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Brain death-induced lung injury is complement dependent, with a primary role for the classical/lectin pathway

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Abstract

In brain-dead donors immunological activation occurs, which deteriorates donor lung quality. Whether the complement system is activated and which pathways are herein involved, remain unknown. We aimed to investigate whether brain death (BD)-induced lung injury is complement dependent and dissected the contribution of the complement activation pathways. BD was induced and sustained for 3 hours in wild-type (WT) and complement deficient mice. $C3^{-/-}$ mice represented total complement deficiency, $C4^{-/-}$ mice represented deficiency of the classical and lectin pathway, and factor properdin $(P)^{-/-}$ mice represented alternative pathway deficiency. Systemic and local complement levels, histological lung injury, and pulmonary inflammation were assessed. Systemic and local complement levels were reduced in $C3^{-/-}$ mice. In addition, histological lung injury and inflammation were attenuated, as corroborated by influx of neutrophils and gene expressions of interleukin (IL)-6, IL-8–like KC, TNF-α, E-selectin, and MCP-1. In $C4^{-/-}$ mice, complement was reduced on both systemic and local levels and histological lung injury and inflammatory status were ameliorated. In $P^{-/-}$ mice, histological lung injury was attenuated, though systemic and local complement levels, IL-6 and KC gene expressions, and neutrophil influx were not affected. We demonstrated that BD-induced lung injury is complement dependent, with a primary role for the classical/lectin activation pathway.

KEYWORDS
basic (laboratory) research/science, complement biology, donors and donation: donation after brain death (DBD), immunosuppression/immune modulation, lung transplantation/pulmonology, translational research/science

Abbreviations: AEC, 3-amino-9-ethylcarbazole; AP, alternative pathway; BD, brain death; CP, classical pathway; DAB, 3,3′-diaminobenzidine; H&E, hematoxylin and eosin; IL, interleukin; KC, keratinocyte chemoattractant; LP, lectin pathway; MAC, membrane attack complex; MAP, mean arterial pressure; MBL, mannose binding lectin; P, factor properdin; PEEP, positive end-expiratory pressure; SNP, single nucleotide polymorphism; WT, wild-type.

Judith E. van Zanden and Neeltina M. Jager contributed equally to this manuscript.

1 | INTRODUCTION

Brain-dead donors are the major source for donor lungs, which are received by patients who suffer from end-stage lung disease.[1] However, unavoidably, the brain death (BD) process deteriorates donor lung quality due to hemodynamic instability, hormonal dysregulation, and activation of the immune system.[2-4] The complement system is part of the innate immune system, which consists of over 50 proteins present in plasma and on cell surfaces. The complement system can be activated through 3 pathways (Figure 1): the classical pathway (CP), the lectin pathway (LP), and the alternative pathway (AP). Activation of each of these pathways leads to central complement component C3 and subsequently C5 activation. Upon C5 activation, a complex is formed from the subunits C5b, C6, C7, C8, and C9 (C5b-9), also known as the membrane attack complex (MAC). The MAC is the end result of complement activation and forms transmembrane pores in the cell membrane. The membrane integrity of the targeted cell is disrupted, which leads to lysis and cell death.[5,6] In brain-dead donors, an increase of C5b-9 is found in plasma.[7] Thereby, local production of complement proteins has been described in kidneys derived from brain-dead donors, which was negatively associated with graft function after transplantation.[8] As for lungs, the presence of complement activation in BD-induced pathophysiology was suggested by Cheng et al, who showed elevated expression of the C3a receptor in lungs upon BD.[9]

Understanding the role of complement activation upon BD might be critical to protect against BD-induced lung injury. The aim of this study was to investigate whether BD-induced lung injury is complement dependent and to dissect the contribution of the complement activation pathways. To this purpose, we subjected mice to 3 hours of BD and compared lungs from wild-type (WT) mice to lungs from complement deficient mice. $C3^{-/-}$ mice represented total complement deficiency, since all complement activation routes signal through central complement component C3. C4 is an important protein in both the CP and LP; therefore, the absence of the CP and LP was represented by $C4^{-/-}$ mice. The AP is stabilized by factor properdin (P); hence, AP deficiency was represented by $P^{-/-}$ mice.[5]

