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Ageing enhances cellular immunity to myeloperoxidase and experimental anti-myeloperoxidase glomerulonephritis

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Abstract

Objectives. Anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis is an autoimmune disease characterised by small blood vessel inflammation, commonly affecting the kidneys and respiratory tract. It is unclear why the incidence of this condition increases with age. Previous studies in a passive antibody transfer system in aged mice have implicated innate effectors. To test the hypothesis that autoimmunity to myeloperoxidase, an autoantigen responsible for ANCA-associated vasculitis, increases with age, anti-myeloperoxidase autoimmunity was studied in murine models of active autoimmunity and disease induced by cellular immunity.

Methods. Young (8 weeks) and aged (either 15 or 22 month) mice were immunised with whole proteins or peptides from ovalbumin, as a model foreign antigen, or myeloperoxidase protein or peptides. Mice were subjected to a model of active anti-myeloperoxidase glomerulonephritis. Cellular and humoral immune responses and tissue inflammation were assessed.

Results. While cellular immunity to ovalbumin was diminished in aged mice, cellular autoimmunity to myeloperoxidase and its immunodominant CD4+ and CD8+ T cell epitopes was increased after immunization with either MPO peptides or whole MPO protein, assessed by peptide and antigen specific production of the pro-inflammatory cytokines interferon-γ and interleukin-17A. MPO-ANCA titres were not increased in aged mice compared with young mice. In experimental anti-MPO glomerulonephritis, cell mediated injury was increased, likely due to CD4+ and CD8+ T cells, innate immunity and the increased vulnerability of aged kidneys.

Conclusion. Heightened cellular immunity to MPO develops with ageing in mice and may contribute to the increased incidence and severity of ANCA-associated vasculitis in older people.

Keywords: vasculitis, MPO-ANCA associated vasculitis, ageing, autoimmunity, cellular immunity, glomerulonephritis, lymphocytes

Key messages:

- T-cell autoreactivity to myeloperoxidase and its immunodominant peptides was increased in aged mice.
- In contrast, immunity to a foreign antigen was impaired with ageing.

- This increased autoimmunity translates into increased disease in an inducible active model of anti-myeloperoxidase glomerulonephritis.

Introduction

ANCA-associated vasculitis (AAV) is characterised by inflammation of small blood vessels, including those in the kidney and respiratory tract, by autoimmunity to one of two neutrophil proteins, myeloperoxidase (MPO) and proteinase 3 (PR3) [1]. Unlike some autoimmune diseases, the incidence of AAV increases with age. In particular, microscopic polyangiitis (MPA), the syndromic clinical presentation usually involving autoimmunity to MPO (MPO-AAV) and glomerulonephritis, typically occurs in the 6th-8th decades of life [1,2]. In addition, AAV in older people tends to be both more severe with poorer outcomes [1,3-5] and more frequent kidney involvement [2]. While the mechanisms underpinning this age-related increase in the incidence and severity of MPA are unknown, key elements of the immune system are substantially altered with ageing [6-8].

Changes to the immune system with age, known as immunosenescence, involve both the adaptive and innate arms of the immune system. In the adaptive immune system, thymic involution results in a smaller pool of naïve T cells, with a corresponding increase in memory T cells [6,9,10]. T-cell immunity to foreign antigens is reduced with increased susceptibility to infection, but chronic antigenic stimulation may increase the chance of autoreactivity. Moreover, innate immune components can exhibit enhanced inflammatory functions, a phenomenon known as inflamm-ageing with a tendency towards low-grade inflammation [7]. Conceivably, persistent low-grade activation of the innate immune system, together with an increased tendency towards autoreactivity, may account for the increasing incidence of AAV with age, as the relevant AAV autoantigens are found within cells of the innate immune system, namely neutrophils and monocyte/macrophages [1].

Both innate and adaptive immunity are important in the pathogenesis of MPO-AAV. Loss of T and B cell tolerance to MPO, abundant in neutrophils, results in T cell dependent production of MPO-ANCA, binding to primed neutrophils that induce microvascular endothelial injury [11], with a predilection for some specialised vascular beds, particularly the glomerular and pulmonary capillaries [1,12]. In addition to inducing injury, MPO-ANCA activated neutrophils release MPO into these microvascular beds [13,14] where MPO-derived immunogenic peptides can be recognised by effector T cells, amplifying local injury [15,16].

The role of ageing in the response of the innate immune system to anti-MPO antibodies has been evaluated in glomerulonephritis induced by passive transfer of anti-MPO antibodies [17].

Here anti-MPO antibodies injected into naïve mice bind to neutrophils that induce glomerular injury [11,18] Monocyte/macrophages also play a role [19]. Anti-MPO antibodies induced more severe injury in aged mice, demonstrating heightened innate immune effectors in response to transfer of anti-MPO antibodies [17]. While these studies shed light on the role of ageing in innate effector responses in AAV, whether ageing promotes active anti-MPO autoimmunity and/or cell mediated effector responses is not known.

The current studies examine the effect of ageing in mice on active T and B cell autoimmune responses to MPO, and the role of ageing in the development of an acute glomerular lesion mediated by a T cell response to MPO. In contrast to the reduced immunity to a model foreign antigen, in aged mice active T cell autoimmunity to MPO is increased, leading to increased glomerular injury and intrarenal proinflammatory leukocyte infiltrates, implicating heightened cellular immunity to MPO in the pathogenesis of AAV in older people.

Methods

Mice

Male C57BL/6 (Monash University Animal Research Platform and the Animal Resources Centre, Western Australia, Australia) were 8-weeks old, or either 15 or 22 months of age [20]. Ages are reported for each experiment in the results section. Studies adhered to the National Health and Medical Research Council of Australia guidelines for animal experimentation and were approved by the Monash University Animal Ethics Committee.

Leukocyte isolation

Lymph nodes (LN) and spleens were minced, filtered (70μM filter, BD Bioscience, San Jose, CA), erythrocytes lysed and cells counted by a Z2 Series Coulter Counter (Beckman Coulter, Brea, CA). For intrarenal leukocyte isolation [21], kidneys were digested (5mg/ml collagenase D and 100μg/mL DNase I, Roche Diagnostics, Indianapolis, IN in HBSS Sigma-Aldrich, St Louis, MO, 20min, 37°C), cells were dissociated, incubated (5min), washed, erythrocytes lysed (NH_4Cl) and samples filtered (40μM filter, BD Bioscience).

