Age-dependent Role of Microvascular Endothelial and Polymorphonuclear Cells in Lipopolysaccharide-induced Acute Kidney Failure

Francis M. Wulfert, M.D.,* Matijs van Meurs, M.D., Ph.D.,† Neng F. Kurniati, M.Sc.,‡ Rianne M. Jongman, B.Sc.,§ Martin C. Houwertjes,‖ Peter Heeringa, Ph.D.,# Michel M.R.F. Struys, M.D., Ph.D.,** Jan G. Zijlstra, M.D., Ph.D.,†† Grietje Molema, Ph.D.‡‡

ABSTRACT

Background: The incidence of acute kidney injury following severe sepsis is higher in the elderly. We hypothesized that microvascular endothelium is "primed" by ageing and that sepsis represents a "second hit," resulting in more severe microvascular complications.

Methods: Three- and 18-months-old mice were intraperitoneally injected with 1,500 EU/g body weight lipopolysaccharide and sacrificed after 8 h. Flow cytometry and myeloperoxidase ELISA determined neutrophils in plasma. Quantitative reverse transcription polymerase chain reaction was used to analyze messenger ribonucleic acid levels of cell adhesion molecules P-selectin and E-selectin, vascular cell adhesion protein-1, intercellular adhesion molecule-1, angiopoietin receptor TIE-2, and angiopoietins Ang1 and Ang2. In kidney tissue we assessed neutrophil influx and E-selectin protein expression. Neutrophils were depleted with the monoclonal antibody NIMP.

Results: At basal conditions, microvascular endothelial cell activation status was similar in both groups, except for a higher Ang-2 expression ($P < 0.05$) in the kidney of aged mice. Lipopolysaccharide-induced increase in neutrophil count was higher in old (3.3-fold change) compared with young mice (2.2-fold change). Messenger ribonucleic acid analysis showed higher upregulation of P- and E-selectin ($P = 0.0004$, $P = 0.0007$) after lipopolysaccharide administration in kidneys of elderly mice, which was confirmed at the protein level for E-selectin. Renal neutrophil influx in lipopolysaccharide-treated aged mice was increased (2.5-fold induction in aged and 2.1-fold in young, $P < 0.0001$). Polymorphonuclear cell depletion exaggerated the lipopolysaccharide-induced kidney injury.

Conclusion: Ang-2 is increased in older mice, which might cause priming of the endothelial cells. Endothelium responded by a more extensive increase in expression of P- and E-selectin in older mice and increased polymorphonuclear cell influx.

What We Already Know about This Topic

- The endothelium of aged but healthy patients is suggested to be more susceptible to inflammatory insults when compared with younger patients. As a result, the kidneys and other organs are more vulnerable in situations such as sepsis.

What This Article Tells Us That Is New

- This study in mice shows that an age-related priming of the microvascular endothelium and dependent responses for a "second hit" such as sepsis may occur, which in turn may be responsible for increased kidney failure in aged septic patients.

AGEING humans are more vulnerable to a wide variety of insults than younger subjects. In some cases this is the result of comorbidities and reduced physiologic reserve. However, in the absence of significant comorbidities, "healthy" ageing still seems to be associated with increased vulnerability to insults associated with systemic inflammation, resulting in significantly worse outcomes in terms of morbidity and mortality.[1,2] In humans, ageing without comor-

* Resident in Training, ‡ Ph.D. Student, § Technician, Departments of Anesthesiology and Pathology and Medical Biology, Medical Biology Section, † Staff Anesthesiologist-Intensivist, Departments of Pathology and Medical Biology and Critical Care, ‖ Animal Technician, ** Professor, Department of Anesthesiology, # Associate Professor in Medical Biology, ‡‡ Professor of Life Sciences, Department of Pathology and Medical Biology, †† Professor, Department of Critical Care, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands.

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Address correspondence to Dr. Zijlstra: Department of Critical Care, University Medical Center Groningen, University of Groningen, P.O. 30.001 9700 RB Groningen, The Netherlands. j.g.zijlstra@umcg.nl. Information on purchasing reprints may be found at www.anesthesiology.org or on the masthead page at the beginning of this issue. ANESTHESIOLOGY's articles are made freely accessible to all readers, for personal use only, 6 months from the cover date of the issue.

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bidity is associated with low-grade inflammatory activity, as measured by a 2–4-fold increase in plasma tumor necrosis factor (TNF-α) and interleukin-6 (IL-6).[3] As a consequence, the microvascular endothelium is likely "primed" by (healthy) ageing, potentially making the elderly more susceptible to the harmful effects of a second inflammatory insult.

Such an increased sensitivity to a second hit could explain the increased incidence of sepsis, and worse outcomes of sepsis, in aged patients.[1,2] Sepsis is a well-described, but poorly understood, condition involving a systemic inflammatory response to infection and leading to multiorgan dysfunction syndrome. Apparently, the kidney is vulnerable because acute renal failure is a frequent complication of sepsis. The current understanding of the pathophysiology of sepsis originates mainly from studies in young animals. From these studies, it has become clear that polymorphonuclear neutrophils (PMNs) are directly engaged in the pathophysiology. The interaction between PMNs and the endothelium plays a pivotal role in facilitating the migration of PMNs into the peripheral tissues. In the majority of organs, PMN migration to a site of injury occurs in postcapillary venules and is mediated by the spatiotemporal expression and activation of adhesion molecules, chemokines, and cytokines by the vascular endothelium as well as by the PMNs.[4] After migration, activated PMNs release proteolytic enzymes and produce reactive oxygen species augmenting tissue injury.[5] It is thought that PMNs play a major role in the induction of organ damage in sepsis.[6–8]

We hypothesized that in elderly the interaction between PMNs and endothelium is increased as a consequence of ageing-related endothelial priming, which in turn is responsible for the increased susceptibility of elderly to a "second hit" like sepsis, resulting in increased organ damage. To investigate this we used lipopolysaccharide-induced acute endotoxemia in mice as a model and compared renal inflammation in young (3 months) and aged (18 months) mice, focusing on expression of a number of endothelial adhesion molecules and members of the angiopoietin/TIE-2 system, and PMN influx. To delineate the contribution of PMNs, similar experiments were performed in neutrophil-depleted mice.

Materials and Methods

Animals

Mice were obtained from Harlan Nederland (Horst, the Netherlands) and maintained on chow and tap water *ad libitum* in a temperature-controlled chamber at 24°C with a 12-h light/dark cycle. All procedures were approved by the local committee for care and use of laboratory animals (DEC, University Medical Center Groningen, Groningen, The Netherlands) and were performed according to strict governmental and international guidelines on animal experimentation.