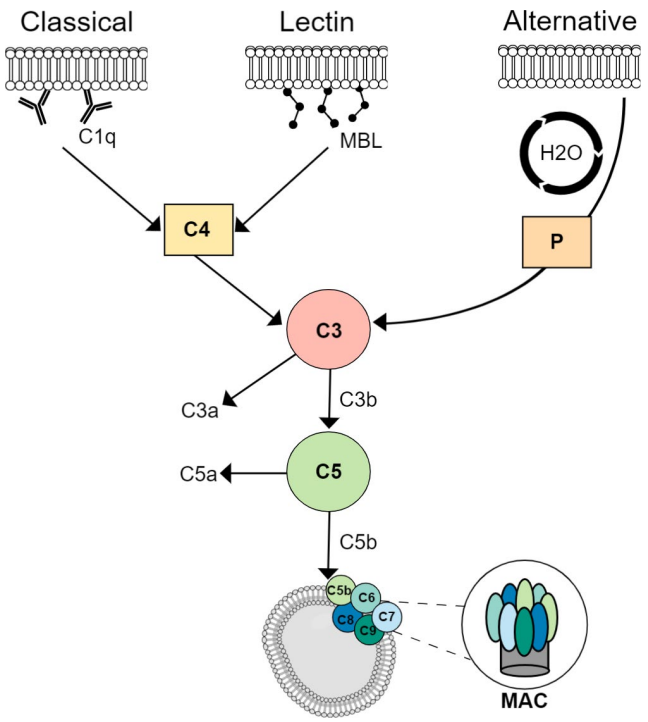

FIGURE 1 Complement system. The complement system can be activated through 3 different pathways: the classical pathway (CP), the lectin pathway (LP), and the alternative pathway (AP). The CP is activated by antigen-antibody complexes binding to C1q, and the LP is activated by binding of mannose-residues on pathogens to mannose binding lectin (MBL). Activation of either the CP or the LP cleaves C4, which leads to downstream activation of C3. The AP is continuously activated due to spontaneous C3 hydrolysis, although only on low levels due to deactivation by complement regulators. However, when activated by external stimuli such as pathogens or surface molecules, AP activation is stabilized by factor properdin (P), which leads to downstream activation of C3. All 3 activation pathways signal through C3, which is cleaved into C3a and C3b. C3b splits C5 into C5a and C5b, and subsequent generation of the membrane attack complex (MAC) C5b-9. The MAC is the end result of complement activation, forming a pore in the cell membrane which induces cell lysis. Split products C3a and C5a are anaphylatoxins, which further stimulate the inflammatory response

2 | MATERIALS AND METHODS

2.1 | Mice

Male WT, C3-, C4-, and P-deficient mice, all on C57Bl/6 background, were provided by C. Stover (University of Leicester, Leicester, UK) and J.S. Verbeek (University of Leiden, Leiden, the Netherlands).[10,11] Mice were bred in the local animal facility in the University Medical Center Groningen and received humane care in compliance with the "Principles of Laboratory Animal Care" and the "Guide for the Care and Use of Laboratory Animals."[12] Mice between 8 and 12 weeks of age, with a weight of 25-28 g were used. The experimental protocol was approved by the local animal ethics committee according to the Experiments on Animals Act.[13]

2.2 | Experimental groups

BD was induced in 4 groups: (1) WT mice (n = 4); (2) $C3^{-/-}$ mice, representing total complement deficiency (n = 8); (3) $C4^{-/-}$ mice, representing CP and LP deficiency (n = 8); and (4) $P^{-/-}$ mice, representing AP deficiency (n = 8). Sham-operated mice (n = 3) served as control.

2.3 | BD induction and lung procurement

The BD procedure was performed according to a previously described model.[14] Mice were anaesthetized with 5% isoflurane/100% O_2. The right carotid artery and left jugular vein were cannulated for mean arterial pressure (MAP) measurements and