Flow cytometry

Cells were incubated with Mouse BD Fc Block (BD Pharmingen, 10min) and surface stained with antibodies (20min, 4°C, Supplementary Table S1, available at *Rheumatology* online). For intracellular Foxp3 staining, cells were stained using the Foxp3/Transcription Factor Staining Buffer Set (ThermoFisher Scientific, Waltham, MA). In these experiments, LN and spleen results are expressed as a proportion (%) of the relevant cell type, as cell numbers were consistently lower in aged mice (naïve mice LN: young $2.66\pm0.30x10^7$, aged $1.29\pm0.25x10^7$, $P<0.01$; spleen: young $8.21\pm0.94x10^7$, aged $4.59\pm0.94x10^7$ $P<0.05$, $n=6$/group). For intracellular cytokine staining, cells were stimulated with Cell Activation Cocktail including Brefeldin A (BioLegend, San Diego, CA, 4h, 5% CO_2, 37°C) and anti-interleukin-17A (IL-17A), anti-interferon-γ (IFN-γ) and anti-tumour necrosis factor (TNF) antibodies using the Foxp3/Transcription Factor Staining Buffer Set. Cells were acquired on the FACSCanto II machine or LSRFortessa X-20 and data analysed using FlowJo 10.7.1 software (TreeStar Inc, Ashland, OR). Flow cytometry gating strategies are shown in Supplementary Fig. S1, available at *Rheumatology* online.

Antigen-specific T cell responses

Mice were immunised with 10μg peptides containing the immunodominant CD4+ and CD8+ MPO or ovalbumin (OVA) epitopes, $MPO_{409-428}$ and $MPO_{431-439}$ or $OVA_{323–339}$ and $OVA_{257-264}$ in Freund's complete adjuvant (FCA; Sigma-Aldrich) subcutaneously (base of the tail). Draining LN were harvested 10 days later, cells cultured (18h, 37°C, 5% CO_2, 5×10^5 cells/well) and restimulated with either 10μg/ml recombinant mouse MPO (rMPO [22]), $MPO_{409-428}$, $MPO_{431-439}$, OVA (Sigma-Aldrich), $OVA_{323–339}$ or $OVA_{257-264}$ in supplemented RPMI media. IFN-γ and IL-17A secretion were detected by enzyme-linked immunospot (ELISPOT; BD Biosciences), enumerated by an automated ELISPOT reader and results expressed as the mean number of spots minus baseline.

Anti-MPO and anti-OVA antibodies

Mice were immunised with 20μg of rMPO or OVA in FCA subcutaneously (base of the tail) and boosted on days 7 (inguinal region) and 14 (axillary region) with 10μg rMPO in Freund's incomplete adjuvant (FIA; Sigma-Aldrich). Sera was obtained on days 0, 14, and 35. For MPO- or OVA-specific IgG, ELISA microplates (Greiner Bio-One, Frickenhausen, Germany) were coated with 5μg/ml rMPO or OVA, incubated (4°C overnight), blocked (2% bovine serum albumin, Sigma-Aldrich, 2h), then incubated with diluted sera (anti-OVA 1:5000, anti-MPO 1:400, 2h, room temperature). MPO- or OVA-specific IgG was detected using horseradish peroxidase-conjugated anti-mouse IgG (1:1000-1:2000; GE Healthcare, Rydalmere, NSW, Australia).

Experimental autoimmune anti-MPO glomerulonephritis

Experimental anti-MPO glomerulonephritis was induced as previously described [23]. Mice were immunized on day 0 with 20μg rMPO in FCA subcutaneously (base of the tail). On day 7, mice were injected with 20μg rMPO in FIA subcutaneously (inguinal). Glomerulonephritis was induced by intravenous injection of sheep anti-mouse basement membrane (BM) globulin on day 17. The dose of sheep anti-mouse BM globulin was adjusted for the increased weight of aged mice, with young mice being injected with 2.5mg and aged mice receiving 3mg. Immunofluorescent staining for glomerular sheep IgG showed similar deposition in young and aged mice, assessed on coded slides (young 1.34±0.06, aged 1.44±0.13 P=0.48, score 0-3+, >30 glomeruli per mouse, n=8/group, antibody dilution 1:4000). Mice were humanely killed 4 days after administration of anti-BM globulin. Control mice were immunised with OVA then injected with sheep anti-mouse BM globulin [23].

Renal injury

Kidney disease was assessed on 3μm-thick formalin-fixed periodic acid-Schiff (PAS)-stained sections [24-26]. A glomerular segmental lesion was defined as an accumulation of PAS-positive material, with or without hypocellularity. At least 50 glomeruli/mouse were examined and results expressed as the percentage of glomeruli exhibiting segmental glomerular lesions per glomerular cross section (gcs). Tubular injury in least 10 high power (x400) fields per mouse was assessed by tubular epithelial cell loss, tubular necrosis, accumulation of cellular debris and tubular cast formation, according to the percentage of affected tubules in a field. The percentage of tubules affected was scored as: 0=normal, 1=<10-25%, 2=26-50%, 3=51-75%, 4=>75%.

Kidney leukocyte staining

Kidney sections were fixed in periodate lysine paraformaldehyde, cryoprotected (20% sucrose) then frozen. A three-layer immunoperoxidase technique was used to detect leukocytes on 4μm sections [24,27], stained via the Vectastain Elite ABCKit (Vector Laboratories, Burlingame, CA) and DAB Brown (Sigma-Aldrich) using rat anti-mouse CD4 (20μg/ml; GK1.5), rat anti-mouse CD8a (20μg/ml; 53–6.72), rat anti-Gr-1 (2.5μg/ml; RB6–8C5) and rat anti-mouse CD68 (10μg/ml; FA/11; all BioXcell, West Lebanon, NH). The secondary antibody was rabbit anti-rat biotin (BD Bioscience). At least 50 glomeruli were counted, and 10 interstitial sections per mouse and results expressed as cells/gcs and cells/high power field (hpf).

