantly in young mice (53.0% increase; $P<$

0.01), but paradoxically, caused a reduced secretion in LT RSV (38.8%, $P<0.05$) and old RSV mice (39.3%, $P<0.05$) when compared to age-matched control mice.

Overall, RSV treatment enhanced intracellular pro-inflammatory cytokine environment in young mice (i.e., increased levels in IL-2 and IFN- γ) but attenuated the age-dependent elevation seen in middle-aged and old mice (i.e., reduction in IFN- γ and IL-6).

In the anti-inflammatory cytokine category, while there were no clear age-related trends in the IL-4 positive CD4+ cells in the hybrid mice (Fig. 3b), T lymphocytes from young RSV mice exhibited a significant 82.7% increase in IL-4 CD4+ level compared to young controls ($P<0.01$). In contrast to young mice, a dramatic reduction in this cytokine level was demonstrated in old RSV mice when compared to old control mice (51.6% reduction, $P<0.05$).

The IL-5-positive CD4+ cells showed a more complex pattern in the control mice where there was a rise from young to middle-aged mice (280.0% increase, $P<0.01$) but a decline from middle-aged to old mice (57.5% reduction, $P<0.05$). The LT RSV treatment partially prevented this raise in the middle-aged mice with a 54.7% lower IL-5 CD4+ level compared to age-matched controls ($P<0.05$).

The IL-10 profile displayed a significant age-related increase with a 170% and 85.2% elevation in young to middle-aged and middle-aged to old cohorts, respectively ($P<0.05$; $P<0.01$). Young RSV-treated mice exhibited a 170% increase ($P<0.01$) in the IL-10 CD4+ level compared to young controls. However, RSV treatment prevented the age-dependent increases of the IL-10 cytokine production in LT RSV and old RSV mice, respectively, when compared to age-matched controls (48.1% reduction, $P<0.01$; 47.0% reduction, $P<0.01$).

Overall, RSV treatment increased IL-4 and IL-10 CD4+ populations in young animals but lowered IL-4 cytokine profile in old mice, while maintaining consistent populations of IL-5 and IL-10 CD4+ in old mice to levels similar to middle-aged mice.

Extracellular cytokines: ageing trends and the effects of chronic RSV intake

In addition to intracellular cytokines, we also examined extracellular cytokine secretions of activated CD4+ splenocytes across different age cohorts

Fig. 3 Dot plots from flow cytometer to assess Th1/Th2 cytokines with CD4+ T cell surface marker phenotyping in PMA-ionomycin activated splenocytes for intracellular cytokine staining of IFN-γ (a) and IL-4 (b) in young and old (control and RSV) mice

Fig. 4 CD4⁺ T cell surface marker phenotyping for activated mice splenocytes for intracellular cytokine staining as analyzed using the flow cytometry (**a**, **b**) for IL-2, IFN- γ , IL-6, IL-4, IL-5, IL-10 and extracellular cytokine secretion profiles using multiplexing bead array technique (**c**, **d**) for IL-2, IFN- γ , TNF-

α , IL-4, IL-6, IL-10 across the different age groups. Values indicate mean $\pm$ SEM. Significant difference: a, b, c P < 0.05 and d, e, f P < 0.01 between the control mice: young and middle-aged, middle-aged and old, old and young; * P < 0.05, ** P < 0.01 between RSV and age-matched control mice

(Fig. 4c, d). In control mice, IL-2 secretion showed a decreasing trend with age with a marked difference recorded between young and middle-aged mice (60.3% reduction, P < 0.05) as well as between young and old mice (60.5% reduction, P < 0.01). In comparison to controls, RSV treatment elevated IL-2 secretions significantly in LT RSV mice (27.7% increase, P < 0.05) and old RSV mice (71.3% increase, P < 0.05).