fluid administration. Intubation was performed after tracheostomy using a 20G intravenous catheter. Mice were lung-protective ventilated on a mouse ventilator Minivent type 845 (Harvard Apparatus, Holliston, MA), with a respiratory rate of 190 breaths/min, a tidal volume of 225 μL/stroke, and a positive end-expiratory pressure (PEEP) of 1 cm H_2O. Body temperature was monitored and maintained at 37°C. In prone position, a frontolateral hole was drilled through the skull and a Fogarty balloon catheter (Edwards Lifesciences, Irvine, CA) was inserted in the epidural space. BD was induced by inflation of the balloon with 14 μL saline/min, until a total of 70 μL was reached. Isoflurane was switched off after confirmation of BD by performing an apnea test. The balloon remained inflated during the experiment. The first 30 minutes after BD induction, mice were ventilated with 100% O_2. Thereafter, the ventilator was switched to 50% O_2/50% medical air. MAP was continuously monitored and maintained above 60 mmHg. To prevent blood pressure drops, 50 μL of a saline/lepirudin mixture (12 μg/mL) was administered every 15 minutes. Lepirudin (Celgene, Summit, NJ) was used as anticoagulant, since heparin can affect complement activity.[15] In case of hypotension despite the standard fluid regimen, extra saline was administered up to a total maximum of 1200 μL. BD was maintained for 3 hours, after which lungs were procured. Sham-operated mice were subjected to the same procedure, without inflation of the balloon catheter, and ventilated for 5 minutes under anesthesia with a mixture of 2.5% isoflurane/100% O_2 before lung procurement. Lungs were partially formalin fixed and paraffin embedded and partially snap-frozen in liquid nitrogen.

2.4 | RT-qPCR

RT-qPCR was performed to detect the level of proinflammatory gene expression in donor lungs. Total RNA was extracted from frozen lungs using TRIzol (Invitrogen Life Technologies, Breda, the Netherlands), according to manufacturer's instructions. RNA integrity was confirmed by gel electrophoresis and DNase I (Invitrogen Life Technologies) was used to remove genomic DNA. RNA to cDNA synthesis was performed according to manufacturer's instructions. The Taqman Applied Biosystems 7900HT RT-qPCR system (Applied Biosystems, Carlsbad, CA) was used to amplify and detect RT-qPCR products, by measuring SYBR green (Applied Biosystems) emission. Thermal cycling was initiated with a hot start on 50°C and increased to 95°C for denaturation. Thereafter, the annealing step and DNA synthesis were achieved after 40 repeated cycles at 60°C. Generation of single, specific amplicons were confirmed by melt curve analyses. CT values were corrected for house-keeping gene β-actin and expressed relative to the mean CT value of WT sham-operated mice.

2.5 | iC3b ELISA

C3b/iC3b/C3c was measured in plasma as described previously, to quantify systemic complement activation at the level of complement C3.[16] A rat anti-mouse monoclonal antibody against C3b/iC3b/C3c was used as capture antibody (Hycult Biotech, Uden, the Netherlands). C3b/iC3b/C3c was detected with a biotinylated rabbit anti-mouse polyclonal antibody against C3 (Hycult). A standard curve was created from zymosan-activated serum and fresh normal mouse serum. C3b/iC3b/C3c in the samples was determined on the basis of the standard curve and expressed in arbitrary units/mL (AU/mL). Samples were analyzed in duplicate and measured at an OD of 450 nm.

2.6 | Lung morphology

Paraffin sections (4 μm) were stained with hematoxylin and eosin (H&E) to assess lung morphology. Tissue areas were quantified according to a lung injury score, as described before.[17] Briefly, 10 snapshots on ×400 magnification were scored for 5 independent variables: (1) neutrophil infiltration in interstitium and alveolar space, (2) alveolar septal thickening, (3) intra- and extra-alveolar hemorrhage, (4) intra-alveolar edema, and (5) overinflation. Neutrophil infiltration scores (1) were derived from automated scoring in Ly6G-stained sections as described next. Sections were graded from 0-4:0 = <10 neutrophils/50 snapshots, 1 = 10-20 neutrophils/50 snapshots, 2 = 20-40 neutrophils/50 snapshots, 3 = 40-60 neutrophils/50 snapshots, and 4 = 60-80 neutrophils/50 snapshots. Variables 2-5 were graded as 0 = negative, 1 = slight, 2 = moderate, 3 = high, and 4 = severe. Lung injury scores were calculated by the sum of the variables after morphological examination was performed by 2 blinded investigators.