Statistical analysis

Student's *t* test was used for analysis of two groups and one-way ANOVA with Tukey's *post hoc* test for multigroup comparisons (GraphPad Prism, GraphPad Software, San Diego, CA). A *P* value of <0.05 was considered significant. Data are expressed as mean±SEM.

Results

Immune systems of normal young and aged mice

Initial experiments examined the differences in the immune systems of otherwise normal, naïve young (8-week old) and aged (22-month-old) mice. All available LN (Fig. 1) and spleens (findings were similar, presented in Supplementary Fig. S2, available at *Rheumatology* online) of normal SPF-housed C57BL/6 mice were studied. Fewer leukocytes were recovered from LN of aged mice, compared with younger mice (young $2.36\pm0.21x10^7$, aged $1.11\pm0.22x10^7$ CD45+ cells, $P<0.01$). Proportions of CD4+ T cells in LN were reduced in older mice, while those of CD8+ T cells were similar. Within the CD4+ and CD8+ subsets, older mice exhibited a lower proportion of naïve $CD62L^+CD44^-$ T cells, with correspondingly increased proportions of $CD62L^+CD44^{hi}$ "central memory" cells and $CD62L^-CD44^{hi}$ effector cells. Proportions of $CD4^+$ T cells that were $Foxp3^+$ were higher in aged mice (similar findings using $CD25^{hi}$). Proportions of $CD19^+$ B cells were similar. In the myeloid lineage (Supplementary Fig. S3, available at *Rheumatology* online), LN from aged mice exhibited a higher proportion of $CD11b^+$ cells, including $CD11b^+Ly6G^+$ neutrophils and $CD11b^+Ly6C^{hi}$ inflammatory monocytes. Proportions of $CD11c^+$ dendritic cells were also increased in aged mice. These findings confirm previous reports of the composition of the immune system in ageing mice and are analogous to humans [9,10,28-30].

Immune responses to immunodominant MPO peptides in aged mice

Mice (8-weeks or 22-months) were immunized either with the immunodominant MPO peptides for MHC $H2^b$ (C57BL/6 mice) $MPO_{408-428}$ ($CD4^+$ T cells) and $MPO_{431-439}$ ($CD8^+$ T cells), or as a control foreign antigen, OVA peptides, $OVA_{323-339}$ and $OVA_{257-264}$ (the $CD4^+$ and $CD8^+$ immunodominant OVA peptides). Immunizations were in FCA and draining LN were studied after 10 days. Younger mice exhibited increased numbers of draining LN cells compared with older mice after immunization (Supplementary Fig. S4, available at *Rheumatology* online), and as in naïve mice, $CD4^+$ T cell proportions were lower in aged mice. Immunization with either self or foreign peptides resulted in higher proportions of effector and central memory T cells in both young and aged mice. $Foxp3^+$ cell and B cell proportions were similar to those seen in naïve mice. However, antigen-specific T cell recall responses (by IFN-γ and IL-17A ELISPOT) showed different patterns between responses to OVA and to the autoantigen MPO (Fig. 2). Compared with young mice, aged mice exhibited diminished OVA-specific T cell responses whether to the whole OVA protein, or to the $CD4^+$ or $CD8^+$ immunodominant OVA peptides.

However, in contrast to the diminished response to this foreign antigen, T cell recall responses to MPO measured by IFNγ production were increased in aged mice. The numerical increase in IL-17A responses to MPO and $MPO_{409-428}$ did not reach statistical significance, and IL-17A responses to the $CD8^+$ epitope $MPO_{431-439}$ were low in both young and aged mice. Therefore, while overall immune stimulation is similar after challenge with adjuvant and antigenic peptides, older mice have weaker T cell responses to a foreign antigen but paradoxically stronger responses to MPO as an autoantigen, consistent with the increasing incidence and severity of MPO-AAV in older people.

Humoral responses in aged mice

Groups of young (8-week-old) and aged (22-month-old) mice were immunized with either OVA or MPO protein in FCA and antibody responses assessed at days 14 and 35. Humoral responses to OVA and to MPO were diminished in aged mice, though there was some increase in MPO-ANCA titres in aged mice at day 35 compared with day 14 (Fig. 2C). Proportions and numbers of splenic $CD19^+$ B cells were similar at day 35. Proportions of marginal zone and T2 B cells were lower in OVA-immunized aged mice (Supplementary Fig. S5, available at *Rheumatology* online).

Immune responses in MPO-immunized aged mice with glomerulonephritis

Young (8-week-old) and aged (15-month-old) mice were immunized twice with MPO or OVA (as a control). After immunisation, serum MPO-ANCA is insufficient to induce disease, so glomerulonephritis is triggered by low dose anti-BM globulins. Injury is mediated by MPO-specific T cells recognising MPO, deposited in glomeruli by neutrophils transiently recruited by the anti-BM globulin. Immune responses and injury are studied four days after disease is triggered (21 days after first immunization). As at 10 days, MPO-stimulated recall responses, measured by IL-17A and IFN-γ on cells from draining LN, were enhanced in aged mice (Fig. 3A), confirming increased responses not only after peptide injection but also after MPO protein immunisation. These findings were confirmed by flow cytometry of PMA/ionomycin-stimulated splenic CD4+ T cells. A higher proportion of CD4+ T cells from aged mice produced IFN-γ after PMA/ionomycin, independent of their specificity (Fig. 3B). There were no significant changes in splenic CD8+ T cell cytokine production (Supplementary Fig. S6A, available at *Rheumatology* online).

Histological glomerular injury is increased in aged mice with anti-MPO nephritis

In mice with anti-MPO glomerulonephritis, glomerular histological changes were increased in aged mice compared with young mice (Fig. 4). While ageing itself causes some glomerular and tubulointerstitial changes [31-33], glomerular lesions due to anti-MPO T cell responses were heightened in aged mice. As this is a short-term model, due to needing to avoid an adaptive immune response to the anti-BM globulin (raised in sheep), tubulointerstitial disease is not prominent. The tubulointerstitial changes seen in older mice were largely age-related, as changes were present in naïve and OVA-immunised mice as well as mice with anti-MPO glomerulonephritis.

