

Characterization of the Hepatic and Splenic Immune Status and Immunoglobulin Synthesis in Aged Male Mice Lacking the Peroxisome Proliferator-Activated Receptor-Alpha (PPAR α)

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Abstract

It is now well established that the nuclear receptor peroxisome proliferator-activated receptor-alpha (PPAR α) is expressed in different types of immune cells and plays a pivotal role in the regulation of age-related production of inflammatory cytokines. However, the role(s) of this receptor in the regulation of immune cell homeostasis in ageing non-lymphoid and lymphoid organs has not yet been resolved. We examine this issue here by evaluating the hepatic and splenic immune status and immunoglobulin (Ig) production in male PPAR α -null mice and their wild-type littermates at one and 2 years of age. In comparison with the age-matched control animals, PPAR α -null mice exhibited age-related elevations in the numbers of total, as well as of phenotypically distinct subpopulations of intrahepatic immune cells (IHIC) and splenocytes. Moreover, at 2 years of age, these alterations in hepatic immune cells were accompanied by significant increases in hepatic levels of the pro-inflammatory cytokines tumour necrosis factor-alpha (TNF- α), interleukin-6 (IL-6) and interferon-gamma (IFN- γ), in combination with the development of hepatic inflammatory foci containing mixtures of leucocytes. Alterations in splenocytes of old PPAR α -null mice were also accompanied by increases in cellularity of both white and red pulps of the spleen. Furthermore, these same animals exhibited pronounced increases in the numbers of splenic plasma cells and enhanced production of Ig of different isotypes, including IgG1, IgG2a and IgE. Thus, our findings indicate that upon ageing, PPAR α plays a crucial role in regulating the total numbers, compositions and functions of immune cells in both lymphoid and non-lymphoid immune organs of mice.

Introduction

The members of the nuclear family peroxisome proliferator-activated receptors (PPAR) are ligand-activated transcription factors that play key roles in global lipid homeostasis [1, 2]. Of these receptors, PPAR-alpha (PPAR α) is expressed at high levels in the liver, kidney, heart and muscles [3, 4], where it acts as a lipid sensor, responding to influxes of fatty acids by stimulating the transcription of genes encoding, e.g. the mitochondrial and peroxisomal β -oxidation systems and fatty acid ω -oxidation in the endoplasmic reticulum [1]. Thus, reduction in the level of expression and/or inactivation of

PPAR α reduces fatty acid catabolism, resulting in accumulation of intracellular lipids in the liver and heart. Indeed, aged mice, which exhibit declining expression of PPAR α , as well as transgenic mice deficient in this receptor (PPAR α -null mice), demonstrate such lipid accumulation [5–8].

PPAR α is also expressed in a number of other tissues and cell types, in particular the cells of the immune system, e.g. macrophages [9], and T and B cells [10]. Several studies have revealed that PPAR α in macrophages and T cells plays a pivotal role in the regulation of inflammation. In macrophages, PPAR α exerts most of its anti-inflammatory properties as an antagonist of the nuclear

factor- κ B (NF- κ B) and activator of the protein-1 (AP-1) signalling pathways [11], thereby repressing the expression of several factors involved in the inflammatory response [11]. These factors include cytokines (e.g. tumour necrosis factor α (TNF- α), interleukin-6 (IL-6) and IL-12), adhesion molecules [e.g. vascular cell adhesion molecule 1 (VCAM1)] and other pro-inflammatory signals [e.g. inducible nitric oxide synthase (iNOS)] [11].

In T cells, PPAR α regulates the synthesis of interferon- γ (IFN- γ) and IL-2, the two classical pro-inflammatory T helper 1 (Th1)-type cytokines [12], via two possible mechanisms. First, this nuclear receptor may suppress IL-2 production by reducing the activity of transcription factor NF- κ B in activated T cells [11]. Secondly, PPAR α can control the production of IFN- γ in these cells by transiently inhibiting the activation of p38 mitogen-activated protein kinase and thereby downregulating the transcription of the T-bet gene [13], which encodes a Th1-specific T box transcription factor that controls the expression of this cytokine [14].

In addition to its regulatory roles in connection with macrophage- and T cell-mediated inflammatory responses, PPAR α plays a pivotal role in the cytokine dysregulation that occurs in the immune system upon ageing [7, 8, 15]. In this context, expression of this nuclear receptor in the spleen, liver and kidney of aged animals has been found to be strongly attenuated [7, 8]. In fact, this reduction in PPAR α in the spleen of aged animals is linked to the constitutive overproduction of cytokines and other mediators whose expression is regulated by NF- κ B (e.g. TNF- α , IL-6, IL-12, migration inhibitory factor and cyclooxygenase) [8, 15]. Furthermore, the splenocytes of PPAR α -deficient mice exhibit an age-related pro-inflammatory phenotype at a younger age than do the corresponding cells from wild-type controls [8].

Despite the extensive documentation that PPAR α exerts regulatory effects on the changes in the production of inflammatory cytokines associated with ageing, the possible involvement of this nuclear factor in immune cell homeostasis in both non-lymphoid and lymphoid organs during ageing is largely unknown. To examine this issue, we compare here the hepatic and splenic immune status of adult (1-year-old) and old (2-year-old) male mice deficient in PPAR α (PPAR α -null mice) to those of age-matched wild-type control animals. We document significant alterations in hepatic and splenic immune cell subpopulations in old mice, and in particular those lacking PPAR α . In the latter animals, these changes are accompanied by substantial elevations in hepatic levels of inflammatory cytokines, as well as enhanced serum levels of various immunoglobulin (Ig) isotypes. Our findings demonstrate that in connection with ageing, PPAR α is an important regulator of immune cell numbers and functions in both lymphoid and non-lymphoid immune organs.