2.7 | Immunohistochemistry

Paraffin-embedded lung sections (4 μm) were stained for neutrophils and local MAC formation. After deparaffinization and antigen retrieval, sections were blocked with endogenous peroxidase for 30 minutes. For neutrophil staining, primary antibody Ly6G (10 μg/mL, eBioscience, San Diego, CA) was incubated for 1 hour at room temperature. Thereafter, sections were incubated for 30 minutes with appropriate horseradish peroxidase-conjugated secondary and tertiary antibodies (Dako, Carpinteria, CA). Reaction was developed by 3,3′-diaminobenzidine (DAB)-peroxidase substrate solution. For MAC staining, primary antibody C9 (2 μg/mL, kindly provided by C. van Kooten, Leiden University Medical Center, the Netherlands) was incubated overnight at 4°C and the secondary horseradish peroxidase–conjugated antibody was incubated for 30 minutes. Reaction was developed by 3-amino-9-ethylcarbazole (AEC; Dako). Sections were counterstained with hematoxylin and embedded in Aquatex mounting agent (Merck, Darmstadt, Germany). For quantification of neutrophils, 50 fields per slide were analyzed with ImageJ software (National Institutes of Health, Bethesda, MD). MAC complex formation was semiquantitative quantified by 2 independent, blinded observers using Aperio ImageScope (Leica Biosystems, Vista, CA). Amount and intensity of staining were graded from 0 to 3 (0 = negative, 1 = mild, 2 = moderate, and 3 = severe).

2.8 | Statistics

Statistical analyses were performed with IBM SPSS Statistics 23 (IBM Corporation, New York, NY). Kruskal-Wallis tests were performed for multiple comparisons between groups. Mann-Whitney *U* tests were used as a post hoc test to compare differences between 2 groups. Outliers were identified by Grubb's test and excluded from analyses. All statistical tests were 2-tailed and $P < .05$ was considered significant. Data are presented as mean ± standard deviations (SD).

3 | RESULTS

3.1 | BD induces systemic and local complement activation

To investigate whether the complement system is involved in the pathophysiology of BD, we assessed both systemic and local complement activation in WT brain-dead vs WT sham-operated mice. Levels of iC3b in plasma were measured as a marker for systemic complement activation, since its presence is a direct result of activation of the complement cascade.[18] When compared to WT sham-operated mice, WT brain-dead mice showed significantly higher levels of iC3b in plasma (Table 1). On a local level, gene expression levels of C3 were assessed in lung tissue and histological deposition of C9 was quantified, of which the latter reflects MAC formation, the final step in the complement activation cascade.[19] Lungs of WT brain-dead mice showed elevated local C3 gene expression levels and more C9 deposition than lungs of WT sham-operated mice (Figure 2A-C). As expected, $C3^{-/-}$ brain-dead mice lacking the central complement component, showed absence of systemic and local complement deposition (Figure 2A,D). Next, we investigated involvement of the CP/LP and AP in BD-induced complement activation. Systemic complement activation was significantly reduced in $C4^{-/-}$ brain-dead mice, representing the CP/LP, compared to WT brain-dead mice (Table 1). In addition, local C3 gene expression levels and deposition of C9 were significantly diminished in $C4^{-/-}$ brain-dead mice (Figure 2A,B,E). In contrast, systemic complement activation did not differ between $P^{-/-}$ brain-dead mice and WT brain-dead mice (Table 1), as well as local gene expression levels of C3 and deposition of C9 (Figure 2A,B,F). These results demonstrate that BD induces systemic complement activation and local MAC formation, primarily via the CP/LP.

TABLE 1 Systemic iC3b levels in plasma

Strain	Pathway	iC3b (AU/mL)	SD (AU/mL)
WT BD	—	20.56	2.59
WT sham	—	10.43**	0.32
$C3^{-/-}$	All	0.00***	0.00
$C4^{-/-}$	CP/LP	15.64*	2.17
$P^{-/-}$	AP	16.35	3.54

Note: Asterisks denote significant differences in comparison to WT brain-dead mice: *$P < .05$, **$P < .01$, ***$P < .001$.