IFN- γ secretions in control mice exhibited significant age-dependent elevations between middle-aged and old mice, and between old and young mice (216% increases, respectively, P < 0.01). Although RSV treatment had no significant effect in young mice, it prevented the age-dependent IFN- γ increase

in the LT RSV and old RSV mice, respectively (62.1% reduction, P < 0.01 and 46.1% reduction, P < 0.01).

Similar to IFN- γ , TNF- α levels were also significantly elevated with age in control mice. A 50.6% and 52.2% increase was seen between the middle-aged and old mice (P < 0.01) and between young and old mice (P < 0.01), respectively. Middle-aged mice with 6 or 12 months RSV intake exhibited consistent attenuation in the TNF- α levels, respectively (29.6% reduction, P < 0.01 and 43.2% reduction, P < 0.01). This profile was also seen in old mice treated with RSV (45.8%, P < 0.01) when compared with age-matched controls.

The decreasing levels of IL-6 secretion with age in controls were significant between the middle-aged and the old mice (57.6% reduction, $P<0.01$) and between the old and young mice (60.7% reduction, $P<0.01$). Young RSV-treated mice exhibited attenuated IL-6 secretion compared to age-matched controls (32.1% reduction, $P<0.01$). However, in old mice, RSV treatment augmented IL-6 levels dramatically (105.1% increase, $P<0.05$) compared to old control mice, to a level similar to that of young control mice.

In general, we found age-dependent decreases in IL-2 and IL-6 levels. RSV partially restored the level in old mice while effectively maintained the IL-6 secretion in old animals. Both IFN- γ and TNF- α secretions exhibited an evident age-related increase and RSV ameliorated these elevated levels in LT RSV and old mice.

Old control mice exhibited a significant decrease of IL-4 secretion with a reduction of 62.8% ($P<0.05$) between middle-aged and old mice. Similarly, a marked decline between young and old mice was also observed for this cytokine (60.9% reduction, $P<0.01$). RSV treatment resulted in a 40.5% ($P<0.05$) and 77.7% ($P<0.01$) increase in young and old mice, respectively, compared to age-matched controls.

IL-10 production showed a strong age-dependent increase of 209.8% ($P<0.01$) between middle-aged and old mice and 184.9% ($P<0.05$) between young and old mice. RSV treatment ameliorated the increasing levels of IL-10 significantly in middle-aged and old mice by a 48.3% ($P<0.01$) and 20.7% ($P<0.05$), respectively.

Overall, RSV treatment fully restored the production of IL-4 in old mice which suffered an age-dependent decrease. The steep increase of IL-10 in old mice was dampened by RSV treatment. LT RSV treatment also resulted in a lower IL-10 production in middle-aged mice.

Age-dependent changes in 8OHdG levels and the effects of RSV treatment

The oxidative DNA damage marker, 8OHdG was evaluated for the three age cohorts in mice spleen. There was a clear age-dependent increase in the basal levels of 8OHdG in control mice (Fig. 5a). Middle-aged spleen demonstrated a marked 282.3% increase compared to young controls ($P<0.01$). Similarly, there was a 69.9% increase in 8OHdG level from

middle-aged to old spleen ($P<0.01$). Overall, a significant difference between the RSV-treated and control mice across the age cohorts ($P<0.05$) was observed. Twelve months RSV treatment markedly decreased the 8OHdG levels in LT RSV mice (50.6%, $P<0.01$). 8OHdG levels in middle-aged and old mice also appeared lower after 6-month RSV treatment although these effects were not significant (18.9% reduction, $P=0.144$ and 21.6% reduction, $P=0.077$, respectively; Fig. 5b).

cDNA microarray analysis

We quantified RSV-mediated gene expression changes related to immune function and inflammation using mouse-specific topic-defined PIQORTM Immunology Microarray assay consisting of 1,076 selected cDNA fragments. For the first time, a cDNA microarray was carried out in purified T lymphocytes from mice which have undergone long-term RSV treatment. Out of the 1,076 genes surveyed, we found robust downregulation (>2 -fold change, p value <0.01) in only one gene (CD72) or 0.1% of the genes surveyed. By slightly relaxing the cutoff criterion (allowing ≥ 1.5 -fold changes), two additional genes, FLAP, and HSF4 were found that were significantly upregulated ($P<0.01$). Interestingly, none of the cytokines identified as exhibiting statistically significant changes at the protein level in response to RSV treatment as mentioned earlier were amongst the differentially expressed genes (Table 1).