Materials and Methods

Animals. Male PPAR α -null 129/Sv mice (originally obtained from Dr. F.J. Gonzalez, National Cancer Institute, National Institutes of Health, Bethesda, MD, [16]) and the corresponding wild-type animals were kind gifts from Professor G. Dallner at our department. These animals were housed at the Wenner-Gren Institute, Stockholm University, with a 12-h dark/12-h light cycle and *ad libitum* access to tap water and RMI (E) FG SQC chow pellets containing 2.71% crude oil (including lauric (0.02%), myristic (0.14%), palmitic (0.31%), stearic (0.04%), myristoleic (0.02%), palmitoleic (0.09%), oleic (0.77%), linoleic (0.69%), linolenic (0.06%) and arachidonic (0.13%) acids), 14.38% crude protein and 61.73% carbohydrate (SDS, Special Diets Services, Essex, UK). These experiments were pre-approved by the Northern Stockholm Ethical Committee for Animal Experimentation (N150/07, N299/05 and N300/05).

Collection of blood, spleen, liver and epididymal fat and preparation of serum and splenocytes. At one and 2 years of age, the mice were weighed, bled by retro-orbital puncture under light isoflurane anaesthesia and sacrificed by cervical dislocation. The thymus, spleen, liver and epididymal fat were removed and weighed. The blood samples were centrifuged to obtain serum, which was stored at -20°C until use for analysis. The spleens were teased apart with forceps in PBS to obtain single-cell suspensions. Animals that exhibited gross internal or external pathology at the time of sacrifice were excluded from the study.

Measurement of serum transaminases, alkaline phosphatase and lipids. Serum alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP), cholesterol and triglycerides were determined employing an autoanalyser (VITROS[®] 5.1 FS Chemistry System, Ortho-Clinical Diagnostics, Johnson & Johnson Company, Sollentuna, Sweden).

Isolation of intrahepatic immune cells. IHIC were isolated employing modified mechanical disruption, as described previously [17]. Briefly, after perfusion with cold PBS (pH 7.2), the liver was excised, minced and gently forced through a 200- μm -gauge stainless steel mesh, and the resulting preparation was suspended in RPMI 1640 medium (Invitrogen AB, Stockholm, Sweden) containing 10% FCS (Invitrogen) (pH 7.4). Thereafter, this suspension was centrifuged (60 g_{av} , 1 min, room temperature), the supernatant containing IHIC was centrifuged (480 g_{av} , 8 min, room temperature) and the pellet was resuspended in 10 ml 37.5% Percoll (GE Healthcare Bio-Sciences, AB Uppsala, Sweden) in HBSS (Sigma-Aldrich Sweden AB, Stockholm, Sweden) containing heparin (Sigma-Aldrich Sweden AB). After re-centrifugation (850 g_{av} , 30 min, room temperature), the new pellet was treated with erythrocyte-lysing buffer and the cell suspension obtained was centrifuged (480 g_{av} , 8 min). The final pellet was resuspended in FACS buffer for cell surface phenotyping by flow cytometry.

Immunofluorescent staining and flow cytometric analysis. Immunophenotyping of IHIC and splenocytes involved the following antibodies (all purchased from BD PharMingen, San Diego, CA, USA): anti-CD45 labelled with (–) fluorescein isothiocyanate (FITC) (clone 30F11), anti-CD4-FITC (clone RM4-4), anti-CD19-FITC (clone 1D3), anti- $\gamma\delta$ T cell receptor-FITC (clone GL3), anti-CD11b-FITC (clone M1/70), anti-CD49b/Pan-NK labelled with phycoerythrin (PE) (clone DX5), anti-T cell receptor β chain-PE (clone H57-597), anti-Gr1-PE (clone H57-597) and anti-CD8a labelled with peridinin chlorophyll protein (PerCP) (clone 53-6.7). To each well of 96-well round-bottom tissue culture plates, 10^5 IHIC or splenocytes in FACS buffer were added (Corning, NY, USA) and then incubated with one or two of these antibodies (in the dark, on ice, 30 min), as recommended by the manufacturer (BD PharMingen). After centrifugation (200 g_{av} , 10 min), the cells were resuspended in FACS buffer for analysis in a single-laser FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA). The signals emitted by FITC, PE and PerCP were detected at 530, 575 and 670 nm, respectively. For each sample, data from 10,000 events (individual cells) were collected and analysed employing the CELLQUEST Software (Becton Dickinson, San Jose, CA, USA).

Measurement of serum and hepatic levels of tumour necrosis factor- α (TNF- α), interleukin 6 (IL-6), IL-4 and IFN- γ . After collection of serum or homogenization of livers in ice-cold PBS-0.1% Tween-20 and centrifugation (5 min, 17,900 g), TNF- α , IL-6, IL-4 and IFN- γ in these samples were quantitated employing commercially available ELISA kits (eBioscience, San Diego, CA, USA; BD Biosciences, San Diego, CA, USA and MABTECH AB, Stockholm, Sweden, respectively) in accordance with the manufacturers' instructions.

Histopathological examination of the liver and spleen.

The livers and spleens were fixed in 4% (v/v) formaldehyde in PBS for 24 h, embedded in paraffin and cut into sections of 5–6 μ m thick. These sections were then stained with haematoxylin and eosin (H&E) and examined by an experienced pathologist (Dr. Ricardo Feinstein, Department of Pathology and Wildlife Diseases, National Veterinary Institute, Uppsala, Sweden).

Measurement of serum levels of IgG1 and IgG2a.