3.2 | BD–induced histological lung injury is reduced in absence of a functional classical/lectin and alternative pathway

To investigate whether BD induces lung injury, we assessed histological lung damage in WT brain-dead mice vs WT sham-operated mice. WT brain-dead mice showed more pronounced histological lung injury when compared to WT sham-operated mice (Figure 3A-C). Next, we investigated whether a dysfunctional complement system attenuated BD-induced lung injury. Since all complement activation pathways signal through C3, $C3^{-/-}$mice represented total complement deficiency.[5] Histological lung injury in $C3^{-/-}$ brain-dead mice was significantly reduced, when compared to lungs from WT brain-dead mice (Figure 3A,B,D). Next, we assessed the involvement of the CP/LP and AP in BD-induced lung injury by comparisons between WT brain-dead mice vs $C4^{-/-}$ and $P^{-/-}$ brain-dead mice. Both $C4^{-/-}$ and $P^{-/-}$ brain-dead mice showed diminished histological damage compared to WT brain-dead mice (Figure 3A,B,E,F). Collectively, these results show that BD-induced lung injury is attenuated in the absence of a functional complement system and shows involvement of both the classical/lectin and alternative complement activation pathways.

3.3 | The classical/lectin activation pathway is mainly involved in BD-induced pulmonary inflammation

Pulmonary inflammation upon BD was assessed by neutrophil influx and cytokine expressions in WT brain-dead vs WT sham-operated mice. WT brain-dead mice showed increased neutrophil influx, compared to WT sham-operated mice (Figure 4A-C). In addition, gene expression levels of proinflammatory cytokines interleukin (IL)-6 and TNF-α, chemokine MCP-1 and adhesion molecules E-selectin and VCAM-1 were significantly higher in WT brain-dead mice than in WT sham-operated mice. IL-8–like keratinocyte chemoattractant (KC) was reduced in sham-operated mice, although not significant (Figure 5A-C). In lungs from $C3^{-/-}$ brain-dead mice, the number of infiltrated neutrophils was significantly lower than in WT brain-dead mice (Figure 4A,B,D). Additionally, proinflammatory gene expressions of IL-6, TNF-α, KC, MCP-1, E-selectin, and VCAM-1 were downregulated in $C3^{-/-}$ brain-dead mice compared to WT brain-dead mice (Figure 5A-C). As for CP/LP activation, less neutrophil infiltration was observed in lungs from $C4^{-/-}$ brain-dead mice than in lungs from WT brain-dead mice (Figure 4A,B,E). Thereby, gene expressions of IL-6, TNF-α, KC, MCP-1, and E-selectin were pronouncedly downregulated in $C4^{-/-}$ brain-dead mice. Nevertheless, VCAM-1 gene expression was not affected in $C4^{-/-}$ brain-dead mice (Figure 5A-C). In $P^{-/-}$ brain-dead mice representing the AP, neutrophil influx was similar to WT brain-dead mice (Figure 4A,B,F). Gene expressions of TNF-α, MCP-1, and E-selectin were significantly downregulated in $P^{-/-}$ brain-dead mice compared to WT brain-dead mice, although IL-6, KC, and VCAM-1 gene expressions were not affected (Figure 5A-C). Taken together, the