Discussion

Advanced age associated with a dysregulation of the immune system, termed ‘immunosenescence’ has been known to contribute to the morbidity and mortality associated with infectious diseases of older adults (McElhaney and Effros 2009; Ostan et al. 2008). One of the consistent changes of the ageing vertebrate immune system is the dramatic involution of the thymus (Miller 2002). The characteristics of age-related thymic involution is a reduction in tissue mass, loss of tissue structure and abnormal architecture and a decline in thymocyte numbers, leading to a decrease in naive T-cell output (Aw et al. 2008). It is the age-associated decline in naive T-cell output that many have argued contributes to the features of

Fig. 5 **a** Oxidative DNA damage measured using the 8OHdG HPLC assay in mice spleen across different age groups. Values indicate mean $\pm$ SEM. Overall, there is significant difference using two-way ANOVA between different age groups, $P < 0.01$,

and an overall significant difference between control and RSV-treated mice, $P < 0.05$; **b** percentage change in 8OHdG levels in spleen between control and RSV-treated mice for 6 and 12 months of treatments, respectively, using t test

immunosenescence, in particular the reduced vaccine efficacy associated with ageing (Aw et al. 2007; Pawelec et al. 2005).

Among these changes, altered T cell function represents one of the most consistent and dramatic

effects during aging (Alberti et al. 2006). To understand the role of T lymphocytes cells during immunosenescence, we analyzed changes in surface markers as well as type 1 and type 2 cytokine production in the CD4 T-lymphocyte subsets from

Table 1 Gene expression changes with >1.2 -fold change ($P < 0.05$) in response to long-term resveratrol (12 months) treatment in middle-aged mice compared to age-matched controls

Name	Fold change	Regulation	<i>p</i> value	Reference sequence
CD72	2.02	↓	0.0221	NM_007654
NM_007654	1.45	↓	0.0045	NM_021501
GADD153	1.32	↓	0.0054	NM_007837
CD79B	1.30	↓	0.0388	NM_008339
IL12RB2	1.25	↓	0.0014	NM_008354
MBD1	1.24	↓	0.0000	NM_013594
DNAJC2	1.24	↓	0.0202	NM_009584
CSF2	1.23	↓	0.0186	NM_009969
TSGA14	1.23	↓	0.0169	NM_031998
SOS2	1.23	↓	0.0455	–
TNFC	1.23	↓	0.0033	NM_008518
PTGFR	1.23	↓	0.0102	NM_008966
FLAP	1.74	↑	0.0061	NM_009663
HSF4	1.62	↑	0.0065	NM_011939
KAI1	1.45	↑	0.0212	NM_007656
IL1R1	1.43	↑	0.0459	NM_008362
STAT3	1.38	↑	0.0193	NM_011486
CCL3	1.32	↑	0.0039	NM_011337
CBLN1	1.32	↑	0.0483	NM_019626
C1QA	1.29	↑	0.0013	NM_007572
PRKCB_4	1.28	↑	0.0363	NM_008855
IFNGR1	1.28	↑	0.0480	NM_010511
ADCY8	1.25	↑	0.0146	NM_009623
TCF3	1.21	↑	0.0298	NM_011548

↓ and ↑ arrows indicate downregulation or upregulation of gene expression, respectively

spleens of hybrid mice of different age groups (Fig. 1). We also evaluated the possible effects of RSV on age-related phenotyping shifts between T-lymphocytes, as well as pro-inflammatory and anti-inflammatory cytokine profiles with respect to the relationship to the oxidative DNA damage marker.