Lipopolysaccharide–Endotoxemia Model

In the study, 96 mice, eight per group (see table 1), were initially entered. Before the experiment, one aged mouse died of unknown cause and was not replaced. Mice were first subdivided in groups of young and old mice, after which we randomly picked eight mice per treatment group. All further experiments were blinded to murine age and treatment.

Table 1. Sample Size of Different Experimental Groups

Groups	Untreated	LPS	IgG	IgG + LPS	NIMP-r14	NIMP-r14 + LPS
3 months old	8	8	8	8	8	8
18 months old	7	8	8	8	8	8

The study initially included 96 mice, eight mice per group; prior to the experiment, one aged mouse died of unknown cause and was not replaced. Three-month- and 18-month-old mice were injected with lipopolysacharide. Control mice were untreated. In a random selection of animals, polymorphonuclear cell depletion was established by polymorphonuclear cell antibody NIMP-r14 24 h prior to the experiment. The control groups received rat immunoglobulin G at the same dose as NIMP-r14.

IgG = rat immunoglobulin G; IgG + LPS = rat immunoglobulin G and lipopolysacharide; LPS = lipopolysacharide; NIMP-r14 = monoclonal rat anti-murine PMN antibody; PMN = polymorphonuclear cells; NIMP-r14+LPS = monoclonal rat anti-murine PMN antibody and lipopolysacharide.

For the induction of endotoxemia, wild-type female C57BL/6 mice, 3 months and 18 months old, were intraperitoneally injected with 1,500 EU/g body weight lipopolysaccharide (lipopolysaccharide; *Escherichia coli*, serotype O26:B6; Sigma-Aldrich, St Louis, MO; for the specific batch of lipopolysaccharide used the dose was 0.5 μg/g body weight). Animals were supplied with water and food during the whole experiment. After 8 h, mice were anesthetized with isoflurane, blood was drawn *via* aortic puncture, and kidneys were harvested. Control mice were, where appropriate, subjected to similar conditions as the lipopolysaccharide-treated mice before and during sacrifice. In a random selection of animals, PMN depletion was established by intraperitoneal injection of monoclonal rat antimurine PMN antibody NIMP-R14 (Hycult Biotech, Uden, the Netherlands) at 0.5 mg in 0.75 ml of phosphate-buffered saline (PBS) 24 h before endotoxaemia induction, as previously described.[9] The control groups received rat immunoglobulin G (IgG) (Sigma-Aldrich) at 0.5 mg of IgG in 0.75 ml of PBS. Rat IgG and NIMP-R14 were both tested for endotoxin contamination by the Limulus Amebocyte Lysate (LAL) test and contained 0.319 EU/ml (IgG) and 0.213 EU/ml (NIMP-r14), respectively.

After harvesting the kidneys they were partly snap-frozen on liquid nitrogen and stored at −80°C for gene and protein analyses, and partly fixed in 4% formaldehyde and embedded in paraffin for histopathological evaluation by periodic acid-Schiff staining according to standard procedures.

PMN and Leukocyte Count

PMN count was assessed in blood of a random selection of animals by flow cytometric analysis. Whole blood samples were collected before and after lipopolysaccharide injection; 500 μl buffer (0.1 5M NH_4Cl with 10 mM $NaHCO_3$) was

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added to 70 μl of whole blood to cause lysis of the red blood cells, after which cells were incubated with primary Abs CD11b-PE (BD Biosciences, Breda, the Netherlands) and Ly6G-FITC (BD Biosciences) for 60 min on ice. After washing three times with PBS supplemented with 2% fetal calf serum, cells were fixed in 2% paraformaldehyde. Cells were analyzed on a Calibur (BD Biosciences) flow cytometer using CellQuest software (BD Biosciences). Total leukocyte counts were also performed (see Supplemental Digital Content 1, http://links.lww.com/ALN/A863, for Materials and Methods, and Supplemental Digital Content 1, fig. 1, which represents total leukocyte counts in control and lipopolysaccharide-treated mice, and lipopolysaccharide-treated mice pretreated with IgG and NIMP-R14, respectively).

Blood Biochemistry

Plasma was collected by aortic puncture at the time of sacrifice. Neutrophil gelatinase-associated lipocalin (NGAL)[10] in plasma was determined by ELISA (BioPorto Diagnostics A/S, Gentofte, Denmark), according to the manufacturer's instructions. Blood urea nitrogen (BUN) analyses were performed by enzymatic degradation assay (Roche, Woerden, The Netherlands). The investigator performing the analyses was blinded to the treatment.

Gene Expression Analysis by Quantitative RT-PCR

Total RNA was extracted from 20 $\times$ 5 μm-thick kidney cryosections and isolated using the RNeasy Mini plus kit (Qiagen, Venlo, the Netherlands), according to the manufacturer's instructions. Integrity of RNA was determined by gel electrophoresis. RNA yield (OD260) and purity (OD260/OD280) were measured by ND-1000 UV-Vis spectrophotometer (NanoDrop Technologies, Rockland, DE). One microgram of RNA was reverse-transcribed using SuperScript III reverse transcriptase (Invitrogen, Breda, the Netherlands) and random hexamer primers (Promega, Leiden, The Netherlands). Quantitative PCR amplifications were performed according to manufacturer's protocol on an ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Applera Nederland, Nieuwerkerk aan den Ijssel, Netherlands). The following Assay-On-Demand primers were used for housekeeping gene, mouse glyceraldehyde 3-phosphate dehydrogenase (assay ID Mm99999915_g1); mP-selectin (assay ID Mm00441295_m1); mE-selectin (assay ID Mm00441278_m1); mouse vascular cell adhesion protein-1 (VCAM-1; assay ID Mm00449197_m1); mouse intercellular adhesion molecule-1 (ICAM-1; assay ID Mm00516023_m1); mAng-1 (assay ID Mm00456503_m1); mAng-2 (assay ID Mm00545822_m1); mTIE-2 (assay ID Mm00443242_m1); TNF-α (assay ID Mm00443258_m1); and IL-10 (assay ID Mm00439614_m1).

The investigator performing the analyses was blinded to the treatment. Calculations were performed as previously described.[11,12] Glyceraldehyde 3-phosphate dehydrogenase was shown not to change with age,[13] and was in our model not affected by lipopolysaccharide administration, as was concluded from the threshold cycle values for this gene that did not differ in the samples generated *via* our standardized protocol. These threshold cycle values were 20.2 $\pm$ 0.7 (young) and 20.5 $\pm$ 1.3 for the control groups, and 20.5 $\pm$ 0.3 (young) and 20.8 $\pm$ 0.6 (old) for the lipopolysaccharide-treated groups.