Serum levels of IgG1 and IgG2a were determined employing sandwich ELISA techniques. Briefly, for this purpose, each well of 96-well, flat-bottomed micro-ELISA plates (Nunc A/S, Roskilde, Denmark) was coated with goat anti-mouse Ig (1 μ g/ml) (Southern Biotechnology, Birmingham, AL, USA) overnight at 4 °C, following which the remaining free binding sites were blocked by incubation for 2 h at room temperature with BSA (1%, v/v) dissolved in PBS. After washing these plates three times with PBS-0.05% Tween, 100 μ l serum diluted 1:4000 in diluent buffer was added to each well and the

plates were subsequently incubated at room temperature for 4 h, washed four times with PBS-Tween, then incubated with alkaline phosphatase-labelled goat anti-mouse IgG1 or IgG2a (at a dilution of 1/2000; Southern Biotechnology) for 2 h at room temperature and thereafter washed three more times. Next, each well was incubated for 30 min at room temperature with a solution of the phosphatase substrate *p*-nitrophenyl phosphate (disodium salt hexahydrate; Sigma) and the absorbance at 405 nm finally determined on a microplate reader. The concentrations of IgG1 and IgG2a in the serum samples were assessed by comparison with a calibration curve generated using an internal standard (Southern Biotechnology).

Measurement of serum level of IgE. Total mouse serum IgE was determined employing a commercially available ELISA kit, BD OptEIA™ (BD Biosciences), in accordance with the manufacturer's instruction.

Statistical analysis. All data are expressed as means $\pm$ standard errors (SE), and differences between the values for wild-type and PPAR α -null mice are examined for statistical significance by the Mann–Whitney *U*-test at the significance level $2\alpha = 0.05$, utilizing the WINSTAT software (R. FITCH Software; Medina AB, Vänerborg, Sweden).

Results

The constitutive phenotype of adult and old male PPAR α -null 129/Sv mice

To elucidate the involvement of PPAR α in the regulation of immune cell homeostasis in aged non-lymphoid and lymphoid organs, male PPAR α -null 129/Sv mice and age-matched wild-type controls were first compared with respect to liver and fat masses, serum activities of hepatic enzymes, and total serum levels of cholesterol and triglycerides at one and 2 years of age. As shown in Table 1, at 1 year of age, the relative liver and fat pad masses, as well as total serum cholesterol were higher in the transgenic than in wild-type animals. At 2 years of age, while total serum cholesterol was still higher in the PPAR α -null mice, the other parameters examined were similar in both strains (Table 1). These results suggest that neither adult nor old male PPAR α -null 129/Sv mice exhibit an obese phenotype.

Old, but not adult male PPAR α -null 129/Sv mice exhibit inflammatory foci in their livers

As a representative non-lymphoid immune organ, we selected the liver, because this organ expresses PPAR α at high levels [3, 4], contains immune cells that differ in composition from those of lymphoid organs and performs a variety of immunological functions, including the generation of immunological tolerance and responses to blood-borne and dietary pathogens [18, 19]. Histopathological

Table 1 Comparison of the changes in body weight, relative masses of the liver and epididymal fat pads, serum activities of aspartate transaminase (AST), alanine transaminase (ALT) and alkaline phosphatase (ALP) and total serum levels of cholesterol and triglycerides in male wild-type and PPAR α -null 129/Sv mice at one and 2 years of age.

	1-year-old mice		2-year-old mice	
	Wild-type (n = 9)	PPAR α -null (n = 9)	Wild-type (n = 8)	PPAR α -null (n = 15)
Body weight (g)	29.8 $\pm$ 0.8 ^a	31.1 $\pm$ 0.7	30.7 $\pm$ 1.3	31.2 $\pm$ 0.6
Liver mass ^b	4.21 $\pm$ 0.13	4.75 $\pm$ 0.14 ^c	5.29 $\pm$ 0.11	5.85 $\pm$ 0.30
Fat mass ^b	1.89 $\pm$ 0.57	2.65 $\pm$ 0.27 ^c	2.45 $\pm$ 0.48	2.24 $\pm$ 0.33
Serum AST (μ kat/l) ^d	1.32 $\pm$ 0.07	1.52 $\pm$ 0.21	3.31 $\pm$ 0.51	4.18 $\pm$ 0.64
Serum ALT (μ kat/l) ^d	0.85 $\pm$ 0.03	1.20 $\pm$ 0.24	1.98 $\pm$ 0.29	2.12 $\pm$ 0.36
Serum ALP (μ kat/l) ^d	1.32 $\pm$ 0.07	1.21 $\pm$ 0.06	1.48 $\pm$ 0.09	1.82 $\pm$ 0.25
Total serum cholesterol (mM) ^d	3.18 $\pm$ 0.13	3.51 $\pm$ 0.14 ^c	3.11 $\pm$ 0.14	3.75 $\pm$ 0.19 ^c
Total serum triglycerides (mM) ^d	1.90 $\pm$ 0.24	2.40 $\pm$ 0.23	3.01 $\pm$ 0.23	3.17 $\pm$ 0.41

^aAll values are means $\pm$ SE. Differences were analysed for statistical significance using the *U*-test.

^bCalculated as [organ weight (g)/body weight (g)] $\times$ 100.

^cStatistically significant ($P \leq 0.05$) in comparison with the wild-type value.

^dBlood samples were analysed for serum levels of hepatic enzymes, cholesterol and triglycerides employing an autoanalyser.

examination of the livers from adult (1-year-old) and old (2-year-old) male PPAR α -null 129/Sv mice and their age-matched wild-type controls revealed, as expected, that the centrilobular hepatocytes of PPAR α -null mice (Fig. 1B,D), but not of wild-type animals (Fig. 1A,C), contained large numbers of macro-vesicular vacuoles. Further staining of the liver sections with red-oil O demonstrated that these vacuoles are fat droplets (not shown). Moreover, at 2 years of age, the livers of PPAR α -null mice contained small regions of inflammation consisting of mixtures of leucocytes and occasional multinucleated giant cells (Fig. 1D), whereas there were no such regions in the liver of this same strain at 1 year of age (Fig. 1B) or in wild-type livers at either age (Fig. 1A,C).