Age-related changes and the effects of RSV on T cell surface marker phenotypes

Twelve to thirty month-old mice used in our experiment exhibited typical age-related changes in the distribution of the main T-lymphocyte populations. With advancing age we observed: (1) decreases in the percentage of CD4 T-lymphocytes and activated CD4+CD69+ cells and (2) increases in the proportion of CD8 T-lymphocytes, activated CD4+CD25+ and activated memory T-lymphocytes expressing CD4+CD44+ (CD4M) and CD8+CD44+ (CD8M) markers.

Distinctive changes in immune cell populations with advanced age that have been reported previously and these include an increase in the proportion of memory T cells, with a corresponding decrease in naive T cells in (Castle 2000; Miller 1996). The increase in the proportion of memory T cells (CD4M and CD8M) and a significant reduction in the proportion of CD4 T-lymphocytes with age in our study mirror findings from previous reports (Harper et al. 2004; Miller 1997; Miller 2001; Witkowski and Miller 1993). Furthermore, the age-associated decline in the proportion of CD4 T-lymphocytes is consistent with some previous results also reporting a decrease with age in spleen, lymph node, and mouse blood CD4+ cells (Callahan et al. 1993; Grossmann et al. 1991). However, others have failed to observe these CD4+ changes in certain inbred mouse strains (Kirschmann and Murasko 1992; Komuro et al. 1990). Similarly, the observed an age-dependent increase in the CD8 T-lymphocytes cell number is consistent with previous reports of increases in this subset (Gonzalez-Quintial and Theofilopoulos 1992; Linton et al. 1996).

RSV treatment restored the age-dependent declining number of CD4 T-lymphocytes and mitigated the increase of CD8 T-lymphocytes and CD8M populations in the LT RSV and old RSV mice, respectively, when compared to age-matched controls. Although we did not perform life span determination in this

study, it has been reported that the higher proportion of CD8M cells at ages 8 and 18 months and lower proportion of CD4+ cells at 18 months in genetically heterogeneous mice are significantly associated with decreased individual life expectancy (Harper et al. 2004). RSV treatment in our study also shifted the phenotype of aged lymphocytes to that of the younger mice (lower CD8M population and a restoration of declined number of CD4 T-lymphocytes). We also found that RSV treatment restored the proliferative capacity of T lymphocytes (stimulated with Concanavalin A) in old mice to a level similar to young mice where there was a significant age-dependent decrease (data not shown). Our observations suggest that RSV may potentially protect T lymphocytes from functionally age-related surface marker changes.

Of particular interest in the context of immunosenescence has been in Foxp3+-expressing CD4+CD25+ T regulatory cells (Tregs) which are implicated in regulation of both transplant related and autoimmune models of disease, and whose numbers and function are reported to alter with age (Dejaco et al. 2006; Sakaguchi et al. 2006). Elevated levels of Tregs may cause increased suppression of pro-inflammatory T-cells and thus contribute to the higher susceptibility to infections, poor vaccine response, and higher risk of neoplasms in aged individuals (Pawelec et al. 2002; Taub and Longo 2005). A growing number of studies suggest that with aging, there is an elevated frequency of Foxp3+ Treg cells in both humans and mice (Gregg et al. 2005; Han et al. 2009; Sharma et al. 2006; Zhao et al. 2007). Consistent with these prior reports, we observed an age-related increase in the expression of CD4+CD25+ cells in the spleen and this was significant when the cells were activated. In contrast to the overall protective effects of RSV, an elevated amount of unstimulated cells CD4+CD25+ in old mice was observed with RSV treatment. Nevertheless, RSV treatment did not produce any significant changes in activated CD4+CD25+ cells when compared to age-matched controls.