Intrarenal inflammation and leukocytes are increased in aged mice with glomerulonephritis

Inflammatory changes were assessed by enumerating leukocytes by immunostaining kidneys (Fig. 5, Supplementary Fig. S7, available at *Rheumatology* online), and by flow cytometry of digested kidneys (Fig. 6). Comparisons between mice with anti-MPO glomerulonephritis showed that in many, but not all instances, there were more leukocytes in glomeruli of older mice (Fig. 5). Aged mice with disease exhibited more glomerular CD8+ cells, macrophages and neutrophils. Except for CD4+ T cells, groups of aged mice tended to have more leukocytes in glomeruli than their corresponding groups of young mice. In the tubulointerstitium, older mice with anti-MPO glomerulonephritis had more CD4+ T cells, CD8+ T cells and macrophages, though numerical increases were also seen in mice receiving OVA and anti-BM globulin (Supplementary Fig. S7, available at *Rheumatology* online). Assessing the capacity of intrarenal leukocytes to produce the pro-inflammatory cytokines IL-17A, IFN-γ and TNF, there was a 4- to 10-fold increase in cytokine producing intrarenal CD4+ T cells (Fig. 6). There was a variable and lesser effect in aged mice immunised with OVA, particularly for IL-17A. Intrarenal CD8+ cells were also shown to produce more cytokines with age, with more IFNγ producing cells between young and aged mice with anti-MPO glomerulonephritis (Supplementary Fig. S6B, available at *Rheumatology* online).

Discussion

The incidence of AAV increases with age and older people with AAV have poorer outcomes [1,3-5]. The current studies show that in mice T cell reactivity to MPO is enhanced after immunisation with MPO or the relevant immunodominant MPO peptides [15,16]. In a T cell dependent model of anti-MPO glomerulonephritis [16,34], older mice not only developed increased T cell autoimmunity to MPO, they also exhibited increased intrarenal leukocyte infiltrates with increased pro-inflammatory capacity and increased glomerular injury. These results, when considered with previous work, mechanistically implicate both autoreactive T cells and the innate immune system in the increasing incidence of AAV with age.

Following experiments in non-immune mice confirming reports of changes in the ageing murine immune system, similar to those in humans, we examined T cell autoreactivity to MPO, using peptides comprising either the MPO CD4+ and CD8+ immunodominant epitopes, or the corresponding immunodominant peptides of OVA. While aged mice had impaired T cell recall responses to OVA as a foreign antigen, anti-MPO T cell responses were heightened. MPO recall responses by IFN-γ ELISPOT were increased. IL-17A responses fell short of statistical significance, likely a type II error. Further studies after immunisation with rMPO in the disease model demonstrated increased IL-17A and IFNγ production in aged mice. The impaired responses to OVA in aged mice align with observations that immunity to infection and vaccines is impaired with age. Conversely, the increased T cell autoreactivity to key MPO peptides spanning an immunological hot spot in the MPO protein [15,16,35,36] may in part explain why this disease increases with increasing age.

While the picture in cellular responses is clear, that of humoral responses is not. Anti-OVA and anti-MPO IgG titres were similarly reduced in aged mice, though we did not directly assess affinity. As the MPO-ANCA generated after immunization with MPO in mice does not induce nephritis, it is unclear to what degree murine MPO-ANCA from MPO intact mice represents human ANCA. It remains unclear whether ANCA production itself in humans is affected by age, or whether the primary abnormalities with ageing exist in the innate immune system and in the degree of loss of T cell tolerance.

Having defined the effects of ageing in loss of tolerance, further experiments examined the effects of ageing on injury in a T cell dependent model of anti-MPO glomerulonephritis.

Previous work using an anti-MPO antibody transfer model examined innate immunity [17]. The model used in the current study involves active anti-MPO autoimmunity, but requires anti-BM antibodies to trigger disease, as MPO-ANCA from MPO intact mice do not themselves induce injury. Transient neutrophil recruitment results in MPO lodging in glomeruli, where it is detected by CD4+ and CD8+ effector T cells [16,23,34]. T cell depletion in the effector phase limits injury [16,34] and injury does not occur in *Mpo*$^{-/-}$ mice [34].

In this active model, aged mice developed not only increased cellular immune responses to MPO, but also increased kidney disease. Experiments included groups of unmodified young and old mice to control for the effects of ageing on the immune system and on kidneys [33] independent of additional immune stimuli, and OVA-immunised mice injected with anti-BM globulin. While the key comparisons were between MPO immunised young and aged mice with nephritis, other observations imply that there are additional reasons why inflammation was increased in aged mice. While MPO-stimulated splenocyte cytokine production was selectively increased in aged mice with anti-MPO autoimmunity, when CD4+ T cells from all groups of aged mice were stimulated with PMA/ionomycin they were more likely to produce IFN-γ, suggesting a generalised pro-inflammatory capacity of aged CD4+ T cells. In some parameters, the effects of ageing itself were observed in the kidney, in line with previous observations [33], consistent with aged glomeruli, a proportion of which already have segmental lesions, being more vulnerable. In addition, there were some changes in kidneys of older OVA-immunised mice injected with anti-BM globulin. This is consistent with reduced renal reserve in ageing and may be due either a reduced threshold for the effects of adjuvant induced immune activation or for inflammation caused by the low dose anti-BM globulin. Adjuvant injection causes arthritis in some rat strains that may also involve the kidney [33,37]. Furthermore, as in the spleen, T cells within the aged kidney may have a more generalised proinflammatory capacity.

Both CD4+ and CD8+ cells play a role experimentally [15,16,34] and likely in human AAV [36,38-40], but the effects of ageing in this model were more prominent in CD4+ T cells. CD4+ T cells in the kidney promote activation of innate leukocytes [41]. This, together with the studies of Wang et al. in the anti-MPO antibody transfer model, and our observations of increased proportions of innate cells in LN of aged mice, position innate effectors as being important in kidney injury in AAV. While it remains to be determined how ageing increases

the likelihood of loss of B cell tolerance, elements of innate immunity are plausibly involved, for example, those involving autoantigen release, availability or processing. Furthermore, clonal haematopoiesis of indeterminate potential, a function of ageing that may influence both myeloid function and autoantigen expression, is more common in people with AAV [42].