Male PPAR α -null 129/Sv mice and wild-type animals exhibit different alterations in their intrahepatic immune cell compositions upon ageing

To further elucidate the role of PPAR α in controlling the composition of IHIC during ageing, the total numbers as

well as phenotypes of isolated IHIC in PPAR α -null and wild-type mice were compared by flow cytometric analysis at one and 2 years of age. As depicted in Fig. 2A, in comparison with the age-matched controls, both adult (1-year-old) and old (2-year-old) PPAR α -null mice exhibited elevations in their total number of IHIC, which more pronounced at 2 years of age. The total numbers of hepatic B (CD19⁺), T helper (CD4⁺CD3⁺) and natural killer (NK) (NKPan⁺CD4⁻) cells, and macrophages (CD11b⁺) were all significantly enhanced, with macrophages being most affected at 1 year of age (Fig. 2B,C,G,H, respectively). At 2 years of age, in addition to enhanced numbers of B, T helper and NK cells (Fig. 2B,C,G), there were also increases in the numbers of T cytotoxic (CD8⁺CD3⁺), T $\gamma\delta$ ($\gamma\delta$ ⁺) and natural killer T (NKT) (NKPan⁺CD4⁺) cells, and granulocytes (Fig. 2D–F,I, respectively). At this age, T helper, T cytotoxic cells and T $\gamma\delta$ cells, and granulocytes were most enhanced (Fig. 2C–E,I, respectively). At the same time, in old control mice, there was no significant elevation in the total numbers of IHIC (Fig. 2A), but significant enhancements in the numbers of hepatic B,

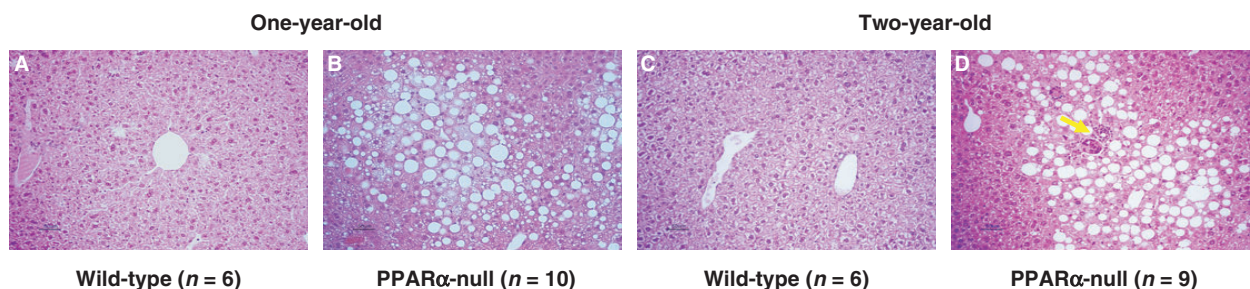


Figure 1 Male PPAR α -null 129/Sv mice, but not wild-type control animals develop hepatosteatosis and hepatic inflammatory foci upon ageing. Representative photographs of liver sections (magnification $\times 200$) from 1-year-old (A and B) and 2-year-old (C and D) male wild-type (A and C) or PPAR α -null (B and D) mice stained with haematoxylin–eosin. The macro-vesicular vacuoles, which apparently contain lipids, are present only in the centrilobular hepatocytes of the PPAR α -null animals (B and D). The yellow arrow in (D) points out an inflammatory foci consisting of large macrophages and lymphocytes in the liver of a 2-year-old PPAR α -null mouse. Virtually identical patterns of histological alterations were observed in all PPAR α -null mice examined. The values in the parentheses indicate the total numbers of the animals used in the experiment.