Cytokine profiles in the aging hybrid mice and the effects of RSV

Cytokines are a key component in the regulatory system of the immune system. They are responsible for differentiation, proliferation, and survival of lymphoid cells and play an important role in immune

responses and inflammation. In young subjects, robust pro-inflammatory condition may be beneficial to combat infections due to exposure to various antigens. However, advanced age is often accompanied by low-grade chronic inflammatory activity including increased levels of pro-inflammatory cytokines (Bruunsgaard 2002; Fagiolo et al. 1993; Franceschi et al. 2007; Krabbe et al. 2004). This inflammatory activity has been suggested to be one prevalent cause of increased incidences of autoimmune diseases, infection, and cancer in elderly people (Bruunsgaard 2002; Franceschi et al. 2007).

Table 2 gives a summary of the changes in cytokine profile with age and the effect of RSV treatment in old mice. IL-2 has been reported to have an essential role in the maintenance of immune system homeostasis and tolerance to self (Fontenot et al. 2005) and in the generation, function and stabilization of Foxp3+CD4+ Tregs (Horwitz et al. 2008). In mice and humans, IL-2 production and the expression of IL-2-receptor have been previously reported to decline with age, leading to a reduced T cell proliferation capacity (Adolfsson et al. 2001; Nagel et al. 1988).

Table 2 Changes in cytokine profiles with age and after RSV treatment

Marker	Age		RSV treatment in old mice	
	Intracellular	Extracellular	Intracellular	Extracellular
IL-2	↔	↓↓	↔	↑
FN-γ	↑	↑↑	↓	↓
IL-6	↔	↓	↓↓	↑
TNF-α	ND	↑	ND	↓
L-4	↔	↓↓	↓	↑
IL-5	↑ ^a	ND	↓ ^b	ND
IL-10	↑↑	↑↑	↓↓	↓

↔, no significant change

↑, <50% increase; $P < 0.05$ or $P < 0.01$

↑↑, >50% increase; $P < 0.01$

↓, <50% decrease; $P < 0.05$ or $P < 0.01$

↓↓, >50% decrease; $P < 0.01$

ND not determined

^a In middle-aged mice

^b In LT RSV middle-aged mice

In our study, extracellular IL-2 production declined with age although IL-2 positive CD4+ cells did not exhibit any age-related trend. This implies that, although the function and the population of the CD4+ IL-2 producing cells remain intact with age, the overall IL-2 production by other splenocytes could be impaired with age.

RSV treatment in our study resulted in an enhanced IL-2 intracellular cytokine level in young mice and partially restored the age-dependent decline of extracellular IL-2 levels in LT RSV and old RSV mice.

Serving as a potential aging biomarker and marker for acute inflammation (Erol 2007), an age-related overproduction of IL-6 has been reported by some and was associated with a spectrum of age-related conditions including low-grade inflammation, frailty and functional decline (Huang et al. 2005; Kiecolt-Glaser et al. 2003). In our study, IL-6 intracellular expression did not exhibit any age-dependent change but a significant decline in the extracellular secretion of IL-6 was observed in control old mice. Consistent with recent findings of RSV's anti-inflammatory properties (Bisht et al. 2009), intracellular IL-6 CD4+ levels were lower in RSV-treated mice compared with control. However, extracellular secretion of IL-6, which declined with age, was restored in old RSV-treated mice to a level similar to that of middle-aged control mice. IL-6 is of special interest in this context as this trend implies that moderate RSV supplementation significantly reduces chronic inflammatory activity in old CD4+ cells while being able to maintain the overall level of IL-6 production in other aging splenocytes.

It has been suggested that there is a non-specific increase in pro-inflammatory protein production in aged populations (Burns and Goodwin 1997; Pawelec 1999). Basal release of IL-6, TNF-α, and IFN-γ increases during aging and is associated with low-grade inflammation (Bruunsgaard and Pedersen 2003; Gabriel et al. 2002). In this study, intra- and extracellular IFN-γ cytokines were indeed found to be elevated with age and TNF-α secretion also increased with age in our hybrid mice. Interestingly, intracellular IFN-γ levels were significantly enhanced by RSV treatment in young mice in our study. However, the observed age-dependent increase of intra- and extracellular IFN-γ were significantly attenuated in LT RSV and old RSV mice. This could indicate early protection in the context of enhanced acute inflammatory responses in the young animals,

while at the same time preventing chronic low-grade inflammation in the old animals.