While most cases occur in older adults, AAV also occurs in children [1]. It is difficult to relate the changes in cellular immune responses with ageing to the pathogenesis of childhood AAV. Early onset disease may be influenced by the presence of one or more inherited or *de novo* damaging mutations, as may be the case in other diseases such as systemic lupus erythematosus [43,44].

AAV has a complex pathogenesis that involves innate immunity in both loss of tolerance and as effectors, as well as cellular immunity. However, a central factor is loss of T cell tolerance to ANCA autoantigens. These studies, showing heightened cellular anti-MPO autoimmunity, implicate cellular immunity to MPO in the increased incidence of MPO-AAV in older people. This heightened cellular immunity, with more active innate effectors [17] and less end organ reserve, renders tissues more vulnerable to immune injury.

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Disclosure Statement

The authors have declared no conflicts of interest.

Data Availability Statement

The data underlying this article are available in the article and in its online supplementary material, available at *Rheumatology* online.

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Figure Legends

Figure 1: Lymphocyte profiles from lymph nodes of naïve young and aged mice.

Assessed by flow cytometry, (**A**) Aged mice had lower proportions of CD4+ T cells, with (**B**) fewer CD62L+CD44- (naïve) CD4+ and CD8+ T cells, and higher proportions of T cells with a central memory (CD62L+CD44+) or effector memory (CD62L-CD44+) phenotype. (**C**) A higher percentage of aged mouse CD4+ T cells were Foxp3+. (**D**) B cell proportions were similar between young and aged mice. FSC-H, forward scatter height. **P*<0.05; ****P*<0.001; *****P*<0.0001, (t test).

Figure 2: Immunity to ovalbumin and autoimmunity to myeloperoxidase in young and aged mice.

(**A**) and (**B**). Mice were immunised with peptides comprising the immunodominant CD4+ and CD8+ epitopes for either ovalbumin ($OVA_{323-339}$, $OVA_{257-264}$) or myeloperoxidase ($MPO_{409-428}$, $MPO_{431-439}$). After 10 days, lymphocytes were restimulated with whole proteins or peptides (as specified above each graph) and responses measured by ELISPOT. (**A**) OVA-specific recall responses were diminished in aged mice. (**B**) Antigen-specific IFNγ producing cells were increased in aged mice, while increases in IL-17A producing cells approached significance. (**C**) Groups of mice were immunised with OVA (young *n*=6, aged *n*=4) or MPO (both *n*=6). Both anti-OVA and anti-MPO IgG titres were lower in aged mice, assessed by ELISA. imm, immunisation, pMPO, mice immunised with both MPO peptides; pOVA, mice immunised with both OVA peptides. **P*<0.05; ***P*<0.01; ****P*<0.001; *****P*<0.0001, (t test).

Figure 3. Immune responses in young and aged mice with anti-myeloperoxidase glomerulonephritis.

Twenty-one days after initial immunisation with myeloperoxidase (MPO) and 4 days post induction of nephritis, draining lymph node cells and splenocytes from young and aged mice were assessed for pro-inflammatory cytokines. (**A**) More cells from draining lymph nodes of MPO-stimulated aged mice produced IL-17A and IFNγ by ELISPOT. (**B**) IL-17A and IFNγ expression, assessed by intracellular cytokine staining of PMA/ionomycin stimulated CD4+ cells from the spleen shows increased IL-17A production in aged mice, reflecting increases after ovalbumin (OVA) immunisation and with disease. For IFNγ there was an increase in all groups of aged mice. Representative flow cytometry plots of IL-17A and IFNγ staining from splenic CD4 T cells of MPO immunised young and aged mice are shown. (**C**) Numbers of

splenic T cells producing IL-17A and IFN-γ. *P<0.05; ***P<0.001; ****P<0.0001, (ANOVA, Tukey's *post hoc* test).

Figure 4. Renal disease in young and aged mice with anti-myeloperoxidase glomerulonephritis.

(**A**) Segmental glomerular lesions were increased in number in aged mice with anti-myeloperoxidase glomerulonephritis. Age-related changed were also observed in naïve aged mice. (**B**) Tubulointerstitial injury was relatively mild in this short-term model of disease. Changes observed were often a function of ageing, although some inflammatory changes were observed in aged mice with anti-myeloperoxidase nephritis. Scale bar is 50μm. The arrow in (**A**) represents a segmental lesion. Arrows in (**B**) indicate examples of proteinaceous material in the tubular lumina, arrowheads indicate leukocytes and areas of interstitial inflammation. αBM, anti-basement membrane globulin; Imm, immunised; MPO, myeloperoxidase; OVA, ovalbumin. *P<0.05; **P<0.01; ***P<0.001, (ANOVA, Tukey's *post hoc* test).

Figure 5. Intrarenal leukocytes in young and aged mice with anti-myeloperoxidase glomerulonephritis.

(**A**) Glomerular leukocytes by immunostaining, showing increased macrophages, neutrophils and CD8+ T cells in glomeruli of aged mice with anti-MPO glomerulonephritis compared with young mice. Some effects of adjuvant and low dose anti-basement membrane globulin on cell numbers were evident in aged mice. Illustrative photomicrographs of CD4+ (**B**) and CD8+ (**C**) T cells in glomeruli of young and aged mice with anti-MPO glomerulonephritis. Scale bar is 50μm. αBM, anti-basement membrane globulin; MPO, myeloperoxidase; OVA, ovalbumin. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001, (ANOVA, Tukey's *post hoc* test).

Figure 6. Pro-inflammatory cytokine production by intrarenal CD4+ cells in young and aged mice with anti-myeloperoxidase glomerulonephritis.