Immunohistochemistry

For the detection of E-selectin and PMNs in tissue slides, an antirabbit peroxidase-based Envision®+ system (DakoCytomation, Carpinteria, CA) was used, according to the manufacturer's instructions. In short, 5 μm cryosections were incubated for 60 min with MES-1 (a gift from Derek Brown, Ph.D., UCB Celltech, Brussels, Belgium), rat antimouse Ly-6G (clone 1A8; BD Biosciences), or isotype control (IgG2a; Antigenix America, Huntington Station, NY). Peroxidase activity was blocked for 5 min by incubation with blocking solution (DakoCytomation), and afterward sections were incubated for 45 min with rabbit antirat secondary antibody (Vector Laboratories, Burlingame, CA), diluted 1:300 in PBS supplemented with 5% fetal calf serum and 2% normal mouse serum, and 30 min with antirabbit polymer-HRP.

After detection of peroxidase activity with 3-amino-9-ethylcarbazole, sections were counterstained with Mayer's hematoxylin. No immunostaining was observed with isotype-matched controls, demonstrating specificity of staining with the antigen-specific antibodies.

Quantification of Myeloperoxidase Levels in Kidney and Plasma by ELISA

Myeloperoxidase protein levels in kidney tissue and plasma from healthy and lipopolysaccharide-treated mice were measured in duplicate using an ELISA kit specific for mouse myeloperoxidase (HK210, Hycult Biotech), according to the manufacturer's instructions and as described previously.[14] Myeloperoxidase levels in the kidney were normalized to total protein concentrations in the tissue homogenate and expressed as pg myeloperoxidase per μg of total protein. The investigators were not blinded to the treatment.

Statistical Analysis

Statistical significance was determined by means of unpaired, independent, and two-tailed Student t test and using one-way ANOVA with Bonferroni correction with selected pairs of groups. All statistical analyses were performed with GraphPad Prism software (GraphPad Prism Software Inc., San Diego, CA). We used two-tailed hypothesis testing with all tests. Several of the variables under study may violate the assumptions of parametric tests, so the results of the statistical tests were confirmed using nonparametric equivalents. Differences were considered to be significant when $P < 0.05$. The sample size was calculated from previously published results from our group.[11]

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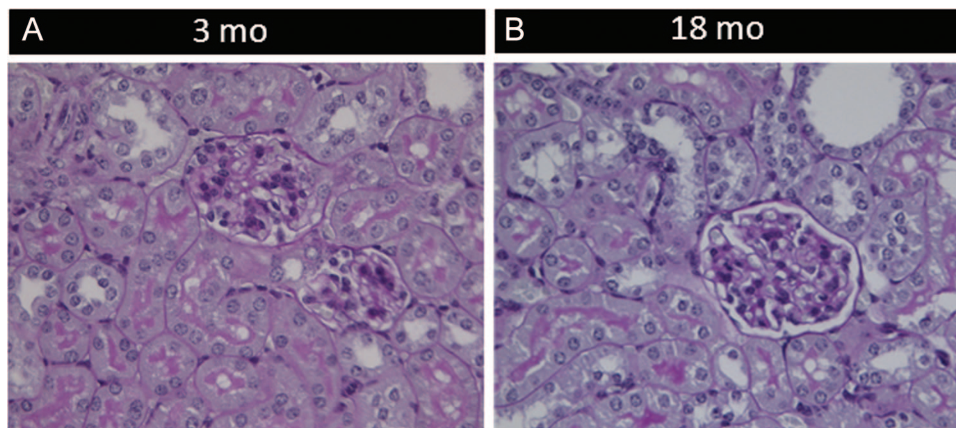

Fig. 1. No morphological differences were found between the kidneys of young and aged mice. Kidney tissue samples of untreated young (*A*) and aged (*B*) mice were fixed in 4% formaldehyde and embedded in paraffin for histopathological evaluation using periodic acid-Schiff staining. Original magnification of ×400.

Results

Morphology

No morphologic differences were found between kidneys of young and of aged mice. Histomorphological assessment of PAS-stained renal sections revealed no differences in glomerular morphology between young and aged mice. Neither the glomerular vascular segments nor any other microvascular segments showed aberrant sclerosis or vascular injury (fig. 1).

The Number of Circulating PMNs Is Increased in Aged Mice after Lipopolysaccharide Challenge

We assessed numbers of circulating PMN in aged and young animals because an overall increase in neutrophil numbers may affect general neutrophil migration in tissue. The increase in PMN count measured in whole blood samples after lipopolysaccharide exposure was higher in old compared with younger mice (fig. 2A).

Myeloperoxidase is abundantly present in azurophilic granules of PMN and is rapidly released when polymorphonuclear leukocytes are activated.[15] Higher levels of serum myeloperoxidase were observed in aged mice after lipopolysaccharide challenge (3.3-fold induction in aged *vs.* 2.2-fold induction in young mice, fig. 2B), indicative of a higher PMN activation status in the older mice.

Increased Expression of Endothelial Adhesion Molecules P-selectin and E-selectin in Kidney in Aged Mice after Lipopolysaccharide Challenge

No differences in basal gene expression were seen between old and young mice before lipopolysaccharide challenge. Endothelial cell activation before and 8 h after lipopolysaccharide challenge were evaluated by analysis of P-selectin, E-selectin, VCAM-1, and ICAM-1 mRNA levels in kidney tissue (fig. 3A) in both young and aged mice. All four adhesion molecules were strongly up-regulated ($P < 0.0001$) in old and young mice 8 h after lipopolysaccharide challenge compared with controls. Eight hours after lipopolysaccharide administration, the fold induction in P-selectin was 87 in old mice compared with 51 in young mice and was significantly higher ($P < 0.0004$). Fold induction of E-selectin in young mice after treatment with lipopolysaccharide was 4.5 compared with 8.5 in old mice, which was significantly higher ($P < 0.0007$). Lipopolysaccharide challenge also increased the levels of soluble endothelial adhesion molecules in plasma of both young and old mice (see Supplemental Digital Content 1, fig. 2, http://links.lww.com/ALN/A863), but the differences between young and old mice were not statistically significant. In neutrophil-depleted mice, lipopolysaccharide challenge did not result in changes in soluble ICAM-1 and soluble VCAM-1, whereas E-selectin increased equally in both age groups. Immunohistochemical staining of E-selectin (fig. 3B) confirmed the mRNA data. An increase in expression of E-selectin protein was seen 8 h after administration of lipopolysaccharide in both groups. This increase was higher in the aged mice compared with the young mice. Immunohistochemical staining of E-selectin furthermore demonstrated striking microvascular heterogeneity in reaction to the stimulus. In young mice expression of E-selectin was mainly found in glomeruli, whereas only minor expression occurred in the peritubular endothelial cells, arterioles, and venules. In aged mice, increased expression of E-selectin was found in both the glomeruli and in the peritubular microvasculature. Lipopolysaccharide injection resulted in an increase in both TNF-α and IL-10 levels in plasma and of mRNA in kidney. Kidney mRNA levels of TNF-α and IL-10 were significantly different ($P < 0.0001$) between young and old mice, yet no differences in plasma TNF-α and IL-10 levels were found between young and old mice (see Supplemental Digital Content 1, fig. 3, http://links.lww.com/ALN/A863).