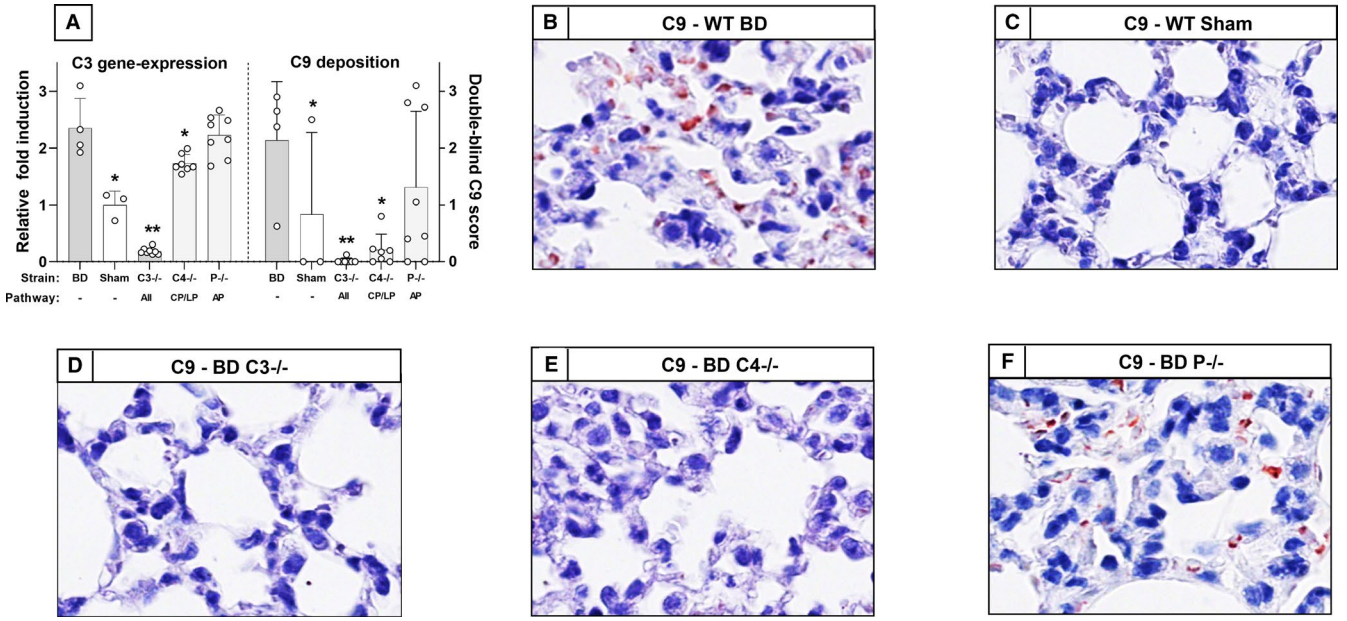

FIGURE 2 Brain death (BD) induces systemic and local complement activation. BD was induced in wild-type (WT) mice, central complement $C3^{-/-}$ mice, $C4^{-/-}$ mice and $P^{-/-}$ mice. $C3^{-/-}$ mice represented total complement deficiency and $C4^{-/-}$ mice and $P^{-/-}$ mice, respectively, represented deficiency of the classical/lectin and alternative activation pathway. Sham-operated mice served as controls. A, Local mRNA gene expressions of C3 and quantification of local C9 deposition lung tissue. C3 mRNA gene expression levels are shown relative to β-actin. Values of sham-operated mice are set at 1, the other values were calculated accordingly. B-F, Representative C9-stained lung slides of brain-dead WT mice, sham-operated controls, and brain-dead complement deficient mice. Data are presented as mean ± SD. $^{*}P < .05$, $^{**}P < .01$. Asterisks indicate significance relative to WT brain-dead mice

CP/LP seems mainly involved in neutrophil influx and pulmonary inflammation upon BD, while the AP seems to be moderately involved.

4 | DISCUSSION

Activation of the immune system upon BD has been widely recognized and described in literature.[2,4] However, the role of complement activation in BD has been underexposed, especially with regard to donor lungs. In this study, we investigated whether BD-induced lung injury is complement dependent, and which pathways are herein involved. We showed that BD-induced lung injury is dependent on activation of the complement system and elucidated a primary role for the CP and/or LP activation pathway.

In both preclinical and clinical studies, the BD process is described to augment cytokine formation, worsen lung morphology, and increase cellular influx.[2,9,20] Consequently the donor lung is injured, which aggravates primary graft dysfunction and graft failure upon transplantation.[2,21] Our model reflected BD-induced lung injury, as corroborated by worsened lung morphology and an increase in neutrophil influx in WT brain-dead mice, compared to WT sham-operated controls. Besides, we observed the BD-induced cytokine storm in line with previous studies, as supported by increased levels of IL-6, TNF-α, MCP-1, E-selectin, and VCAM-1 in brain-dead mice, compared to sham-operated controls.[2,9,20]

The presence of complement activation in BD-induced pathophysiology was previously suggested by Cheng et al. They found elevated mRNA and protein expressions of the C3a receptor in lungs donated after BD, compared to lungs derived from living mice.[9] In our study, we showed that complement is activated on a systemic level, as corroborated by increased plasma levels of iC3b in WT brain-dead mice compared to WT sham-operated mice.