Numbers of CD4+ T cells expressing (**A**) IL-17A, (**B**) IFNγ and (**C**) TNF were increased in aged mice with anti-MPO glomerulonephritis compared with young mice. CD4+ cells from kidney digests were assessed by flow cytometric intracellular cytokine staining after PMA/ionomycin stimulation, with quantitative analyses (numbers per digested whole kidney) in the upper part of each panel, followed by flow cytometric plots from an individual mouse in the anti-MPO glomerulonephritis groups (with proportions of cytokine secreting cells

expressed as a percentage). Some effects of ageing itself were also apparent, for example in the numbers of TNF producing cells from kidneys of unmodified aged mice and the numbers of IL-17A producing cells in mice receiving OVA and anti-basement membrane globulin. αBM, anti-basement membrane globulin; Imm, immunised; MPO, myeloperoxidase; OVA, ovalbumin. *P<0.05; ***P<0.001, (ANOVA, Tukey's *post hoc* test).

Figure 1. Lymphocyte profiles from lymph nodes of naïve young and aged mice.

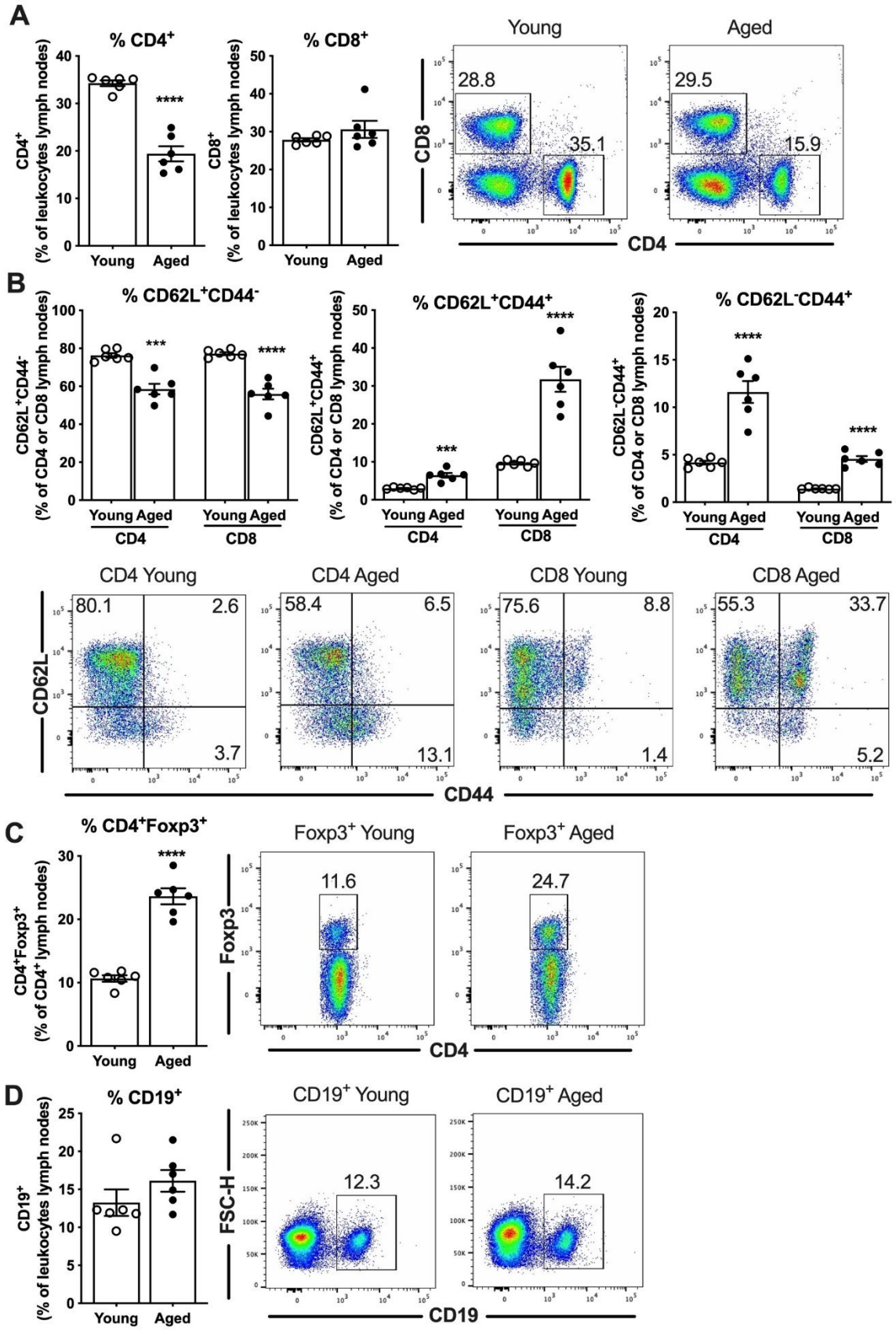


Figure 2. Immunity to ovalbumin and autoimmunity to myeloperoxidase in young and aged mice.