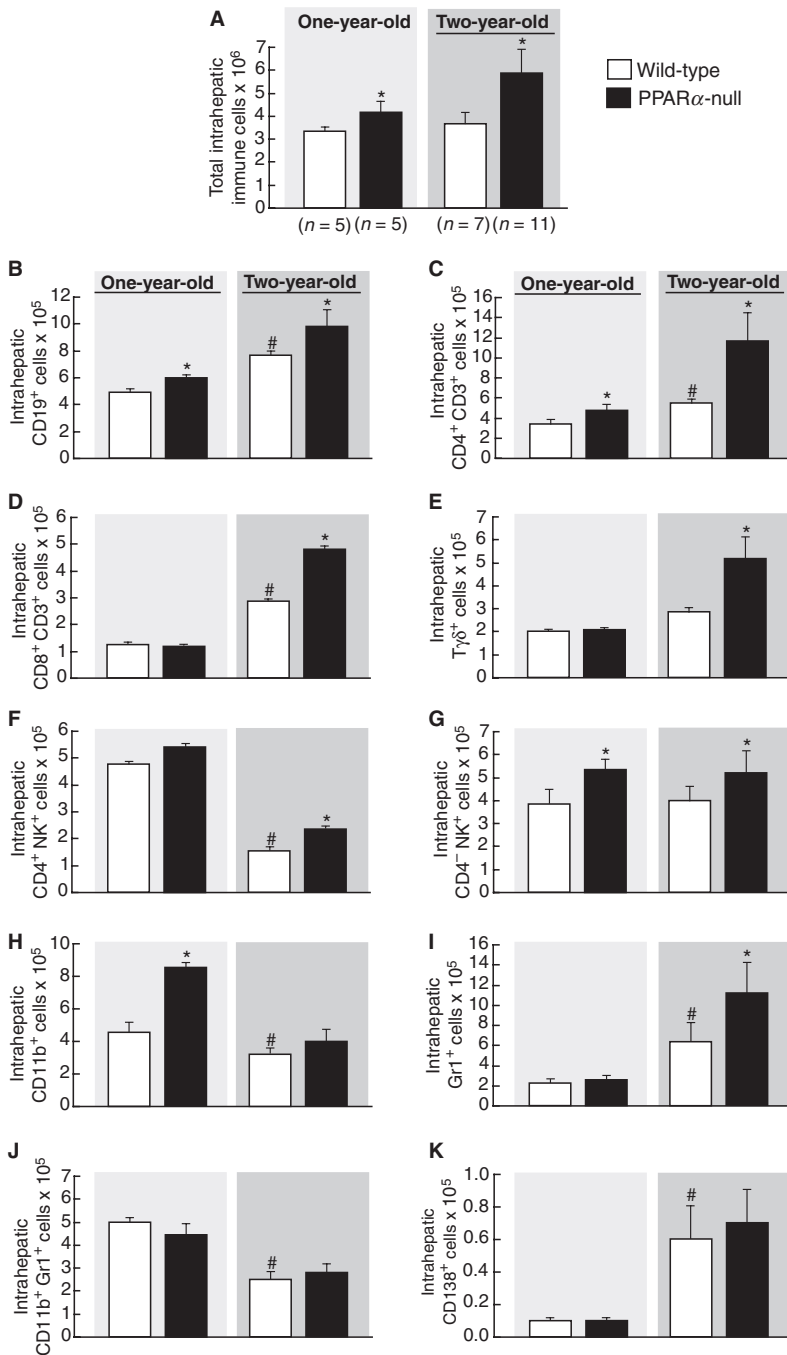


Figure 2 Age-related alterations in immune cell subpopulations in the livers of male PPAR α -null 129/Sv mice and their wild-type control animals. Male wild-type (open bars) and PPAR α -null (solid bars) 129/Sv mice were sacrificed by cervical dislocation at one (light grey rectangles in A–K) and two (dark grey rectangles in A–K) years of age and their livers removed by dissection. After isolation of the intrahepatic immune cells, their total numbers (A) were determined by light microscopy with Trypan blue staining, and the different subpopulations including B cells (CD19 $^+$) (B), T helper cells (CD4 $^+$ CD3 $^+$) (C), T cytotoxic cells (CD8 $^+$ CD3 $^+$) (D), T $\gamma\delta$ cells ($\gamma\delta^+$) (E), natural killer T (NKT) cells (CD4 $^+$ NKPan $^+$) (F), natural killer (NK) cells (CD4 $^+$ NKPan $^+$) (G), macrophages (CD11b $^+$) (H), granulocytes (Gr1 $^+$) (I), Myeloid suppressor cells (CD11b $^+$ Gr1 $^+$) (J) and plasma cells (CD138 $^+$) (K) were evaluated employing flow cytometry. All values presented are means $\pm$ SE (standard error) for 5–11 animals in each group (as indicated in parentheses). *Statistically significant ($P \leq 0.05$) in comparison with the corresponding age-matched wild-type value. #Statistically significant ($P \leq 0.05$) in comparison with the corresponding wild-type animals at 1 year of age.

T helper and T cytotoxic cells, granulocytes and plasma (CD138 $^+$) cells (Fig. 2B–D,I,K, respectively) along with substantial reductions in the numbers of hepatic NKT cells, macrophages and myeloid suppressor (CD11b $^+$ Gr1 $^+$) cells were observed (Fig. 2F,H,J, respectively).

Age-associated increases in hepatic levels of inflammatory cytokines are exacerbated in PPAR α -null 129/Sv mice

To determine whether, upon ageing, these changes in the morphology and immune cell composition of the liver

are accompanied by alterations in the hepatic immune status, total hepatic levels of TNF- α , IL-6, IFN- γ and IL-4 were quantified (Table 2). Both the PPAR α -null and wild-type strains exhibited an elevation in the levels of these cytokines with advancing age. However, this elevation was more pronounced in the former strain: at 1 year of age, the liver of the PPAR α -null animals contained significantly higher levels of TNF- α (≥ 1.6 -fold) and IL-6 (≥ 1.3 -fold) than the wild-type controls (Table 2). More strikingly, at 2 years of age, the corresponding differences were ≥ 2.6 - and ≥ 5.5 -fold, respectively, and

Table 2 Total levels of cytokines in liver homogenates from male wild-type and PPAR α -null 129/Sv mice at one and 2 years of age.