In Aged Mice, Ang2 mRNA Levels in Kidney Were Higher before and after Lipopolysaccharide Challenge

Because competition with Ang1 for binding to the receptor TIE-2 by Ang2 induces inhibition of TIE-2 signal transduc-

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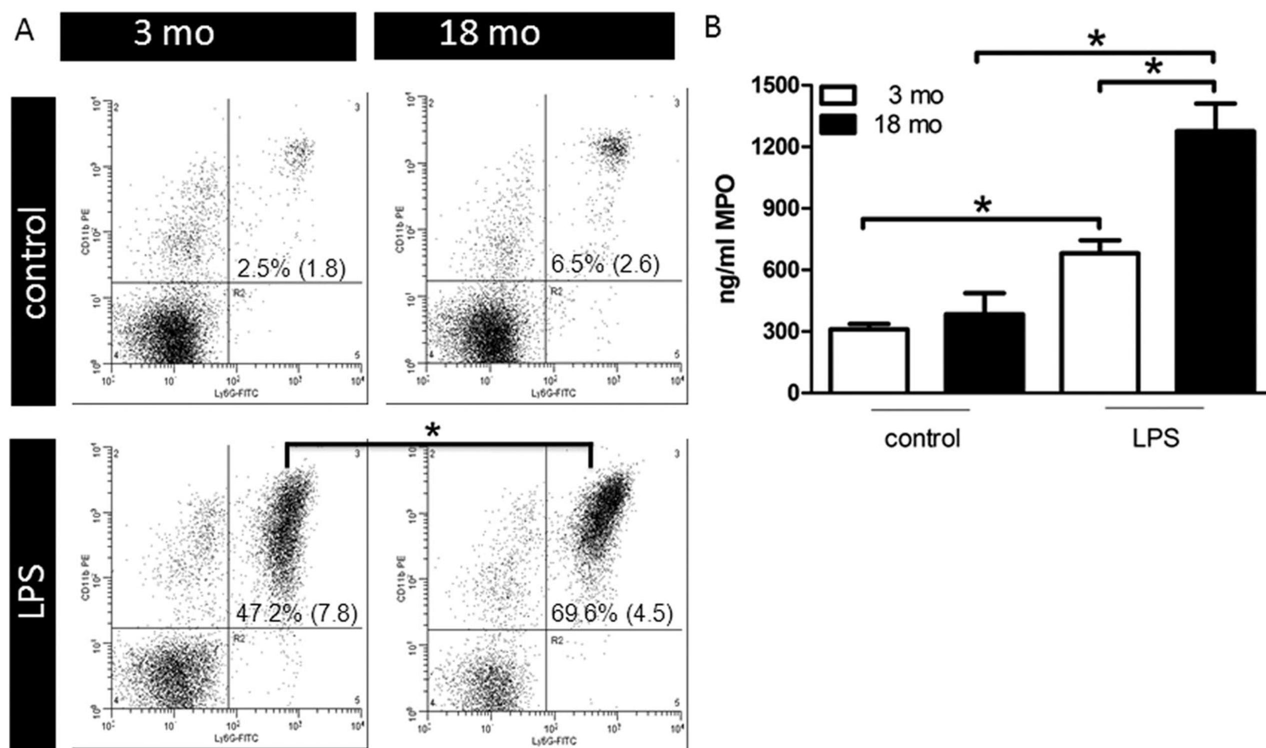

Fig. 2. In aged mice, the increase in number of circulating polymorphonuclear cells after lipopolysaccharide challenge is higher than in young mice. (*A*) Flow cytometrical analysis of representative blood samples of a 3-month and 18-month-old mouse with and without lipopolysaccharide injection. In each scatter plot, the right upper quadrant represents polymorphonuclear cells (Ly-6G and CD11b-positive). The relative increase in polymorphonuclear cells count after lipopolysaccharide administration is lower (*$P < 0.05$) in 3 months compared with aged mice. Value as % of polymorphonuclear cells of total leukocyte count (±SD) of n = 3. (*B*) Quantification of myeloperoxidase (MPO) levels in plasma assessed by ELISA (mean [SD], n = 8 per group; *$P < 0.05$, by ANOVA with *post hoc* comparison of all groups using Bonferroni correction). LPS = lipopolysaccharide.

tion and is associated with inflammation and vascular leakage, we quantified Ang1, Ang2, and TIE-2 mRNA levels in kidney tissue.[12,16] No differences in basal gene expression of Ang1 was seen between the age groups (fig. 4A). Basal expression of Ang2, however, was significantly higher ($P < 0.05$) in aged mice compared with young mice (fig. 4B). Basal expression of TIE-2 (fig. 4C) showed no differences between the age groups; however, after lipopolysaccharide challenge, TIE-2 expression was down-regulated significantly ($P < 0.0006$) in both groups, as previously reported in young mice.[12] In both groups Ang1 was not significantly different; Ang2 was significantly higher expressed after lipopolysaccharide challenge in the aged mice compared with the young mice, although in both groups the absolute increase induced by lipopolysaccharide was approximately 10-fold.

Higher PMN Count and Higher Expression of E-selectin and P-selectin in the Aged Mice after Lipopolysaccharide Challenge Is Paralleled by a Higher PMN Influx in Kidney

Immunohistochemical staining of leukocytes using Ly-6G antibody showed that, in contrast to young mice, in old mice a small number of PMNs is present in glomeruli already before lipopolysaccharide exposure. The influx of PMNs in kidney tissue 8 h after lipopolysaccharide administration (fig. 5A) is moreover markedly higher in aged mice. The increased influx of PMNs in aged mice was associated with increased myeloperoxidase levels (fig. 5B) with a 2.5-fold induction in aged mice after lipopolysaccharide and a 2.1-fold induction in young mice after lipopolysaccharide.