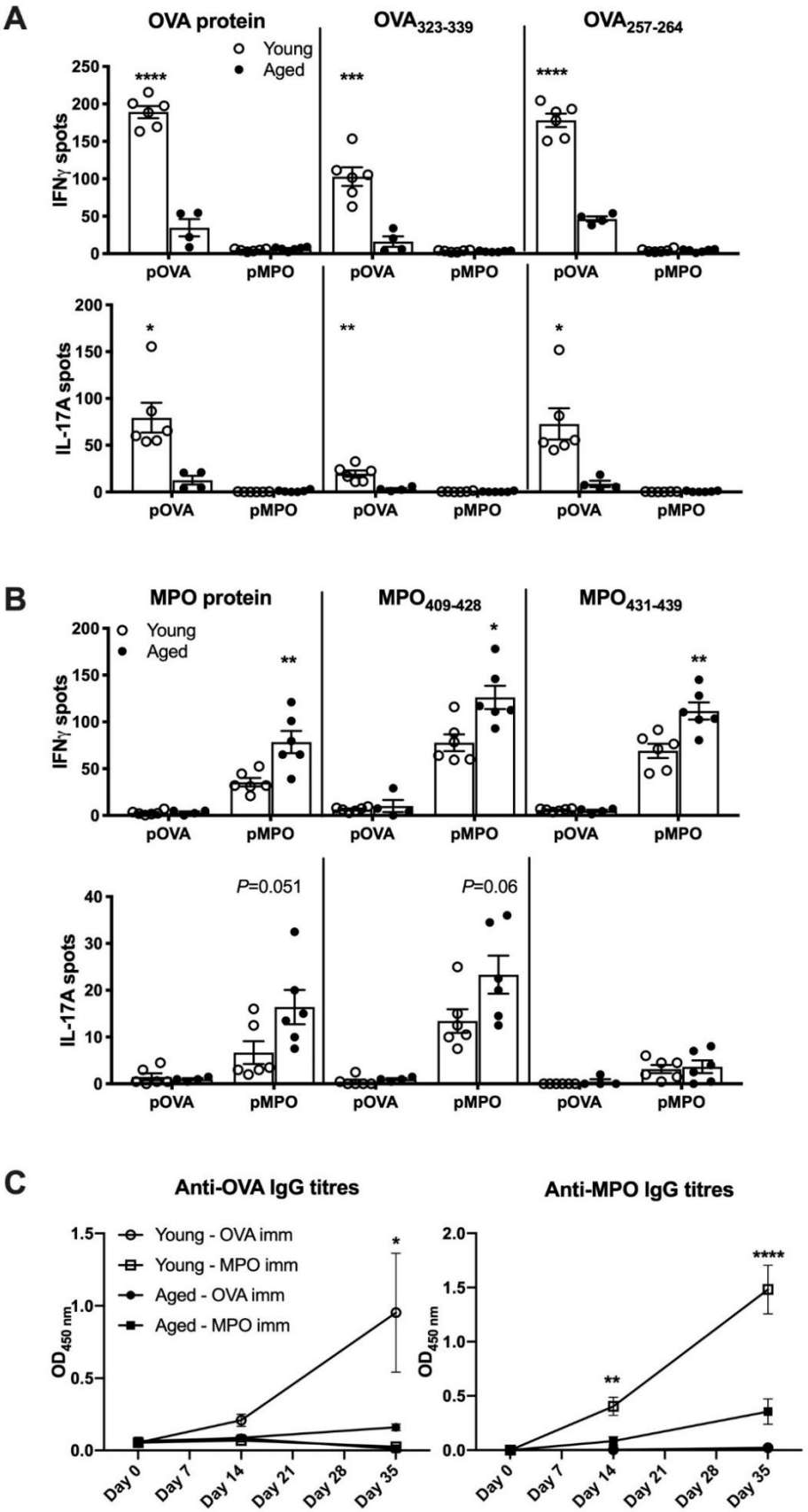
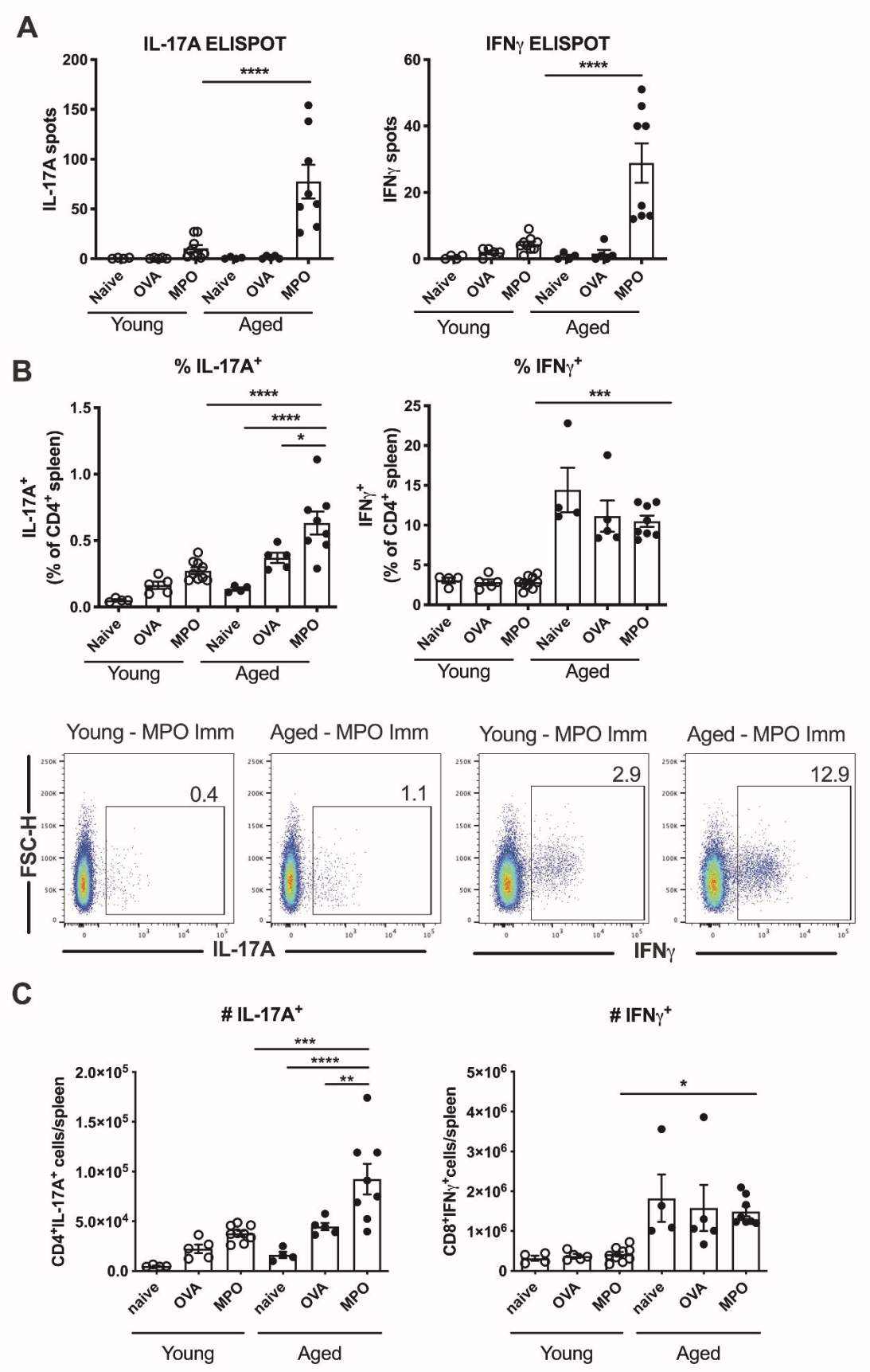


Figure 3. Immune responses in young and aged mice with anti-myeloperoxidase glomerulonephritis.