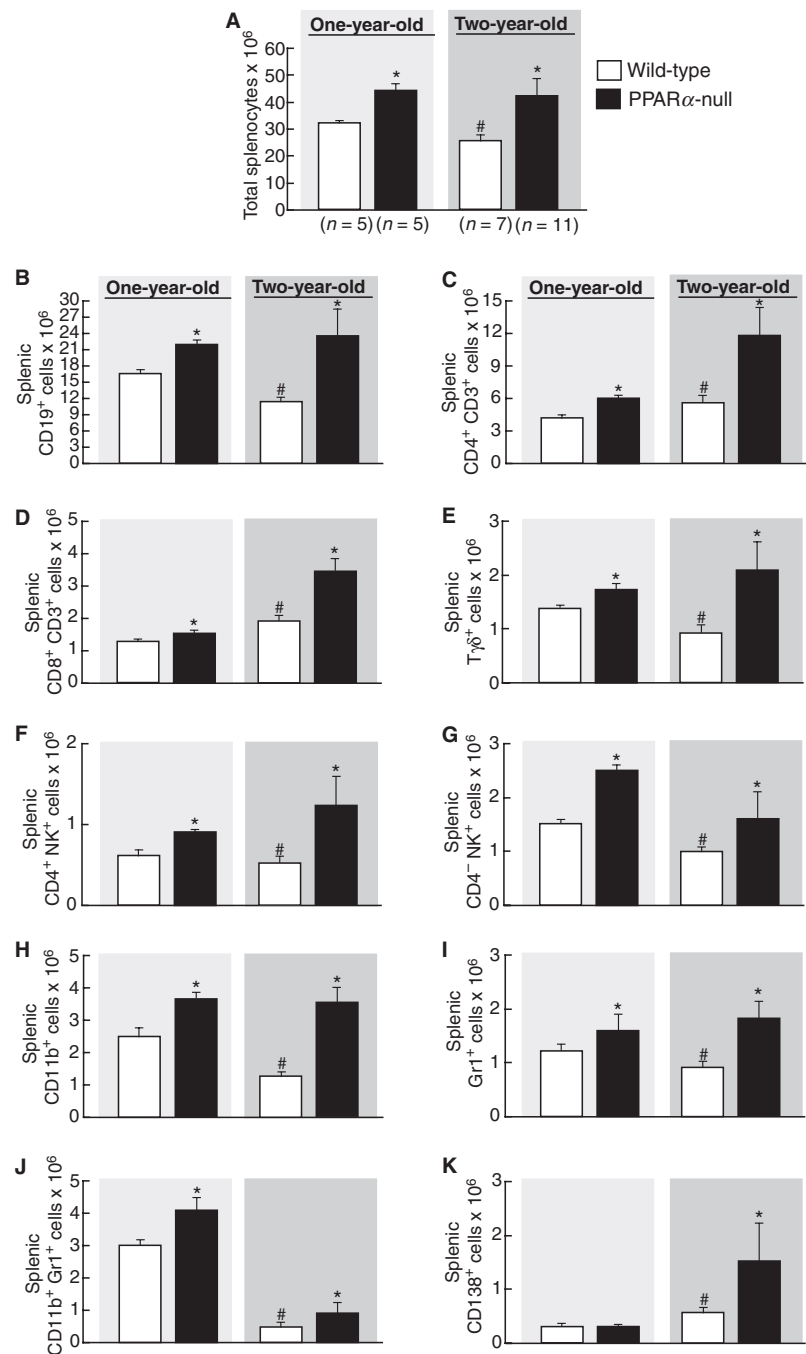
Hepatic level	1-year-old mice		2-year-old mice	
	Wild-type (n = 5)	PPAR α -null (n = 5)	Wild-type (n = 4)	PPAR α -null (n = 7)
TNF- α (ng/ml)	0.33 $\pm$ 0.05 ^a	0.45 $\pm$ 0.03 ^b	0.40 $\pm$ 0.04	1.07 $\pm$ 0.13 ^b
IL-6 (ng/ml)	0.09 $\pm$ 0.01	0.12 $\pm$ 0.01 ^b	0.32 $\pm$ 0.18	1.78 $\pm$ 0.26 ^b
IFN- γ (ng/ml)	1.58 $\pm$ 0.07	1.64 $\pm$ 0.10	4.92 $\pm$ 0.43	7.23 $\pm$ 0.66 ^b
IL-4 (ng/ml)	0.15 $\pm$ 0.01	0.12 $\pm$ 0.01	0.14 $\pm$ 0.01	0.16 $\pm$ 0.02

PPAR α , proliferator-activated receptor-alpha.

^aAll values are means $\pm$ SE. Differences were analysed for statistical significance using the *U*-test.

^bStatistically significant ($P \leq 0.05$) in comparison with the corresponding wild-type value.

Figure 3 Age-related alterations in immune cell subpopulations in the spleens of male PPAR α -null 129/Sv mice and wild-type controls. Male wild-type (open bars) and PPAR α -null (solid bars) 129/Sv mice were sacrificed by cervical dislocation at one (light grey rectangles in A–K) and two (dark grey rectangles in A–K) years of age and their spleens were removed by dissection. Following disruption into single-cell suspensions, the total numbers of splenocytes (A) were determined by light microscopy with Trypan blue staining, and different cell populations including B cells (CD19⁺) (B), T helper cells (CD4⁺CD3⁺) (C), T cytotoxic cells (CD8⁺CD3⁺) (D), T $\gamma\delta$ cells ($\gamma\delta$ ⁺) (E), natural killer T (NKT) cells (CD4⁺NKPan⁺) (F), natural killer (NK) cells (CD4⁺NKPan⁺) (G), macrophages (CD11b⁺) (H), granulocytes (Gr1⁺) (I), Myeloid suppressor cells (CD11b⁺Gr1⁺) (J) and plasma cells (CD138⁺) (K) were examined employing flow cytometry. All values presented are means $\pm$ SE (standard error) for 5–11 animals in each group (as indicated in parentheses under the bars). *Statistically significant ($P \leq 0.05$) in comparison with the age-matched wild-type value. #Statistically significant ($P \leq 0.05$) in comparison with the corresponding wild-type animals at 1 year of age.



the level of IFN- γ was also higher in the PPAR α -deficient animals (≥ 1.4 -fold) (Table 2).

Male PPAR α -null 129/Sv mice and wild-type control animals demonstrate different alterations in their splenic immune cells upon ageing

To evaluate the regulatory effects of PPAR α on the composition of immune cells in an ageing lymphoid immune organ, the phenotypes of splenocytes in the PPAR α -null and wild-type strains were compared by flow cytometric analysis at one and 2 years of age. As expected, wild-type animals exhibited a significant reduction in the total numbers of splenocytes with advancing age (Fig. 3A), and this reduction involved most of the subpopulations including B, $\gamma\delta$ T, NKT and NK cells (Fig. 3B, E–G, respectively), macrophages (Fig. 3H), granulocytes (Fig. 3I), and myeloid suppressor cells (Fig. 3J), but not T helper, T cytotoxic and plasma cells, the numbers of which were actually increased (Fig. 3C,D,K, respectively). In contrast, the total numbers of splenocytes in 2-year-old PPAR α -null mice were higher than in the 1-year-old animals (Fig. 3A) and, furthermore, at both ages, these mice contained more splenocytes than the age-matched wild-type animals (Fig. 3A). More importantly, both adult and old PPAR α -null mice showed significant enlargements in most, if not all, subpopulations of splenocytes, i.e. B, T helper, T cytotoxic, $\gamma\delta$ T, NKT and NK cells, macrophages, granulocytes, and myeloid suppressor cells (Fig. 3B–J, respectively). Moreover, the old PPAR α -null mice had a strikingly larger numbers of plasma cells than the age-matched controls (Fig. 3K).