Loss of Kidney Function in Aged Mice after Lipopolysaccharide Challenge Cannot Be Prevented by PMN Depletion

To study whether increased PMN influx in kidney of aged mice was accompanied by an increase in functional loss, we measured NGAL and BUN levels in plasma. In quiescent conditions, no differences in NGAL and BUN levels between young and aged mice were observed. After lipopolysaccharide challenge, there was a significantly higher ($P < 0.0001$) increase of NGAL and BUN concentration in plasma of older mice (fig. 6A and B). It is of note that not for all mice enough blood or urine was available for kidney function measurements.

Because PMNs are associated with increased loss of vascular integrity and kidney damage, we next depleted the PMNs before lipopolysaccharide administration to examine the role of neutrophil-endothelial interaction in increased

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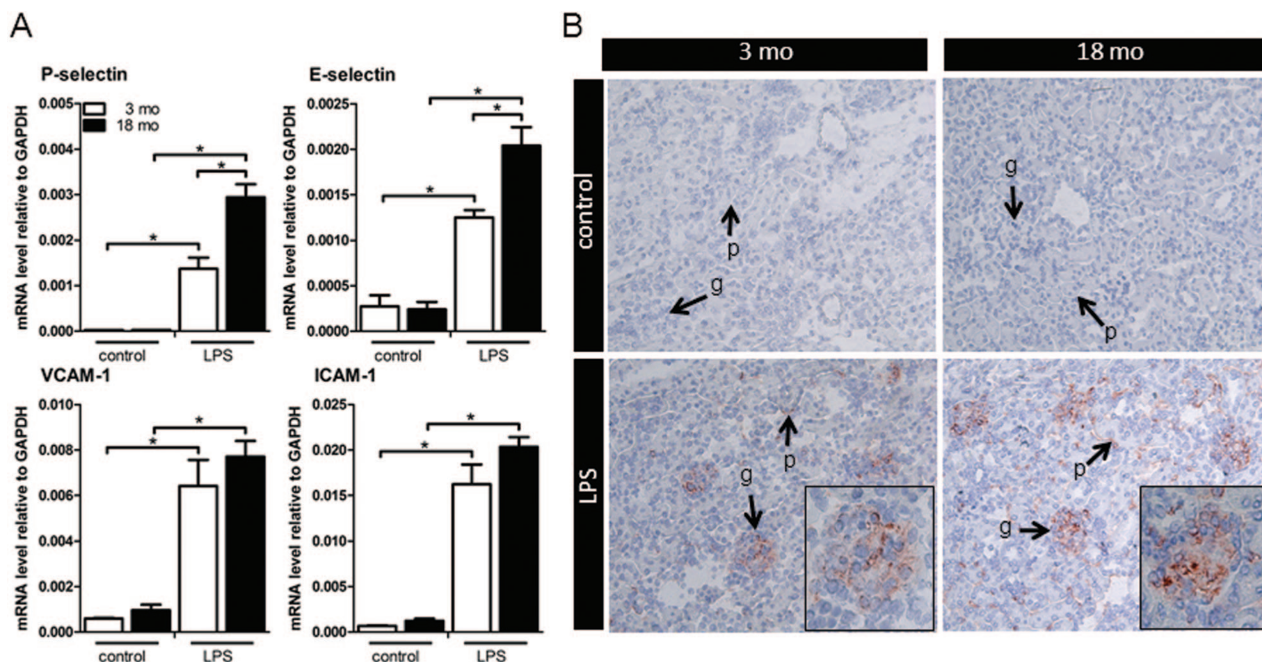

Fig. 3. In the kidney, the expression of P- and E-selectin was more extensively induced by lipopolysaccharide in aged mice compared with young mice. (*A*) Expression of renal messenger RNA (mRNA) of P-selectin, E-selectin, vascular cell adhesion molecule-1 (VCAM-1), and intercellular adhesion molecule-1 (ICAM-1) in young, 3-month-old (*white bars*) and elderly, 18-month-old (*black bars*) mice subjected to lipopolysaccharide. Young and elderly mice were treated with lipopolysaccharide and sacrificed 8 h later. mRNA levels shown are relative to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as housekeeping gene and were determined by quantitative reverse transcription polymerase chain reaction. Mean (SD), n = 6 per group; $^{*}P <$ 0.0001, by ANOVA with *post hoc* comparison of all groups using Bonferroni correction. (*B*) Young and elderly mice were treated with lipopolysaccharide and sacrificed 8 h later. Frozen kidney tissue section were immunohistochemically stained for E-selectin (*red*); expression is visible in glomeruli (g) and peritubular (p) vasculature. Original magnification ×200; insets: ×400.

kidney function loss in aged mice.[5] Fluorescent-assisted cell-sorting analysis of whole blood of mice 24 h after injection of a NIMP antibody demonstrated that the mice had become severely neutropenic. In PMN-depleted mice, NGAL and BUN levels (figs. 6C and D) were higher than in untreated controls. After PMN depletion, there was a significantly higher increase in NGAL in aged mice compared with younger mice after lipopolysaccharide administration, with a 12-fold increase in younger mice and a 19-fold increase in older mice (fig. 6B). It is of note that both IgG and NIMP injection raised NGAL levels in young and old mice. However, this observation was not seen in the BUN measurements.

Discussion

Sepsis is a disease striking hardest in the elderly, and the aim of this study was to investigate the differences in renal microvascular endothelial and PMN responsiveness to lipopolysaccharide challenge between young and aged mice, in order to study differences in susceptibility of the kidney to lipopolysaccharide. This model shows that lipopolysaccharide in mice increased NGAL and BUN, which is suggestive of acute kidney injury. In older mice this phenomenon is more pronounced. We demonstrated that at basal conditions, microvascular endothelial cell activation status was similar in both groups of mice, except for a higher Ang2 expression in the kidney of aged mice. The PMN increase in plasma is higher and P-selectin and E-selectin levels in the kidney are higher in aged mice after lipopolysaccharide challenge compared with those in young mice. In older mice there was also involvement of the peritubular endothelial cells with up-regulation of E-selectin not only in the glomeruli. The higher PMN count in plasma and higher expression of P-selectin and E-selectin after lipopolysaccharide challenge was paralleled by a higher renal PMN influx. We also demonstrated that older mice are more prone to kidney failure as measured by NGAL. Loss of kidney function in aged mice after lipopolysaccharide challenge was not counteracted in PMN-depleted mice, implying that the PMNs are not major participants in kidney dysfunction.