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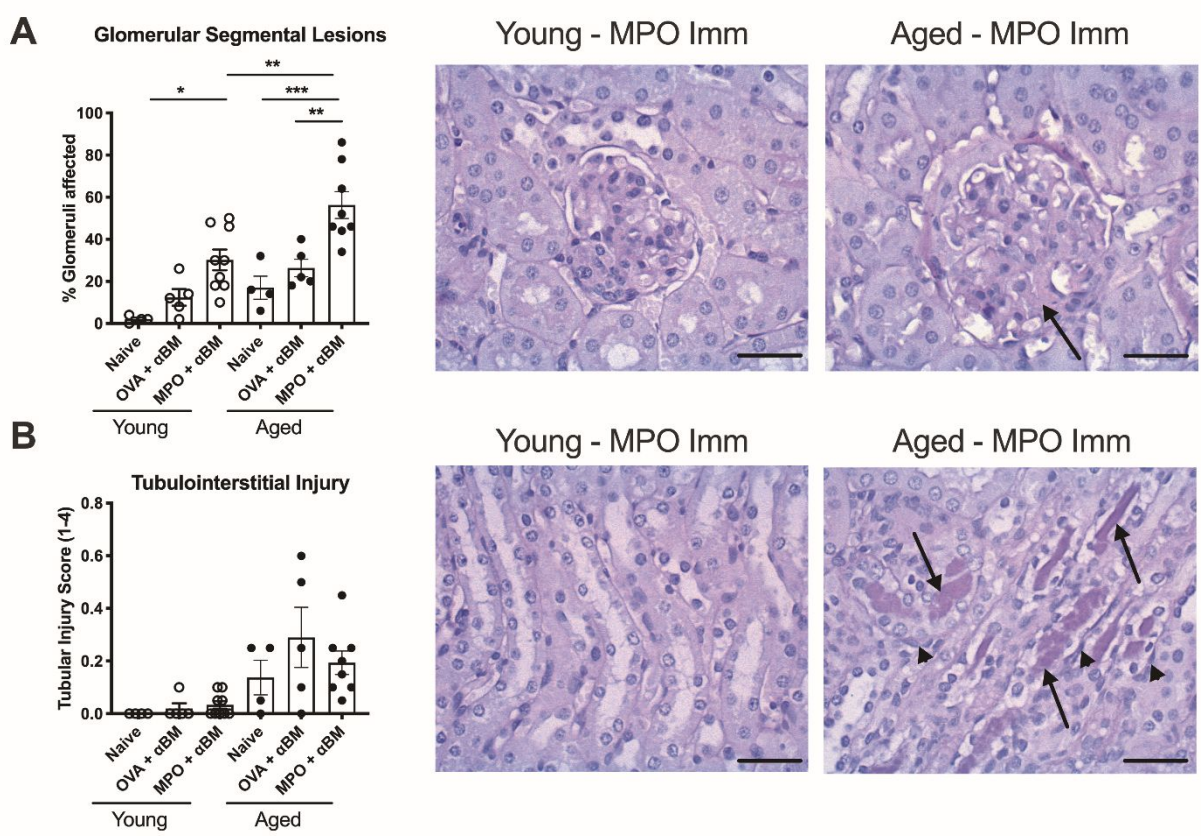

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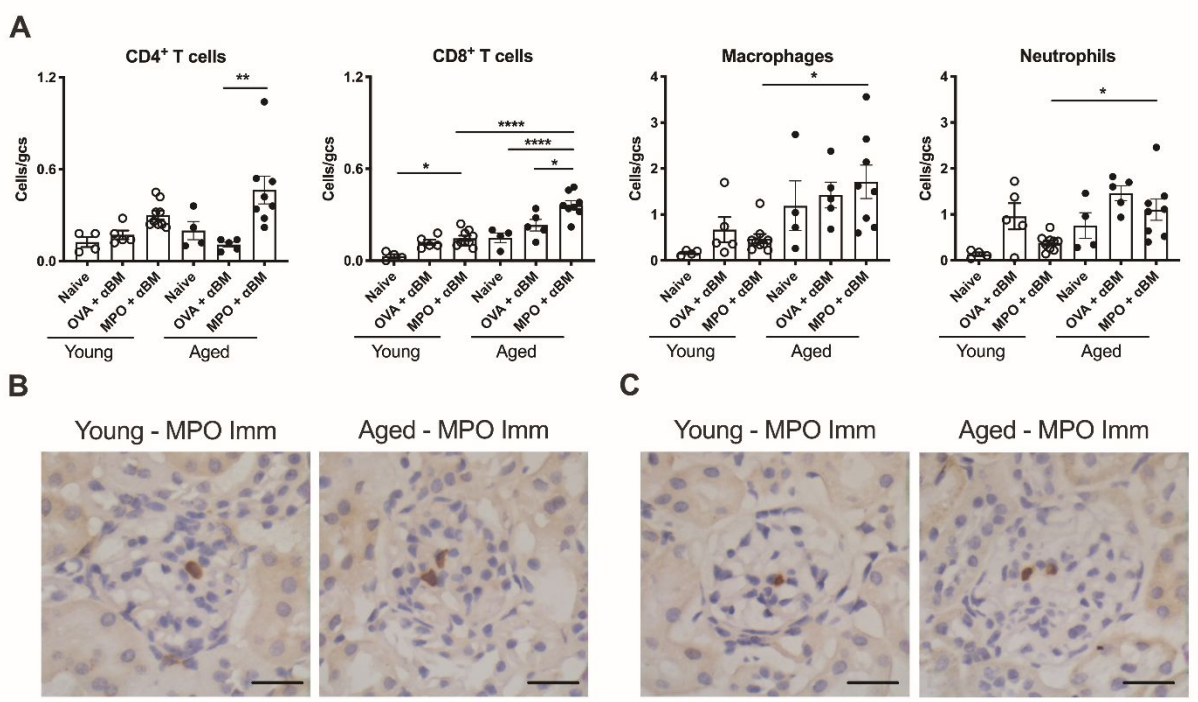

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