To determine whether alterations in the immune cell composition of the spleen in old PPAR α -null mice are accompanied by the changes in the morphology of their spleens, we examined the histology of the spleens from

adult old (2-year-old) male PPAR α -null 129/Sv mice and their age-matched wild-type controls. As shown in Fig. 4B, the spleens of old PPAR α -null mice exhibited enhanced cellularity in both white and red pulps compared to those of wild-type controls (Fig. 4A).

Old, but not adult male PPAR α -null 129/Sv mice produce high levels of IgG1, Ig2a and IgE

This substantial increase in the numbers of splenic plasma cells in the two-year-old, but not in 1-year-old, male PPAR α -null mice indicated that PPAR α might play a role in regulating the production of Ig by these cells. Indeed, old, but not adult PPAR α -null mice exhibited significantly higher serum levels of IgG1, IgG2a and IgE than the age-matched wild-type animals, which in turn tended to demonstrate enhanced serum levels of IgG1 and IgG2a compared to those aged 1 (Fig. 5A–C).

Discussion

The deleterious alterations that occur in both the innate and adaptive immune systems upon ageing are referred to as immunosenescence [20]. The major change in the case of innate immunity involves enhanced production of pro-inflammatory cytokines in the absence of insult, which increases susceptibility to infections [21]. As for the adaptive immune system, ageing involves deterioration of the thymus and bone marrow, the two major primary immune organs [20, 22], as a consequence of which the numbers of naïve T (both helper and cytotoxic T cells) and B cells exiting from these organs are substantially reduced [20, 22]. In addition, T and B cells from elderly animals exhibit several functional defects, including reduced IL-2 production, enhanced release of pro-inflammatory cytokines by T cells, and attenuated

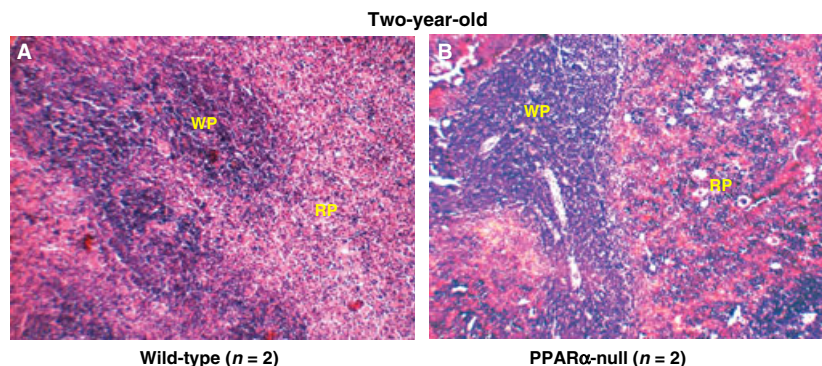


Figure 4 Increased cellularity of both white and red pulps in the spleens of old PPAR α -null mice. Representative photographs of spleen sections (magnification $\times 100$) from 2-year-old male wild-type (A) or PPAR α -null (B) mice stained with haematoxylin–eosin. An increased cellular density is present in both white and red pulps (WP and RP, respectively) of the spleen from a PPAR α -null mouse (B) compared to that from the wild-type control (A). Virtually identical patterns of histological alterations were observed in all PPAR α -null mice examined. The values in the parentheses indicate the total numbers of the animals used in the experiment.

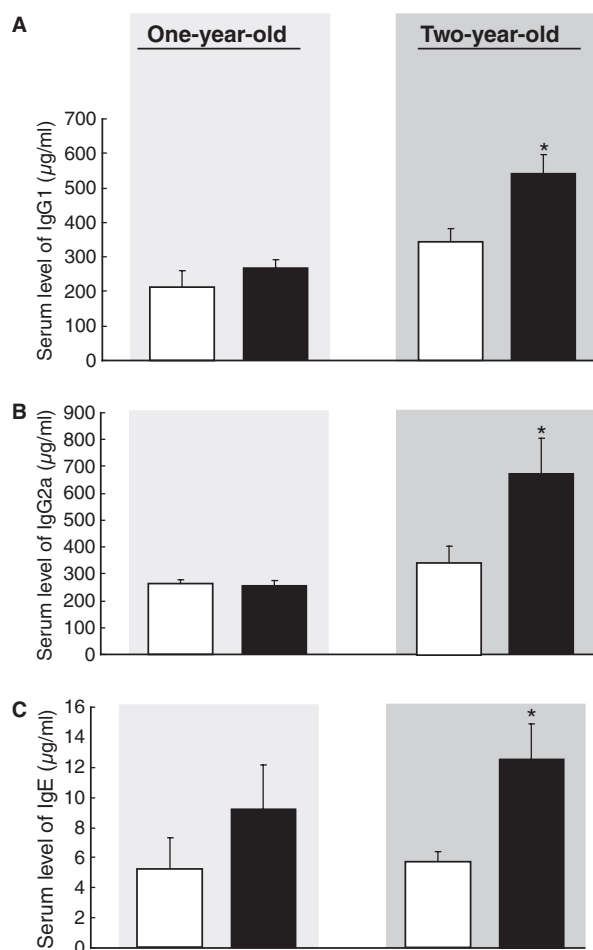


Figure 5 Age-related alterations in serum levels of IgG1, IgG2a and IgE in male PPAR α -null 129/Sv mice and wild-type controls. Male wild-type (open bars) and PPAR α -null 129/Sv (solid bars) mice were bled at one (light grey rectangles in A–C) and two [dark grey rectangles in A–C] years of age, and serum levels of IgG1 (A), IgG2a (B) and IgE (C) were determined employing ELISA procedures. All values presented are means $\pm$ SE (standard error) for six animals in each group. *Statistically significant ($P \leq 0.05$) in comparison with the corresponding wild-type value.

susceptibility to apoptosis, defects in Ig class switching and elevated production of autoreactive antibodies by B cells [20, 22]. These age-associated alterations in the adaptive immune system are manifested as an overall decline in antigen-specific immune responses [22].