Acute renal failure in sepsis patients is an independent predictor of mortality.[17] Therefore we focused on renal injury. Although lipopolysaccharide injection is a model with limited resemblance with sepsis, we were able to induce renal failure in this model, which might share pathophysiological mechanisms with sepsis. We hypothesized that a "set point change" of endothelial cells by "healthy ageing" might play a role in the increased vulnerability of the aged. To determine

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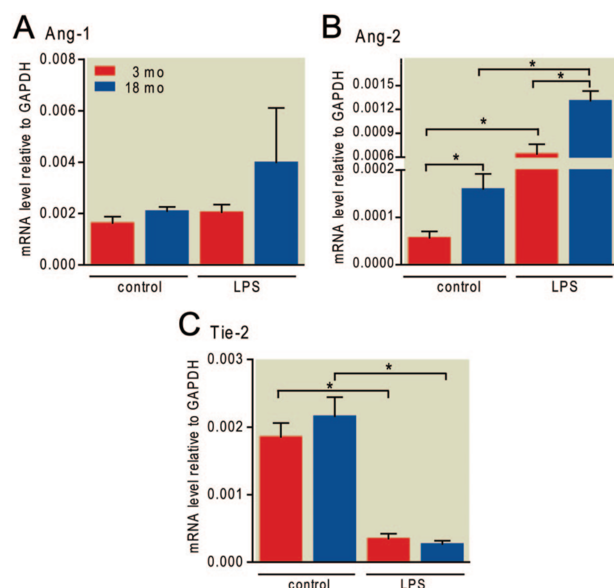

Fig. 4. In aged mice, angiopoietin-2 messenger RNA (mRNA) levels in kidney were higher before and after lipopolysaccharide challenge. Angiopoietin-1 (*A*), angiopoietin-2 (*B*), and TIE-2 (*C*) in young, 3-month-old (*red bars*) and elderly, 18-month-old (*blue bars*) mice subjected to lipopolysaccharide. Young and elderly mice were intraperitoneally injected with lipopolysaccharide and sacrificed 8 h later. mRNA levels shown are relative to glyceraldehyde 3-phosphate dehydrogenase as housekeeping gene and were determined by quantitative reverse transcription polymerase chain reaction. Mean (SD), n = 6 per group; *$P < 0.0001$, by ANOVA with *post hoc* comparison of all groups using Bonferroni correction. Ang-1 = angiopoietin-1; Ang-2 = angiopoietin-2; Tie-2 = endothelium-specific receptor tyrosine kinase.

whether this "priming" exists in the microvascular compartment, we studied the expression of proinflammatory adhesion molecules that are used by the endothelial cells to recruit PMN into tissue. A higher increase of P-selectin and E-selectin in aged mice after lipopolysaccharide challenge was observed. P-selectin and E-selectin mediate leukocyte rolling and thereby represent the first step in neutrophil migration across the wall of microvessels into tissue.[4] ICAM-1 and VCAM-1 were not significantly different in aged mice. This observation seems in contrast to data published by Saito *et al.*, who observed a higher increase in ICAM-1 in the heart of old mice compared with young mice.[18] This possibly can be explained by endothelial heterogeneity between organs, as we previously showed in a hemorrhagic shock model.[11]

A dual role of PMNs and macrophages has been described before, *e.g.*, humans with signs of down-regulation of the cellular components of the innate immune system have a higher mortality in sepsis.[19] This was explained by a deficient immune system resulting in a lower clearing capacity for infection. However, our current study and others suggest that leukocyte activation can also have a protective role other than in elimination of an infection. The classic innate immune response does not seem to be the explanation for vulnerability of the kidney. The finding that PMN depletion does not save kidney function also supports this. On the contrary, in PMN-depleted mice, kidney function after lipopolysaccharide challenge was significantly worse. The increase in myeloperoxidase and PMN in kidney tissue after lipopolysaccharide in aged mice compared with young mice has an analog. Gomez *et al.* did a similar observation in lung tissue in aged mice after lipopolysaccharide challenge.[20] We observed that the kidney function, measured as plasma BUN levels, in PMN-depleted mice after lipopolysaccharide challenge was significantly worse both at young and older age, whereas NGAL was statistically not different. Polymorphonuclear leukocytes are an essential component of the innate immune system. Pathogens are ingested and killed by means of phagocytosis and the generation of reactive oxygen species and the release of toxic granular enzymes.[21] Kasten *et al.* have shown that during sepsis neutrophils are producers of IL-10, an antiinflammatory cytokine.[22] In a lipopolysaccharide model employing young mice, Wu *et al.* furthermore showed an increase in TNF-α and, in accordance with our data, in BUN in PMN-depleted mice after lipopolysaccharide challenge.[23] Depletion of PMNs might therefore lead to decreased IL-10 and increased TNF-α production, both having been suggested to contribute to organ failure. PMN depletion possibly prevented the occurrence of the antiinflammatory reactions, and as a consequence an increase in kidney injury occurred in both young and aged mice after lipopolysaccharide challenge.

On the other hand, PMN may be a significant contributing factor to organ dysfunction because of an exaggerated inflammatory response by release of reactive oxygen species after migration into tissue facilitated by adhesion molecules.[21] Hoesel *et al.* showed that in a caecal legation and puncture model in young mice, delayed depletion of PMN after caecal legation and puncture resulted in improved liver and kidney function. This implies that, by depleting the PMNs before lipopolysaccharide challenge, we may have blocked both functions of the PMN, accelerating and resolving inflammation. Important to note is that the antibody used in the study of Hoesel *et al.* (RB6–8C5) is not PMN-specific but also depletes the monocyte/macrophage populations.[24] As macrophage function involves release of mediators such as cytokines and phagocytosis of microorganisms, they also play a major role in organ function, and hence it is not surprising that their depletion played a role in improved organ outcome.[25] We saw an increase in macrophages in the kidney in aged mice before lipopolysaccharide treatment (data not shown), rendering it possible that macrophages play a role in loss of kidney function.