Similarly, age-dependent elevation of TNF- α production was also mitigated by the RSV intake in the middle-aged, LT RSV, and old mice. This is consistent with previous report on RSV's anti-inflammatory properties associated with the inhibition of NF- κ B in LPS-, TNF- α , or PMA-mediated macrophages, myeloid, Jurkat, and epithelial cells (Gao et al. 2001; Manna et al. 2000). RSV also suppressed the secretions of TNF- α , IL-1, IL-6, IL-12, and IFN- γ by splenic lymphocytes and macrophages (Gao et al. 2001; Kowalski et al. 2005).

Contrary to the some studies which reported enhanced expression and production of Th2 cytokine such as, IL-4 with age (Alberti et al. 2006; Rink et al. 1998), hybrid mice in our study exhibited reduced IL-4 secretion with age. RSV intake ameliorated this age-dependent decrease by maintaining the level of IL-4 produced by the splenocytes in the old mice. In old CD4+ cells, however, intracellular IL-4 was reduced by RSV treatment. This suggests the possibility of RSV affecting the intracellular components of CD4+ cells but not the production of IL-4 in other splenocytes.

We also observed age-dependent elevations of IL-10 CD4+ expression and extracellular IL-10 secretion which is consistent with previous studies that have identified an age and chronic illness-associated increase in IL-10 production (Castle et al. 1997, 1999; Pawelec 1999). Interestingly, RSV treatment was able to ameliorate the increasing IL-10 levels in the old animals. Overall, we observed that RSV treatment resulted in a concerted effect of attenuating the escalating pro-inflammatory profile while maintaining the anti-inflammatory status in the aged animals. On the other hand, RSV increased the production of IL-2, IFN- γ , IL-4, and IL-10 in young mice which suggests an enhanced cell-mediated environment that might provide effective defense against intracellular pathogens.

Elevation of 8OHdG and IFN- γ cytokine with age

The study of oxidative DNA damage has become clinically important in recent years (Valavanidis et al. 2009). Studies have shown that oxidative DNA damage correlates to a variety of aging-associated degenerative diseases such as coronary heart disease,

diabetes, and cancer (Sander et al. 2008; Wu et al. 2004). Upon oxidation, a hydroxyl group is added to the eighth position of the guanine molecule and the oxidatively modified product, 8OHdG is one of the predominant forms of free radical-induced lesions of DNA (Wu et al. 2004).

We observed a consistent age-dependent increase in the levels of 8OHdG, a biomarker for oxidative DNA damage in ageing splenocytes, and RSV treatment attenuated this elevation in the middle-aged and old hybrid mice. This profile is parallel to the IFN- γ pattern observed with increasing pro-inflammatory condition in old mice. This implies that pro-inflammatory status of the splenocytes correlates to the increasing oxidative DNA damage in spleen of aging mice which can be reduced by low dose, chronic RSV intake. However, this data alone is not conclusive regarding causality as pro-inflammatory signals could in principle cause elevated oxidative damage or vice versa (Kulinsky 2007). In our earlier study, we have shown that RSV treatment reduced the observed age-dependent accumulation of 8OHdG in liver and heart of aging hybrid mice (Wong et al. 2009). It is clear that the level of RSV in our study is too low for it to act as a direct antioxidant (Wong et al. 2009). Several studies have cited RSV as having anti-inflammatory properties and the ability to down-regulate pro-inflammatory cytokines through various mechanisms of action that include induction of apoptosis, inhibition of signal transduction and promotion of cellular differentiation (Baur and Sinclair 2006; Das and Das 2007; Pervaiz et al. 2009). Free radicals have been demonstrated to play a role in a highly interconnected microenvironment of pro-inflammatory/anti-inflammatory responses in balancing immune homeostasis (De la Fuente and Miquel 2009).