The mechanisms underlying such immune alterations in ageing animals are poorly understood. It has been proposed that both intrinsic defects and extrinsic factors such as hormones and cytokines play important roles in this context [21–23]. In connection with this proposal, we demonstrate here that as an intrinsic factor, PPAR α can play an important role in the process of ageing in both non-lymphoid and lymphoid immune organs.

Regarding the influence of PPAR α on the senescence of non-lymphoid immune organs, we show that upon

ageing, the total numbers of immune cells in the livers of wild-type animals remain relatively unchanged, whereas in PPAR α -null mice, the numbers of hepatic immune cells increase constantly. Furthermore, this enhancement of total numbers of IHIC is accompanied by continuous increases in the numbers of various hepatic immune cell subpopulations, in particular granulocytes, T helper, T cytotoxic and T $\gamma\delta$ cells in their livers at old age. More strikingly, at this age, PPAR α -null mice develop inflammatory foci containing different immune cell types in combination with substantial increases in the levels of inflammatory cytokines in their livers. These observations lead us to conclude that PPAR α may play a regulatory role in maintaining the total numbers and composition of hepatic immune cells in a constant rate and in a balanced pattern during the process of ageing. Thus, permanent abrogation of this regulatory element (PPAR α deficiency) may result in a gradual accumulation of both naïve and endogenously activated immune cells in the liver upon advancing of age and consequently induce a significant alteration in the hepatic immune system at old age (as supported by our findings in here).

The enhanced inflammatory background of the livers of both adult and old PPAR α -null mice suggests that in the absence of PPAR α , the liver is more susceptible to inflammatory-mediated injuries caused by hepatotropic insults. We are now in the process to test this suggestion and to characterize the types of hepatic immune cells that are responsible for the aberrant production of inflammatory cytokines in ageing PPAR α -null mice.

Considering the involvement of PPAR α in the process of ageing of lymphoid immune organs, we demonstrate that while wild-type control animals exhibit a significant reduction in the total numbers of splenocytes in their spleens by advancing of age, PPAR α -null mice do not show such a reduction in their total numbers of splenocytes upon ageing. In fact, in comparison with old wild-type animals, PPAR α -null mice show marked elevations not only in the total numbers, but also in the numbers of various subpopulations of splenocytes including macrophages, granulocytes, B, T helper, T cytotoxic, T $\gamma\delta$, NKT and plasma cells. These enhancements in the numbers of splenocytes and subpopulations thereof were also accompanied by the enhanced cellularity of both white and red pulps of the spleen. Together, these findings strongly suggest that during the life time, PPAR α exerts regulatory effects on the spleen to sustain the cellularity and cellular composition of this organ in accordance with the age of animal. Hence, permanent cessation of these regulatory effects (PPAR α deficiency) might interfere with normal process of ageing of the spleen and thereby lead to a constant accumulation of various naïve and activated immune cells in this organ by advancing of age (as evidenced by our observations in here). In fact, such an

accumulation of immune cells in the spleen will be more evident at old age, because at this age and under normal circumstances (PPAR α sufficiency), the cellularity of the spleen is markedly reduced [20, 22]. Whether these enhancements in the cellularity and immune cell composition of the spleen in old PPAR α -deficient mice are immunologically beneficial or detrimental remains to be elucidated.

Our observations that the number of splenic plasma cells in old PPAR α -null mice is highly elevated and that this elevation is accompanied by significant increases in serum levels of IgG1, IgG2a and IgE are of interest. These findings constitute strong evidence that PPAR α also exerts regulatory effects on the age-related development of plasma cells as well as the production of Igs of different isotypes by these cells. In fact, two possible mechanisms can explain these PPAR α -mediated regulatory effects. First, because the splenic plasma cells that produce different Ig isotypes are primarily short-lived and generated as a consequence of continuous activation of follicular B cells by T cell-dependent antigens [24], it is possible that PPAR α modulates specific antibody responses to endogenous antigens, e.g. autoantigens at old age. Thus, characterization of the specificities of the antibodies produced in old PPAR α -null mice should be highly informative and is presently being carried out in our laboratory. Secondly, it has been known that the bone marrow is the main site for the synthesis of large amounts of specific antibodies during anamnestic immune responses [25] and that these antibodies are produced by long-lived plasma cells that accumulate in the bone marrow compartment after every antigenic challenge [24–26]. Thus, as the second possibility, we speculate that in the mouse, the generation or accumulation of long-lived plasma cells in the bone marrow is accelerated by the absence of PPAR α . Obviously, additional studies are required to support this speculation.

Together, our findings here indicate that lack of PPAR α dramatically alters the hepatic and splenic immune status, and endogenous production of Igs in aged animals. In this connection, it is of considerable importance to determine whether the development of immunological alterations in old PPAR α -null mice are directly associated with the lack of expression of PPAR α in immune cells or that the effects are indirect, i.e. caused by functional alterations of the microenvironment in which the immune cells reside. We are presently performing studies to answer these questions.

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