We investigated the angiopoietin/TIE system, because an imbalance of this system is associated with inflammation and vascular leakage. In quiescent conditions, Ang2 mRNA levels were higher expressed in aged mice compared with young mice, suggesting a change in basal endothelial activation status in aged mice. Competition with Ang1 binding for the receptor TIE-2 by Ang2 induces inhibition of TIE-2 signal

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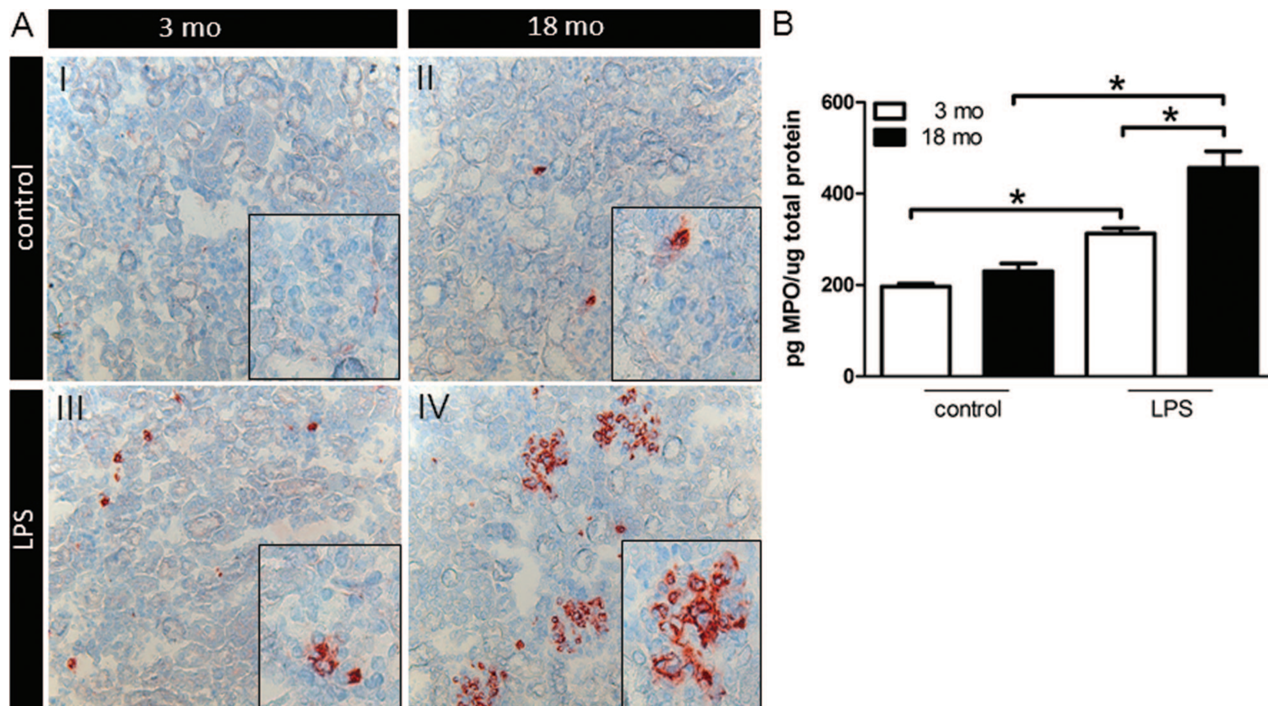

Fig. 5. Polymorphonuclear cell infiltration in the kidney 8 h after lipopolysaccharide injection is higher in aged mice. Young and elderly mice were treated with lipopolysaccharide and sacrificed 8 h later. (*A*) Frozen kidney tissue sections were immunohistochemically stained for polymorphonuclear cell infiltration (*red*) using Ly-6G antibody. Original magnification ×200; insets: ×400. (*B*) Quantification of polymorphonuclear cell infiltration in kidney tissue was performed using myeloperoxidase (MPO) ELISA. Mean (SD), n = 8 per group; *$P < 0.05$, by ANOVA with *post hoc* comparison of all groups using Bonferroni correction. LPS = lipopolysaccharide.

transduction and is associated with inflammation and vascular leakage.[16] In humans an increased Ang2 level in blood is associated with more severe disease and worse outcome.[26] The higher expression of Ang2 could imply that the vasculature is more prone to inflammation, as reflected by the higher number of PMN present in the glomeruli of aged mice. Fiedler *et al.* showed that Ang2 sensitized endothelial cells to TNF-α.[27] Since we demonstrated in our study that aged mice have higher levels of Ang2, it can be hypothesized that aged mice may be more sensitive to TNF-α exposure.[27] To investigate a possible role of Ang2 in sensitizing microvascular endothelial cells in older mice, local and systemic Ang2 levels would be informative. Unfortunately, methods to measure these protein levels in mice are at present not available. Future studies in aging mice in which Ang2 is neutralized, *e.g.*, by treatment with antibodies,[28] will provide additional insight in the role of Ang2 in endothelial responses in endotoxemia and sepsis during aging.

Others have shown that aging is associated with higher levels of TNF-α in healthy elderly, in mice after lipopolysaccharide challenge, and in mice after caecal legation and puncture.[3,29–32] TNF-α induces endothelial cell activation with an increased expression of adhesion molecules,[33] which could explain the increased P-selectin and E-selectin expression in aged mice in our study. Cunningham furthermore showed that TNF-α is a key mediator of acute renal failure in mice.[34] Taken together, higher levels of TNF-α in the aged mice might cause more endothelial activation and kidney injury through Ang2 sensitization of endothelial cells for TNF-α. Although we did not find any differences in TNF-α and IL-10 levels between young and old mice 8 h after lipopolysaccharide injection (see Supplemental Digital Content 1, fig. 3, http://links.lww.com/ALN/A863), this does not rule out organ-specific differences in cytokine production nor differences in proinflammatory cytokine levels in plasma at earlier time-points, as suggested by Chorinchath et al.[30]

Our mouse model, to represent patients with sepsis, has some shortcomings. Although lipopolysaccharide-induced shock is a widely used laboratory model for sepsis, there are more clinically relevant animal models. We chose to use our model based on the fact that these highly standardized and frequently used single-hit animal models are reproducible and make comparison with the published research possible. However, in mouse lipopolysaccharide models, lipopolysaccharide dosing is highly variable.