Long-term RSV treated mice exhibits minimal immunological gene expression changes

To further explore possible mechanisms explaining the mode of action of RSV in immune cells, we conducted gene expression analysis on T lymphocytes from 12-month LT RSV middle-aged mice and compared this with the respective controls. Somewhat unexpected, given the clear functional efficacy of RSV in terms of age-dependent cytokine changes, none of the affected cytokines showed significant transcriptional changes. Furthermore, only one

(CD72) out of 1,076 genes was downregulated by at least a factor of 2. In addition, the FLAP and HSF4 genes showed significant upregulated expressions of >1.6-fold change. It should be noted, however, that due to the moderate gene expression changes and the limited sample amount available we did not confirm these results by real-time-PCR. Given the low (nM) plasma levels of RSV that we have previously detected in these mice (Wong et al. 2009), the relatively weak transcriptional effects of RSV treatment may not be surprising. However, despite these low plasma levels, significant changes in terms of age-dependent effects of RSV treatment on cytokine profiles were observed in these animals. Therefore, the absence of more robust gene expression changes maybe the most significant result from this experiment. CD72, the only gene that showed a robust expression change was downregulated two-fold in response to 12-month RSV treatment. CD72 is a 45 kDa transmembrane protein known as a B-cell co-receptor (BCR; Wu and Bondada 2009). CD72 has been implied as a modulator of BCR dependent activation of a number of targets, including NFATc1, NF- κ B, MAPK as well as Akt (Li et al. 2008). This complexity in function makes it difficult to predict the effect (if any) of the observed down regulation of CD72 without further experiments. The apparent impact of low dose (nM) RSV on CD72 function in the context of BCR activation might be of interest for future mechanistic work, but this type of investigation goes beyond the scope of this article.

In summary, our microarray data does not explain the observed immune-modulatory efficacy of chronic low-dose RSV. In contrast, it has recently reported that several genes involved in cellular immune and inflammatory responses were induced in the heart and cerebellum of old mice of multiple inbred strains which suggests a heightened immunity in the aged animals (Park et al. 2009) and that high RSV intake at 50 mg/kg of diet significantly inhibited the gene expression changes related to cardiac aging in the study in these mice (Park et al. 2009). Given that RSV plasma levels in our study were in the nM range, it is possible that functionally significant gene expression changes may be below the detection limit of the array experiment but still contributed to the overall RSV efficacy. Alternatively, low dose RSV might affect immunological processes without causing transcriptional change in lymphocytes.

Conclusions

Our results suggest that the T cell compartment of old hybrid mice undergoes changes involving not only the proportions of different surface phenotype subsets, but in the immuno-inflammatory network systems. Taken together, our study shows that low dose supplementation of RSV can lead to distinct changes in T cell responses, including a shift in T cell subset phenotypes that reminisced that of young mice while attenuating the age-dependent increase of pro-inflammatory condition. Overall, RSV treatment preserved an anti-inflammatory cytokine environment (IL-4 and IL-10) with age, without inducing any major immunological gene expression changes. At the same time, RSV treatment resulted in the suppression of pro-inflammatory cytokines such as IFN- γ , IL-6, and TNF- α thus regulating homeostatic cytokine balance in the aged while attenuating the elevation of 8OHdG levels in the aging spleen. These immunological profiles observed in RSV-treated mice were also similar to results found in caloric restriction studies (Pahlavani 2004). Although the current understanding of these data is preliminary, there is a possibility that age-related oxidative stress, changes in cell surface receptors, chronic low-grade inflammation, and caloric restriction have common factors that regulate the aging mechanisms. Future work should be aimed at identifying what other cell sub-populations are responsible for the production of these cytokines and how these cells are influenced by potential age-related factors such as oxidative stress.

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