Previously, others have shown that lipopolysaccharide-induced shock causes renal injury, including decreased glomerular filtration rate, increased BUN, and increased renal PMN infiltration.[35,36] We had several reasons for choosing the mouse model employed. First, most of the antibodies to knock out PMNs, including NIMP-r14, cannot be used in rats. Second, the availability of knockout mice is of importance for future follow-up studies. Third, in our previous experiments with a lipopolysaccharide model in young mice

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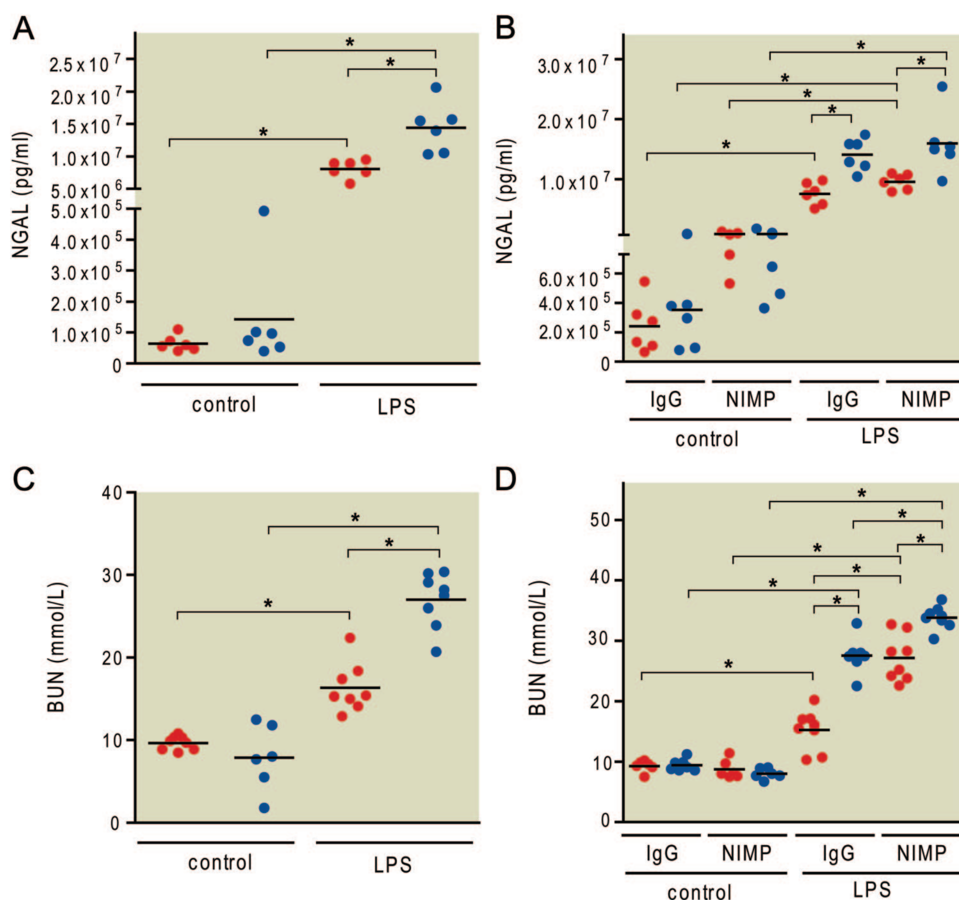

Fig. 6. Plasma neutrophil gelatinase-associated lipocalin (NGAL) and blood urea nitrogen (BUN) levels in young and old mice. NGAL (*A*) and BUN (*B*) were measured, as described in Materials and Methods, in plasma of 3-month-old (*red circles*) and 18-month-old (*blue circles*) mice. Subset of mice were treated with anti-NIMP antibody 24 h before lipopolysaccharide injection to deplete polymorphonuclear cells (neutrophils) or control immunoglobulin G antibody that did not affect polymorphonuclear cell count, whereafter NGAL (*C*) and BUN were measured (*D*). At time 0, mice were intraperitoneally challenged with lipopolysaccharide at 0.5 μg/g and killed 8 h later. Untreated mice served as controls. Values are means $\pm$ SD; n = 8; **P* $\leq$ 0.05 by ANOVA with *post hoc* comparison of all groups using Bonferroni correction. IgG = Immunoglobin G; LPS = lipopolysaccharide.

with a relatively low dose of lipopolysaccharide (0.5 μg/g), we measured an increase in renal PMN infiltration and BUN.[12]

The new observations in this model now call for further studies in more complex models, such as those employing multiple insults, which is seen in sepsis patients.[37] In addition, several morphologic changes that are reported in literature to occur with aging are in fact caused by associated comorbidities such as diabetes, arteriosclerosis, and hypertension. Moreover, evidence exists that structural as well as functional changes occur in the human aging kidney, independent of related illnesses.[38] In contrast to findings in the human situation, where as a result of aging an increasing number of glomeruli are affected by glomerulosclerosis,[39] in our animal model no difference in morphology between young and old mice was observed. This suggests that whereas in the clinical situation a secondary hit, for example sepsis, might have more extensive effects, this is not expected to occur in the mouse model we have employed. An important role in the development of glomerulosclerosis in humans is the high-caloric Western diet. Mice subjected to a high-caloric Western diet were recently shown to develop vascular and renal activation and inflammation,[40] which provides an interesting perspective for the design of future experiments to combine Western-style diet in ageing mice with a lipopolysaccharide challenge. A limitation of our depletion study is the possible confounding effect of the IgG and NIMP-r14 antibody, as these might impact NGAL levels.

Although animals were supplied with water during the experiment we cannot rule out that elderly mice were more ill and therefore drank less and had more fluid extravasation, leading to increased volume depletion. As dehydration is considered a factor for acute kidney injury, this could have influenced the higher NGAL and BUN levels in elderly mice. It would be interesting for follow-up studies to explore the role of fluid suppletion.

A number of limitations may be taken into account for the use of NGAL as a marker of kidney injury. First of all, large-scale population-based "normal ranges" of NGAL are currently lacking. Furthermore, the preciseness of NGAL as

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a marker need to be further substantiated because published studies of NGAL show wide variation in levels and CIs.[41]

In summary, in aged mice no obvious proinflammatory endothelial cell activation was found in the microvascular segments of the kidney, as measured by classic markers of endothelial activation. In contrast, Ang2, an autocrine activator of endothelial cells, was significantly increased ($P < 0.05$) in older mice before lipopolysaccharide exposure, possibly priming the endothelial cells for an exaggerated response to an inflammatory stimulus. Upon endotoxaemia, representing a "second hit," the endothelium responded with a more extensive increase in expression of P-selectin and E-selectin in comparison with young mice. Moreover, in aged mice, circulating PMN count increased significantly upon lipopolysaccharide exposure (3.3-fold), which was paralleled by an increase in PMN in glomeruli. Surprisingly, these PMNs might have a protective role, in young as well as older mice, as demonstrated in PMN-depletion experiments. The observed striking differences between young and aged mice in our sepsis model and other studies[30,32,42] have an important impact for future drug development in sepsis research because the underlying pathophysiological mechanisms between young and old subjects might show important differences. Detailed clinical observations and analyses of biochemical parameters in aged patients are needed to understand age-dependent mechanisms of organ dysfunction.

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