On a local level, we demonstrated MAC formation in lungs from brain-dead mice by the presence of C9 deposition. In contrast, C9 deposition was absent in sham-operated mice. Clinical importance of the MAC in brain-dead donors and its detrimental effect on recipient graft survival has previously been emphasized by Budding et al.[22] In the mentioned study, they described that lung transplant recipients are at higher risk for chronic rejection, when receiving donor lungs with a CD59 single nucleotide polymorphism (SNP) configuration. Under normal circumstances, CD59 acts as a potent MAC-regulatory protein.[5] However, in donor lungs with a CD59 SNP expression, the regulatory function of CD59 is disturbed, which lowers the threshold for MAC activation and cell lysis. Based on the mentioned study, dysregulation of the complement system in the donor seems an important contributing factor to donor lung quality and survival. In this study, we showed that BD-induced lung injury is indeed complement dependent. This was corroborated by improved lung morphology scores, attenuated neutrophil infiltration and reduced proinflammatory gene expressions in brain-dead $C3^{-/-}$ mice, which represented total complement deficiency. Thereby, C9 deposition was absent in lungs from $C3^{-/-}$ mice, which supports that MAC formation is prevented in absence of central component C3. No studies have

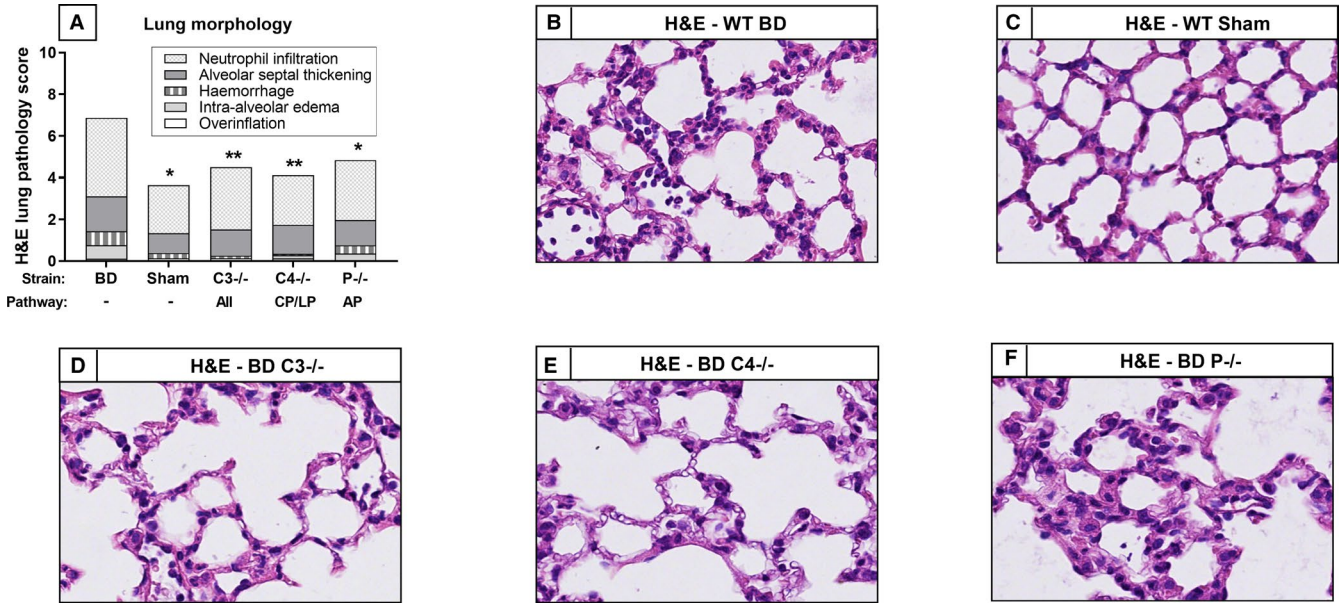

FIGURE 3 Brain death (BD)-induced histological lung injury is reduced in absence of a functional classical/lectin and alternative pathway. BD was induced in wild-type (WT) mice, central complement $C3^{-/-}$ mice, $C4^{-/-}$ mice, and $P^{-/-}$ mice. $C3^{-/-}$ mice represented total complement deficiency and $C4^{-/-}$ mice and $P^{-/-}$ mice, respectively, represented deficiency of the classical/lectin and alternative complement activation pathway. Sham-operated mice served as controls. A, Quantification of lung morphology scores in hematoxylin and eosin (H&E)-stained lung slides. B-F, Representative H&E-stained lung slides of WT brain-dead mice, WT sham-operated controls and brain-dead complement deficient mice. Data are presented as mean ± SD. * $P < .05$, ** $P < .01$. Asterisks indicate significance relative to WT brain-dead mice

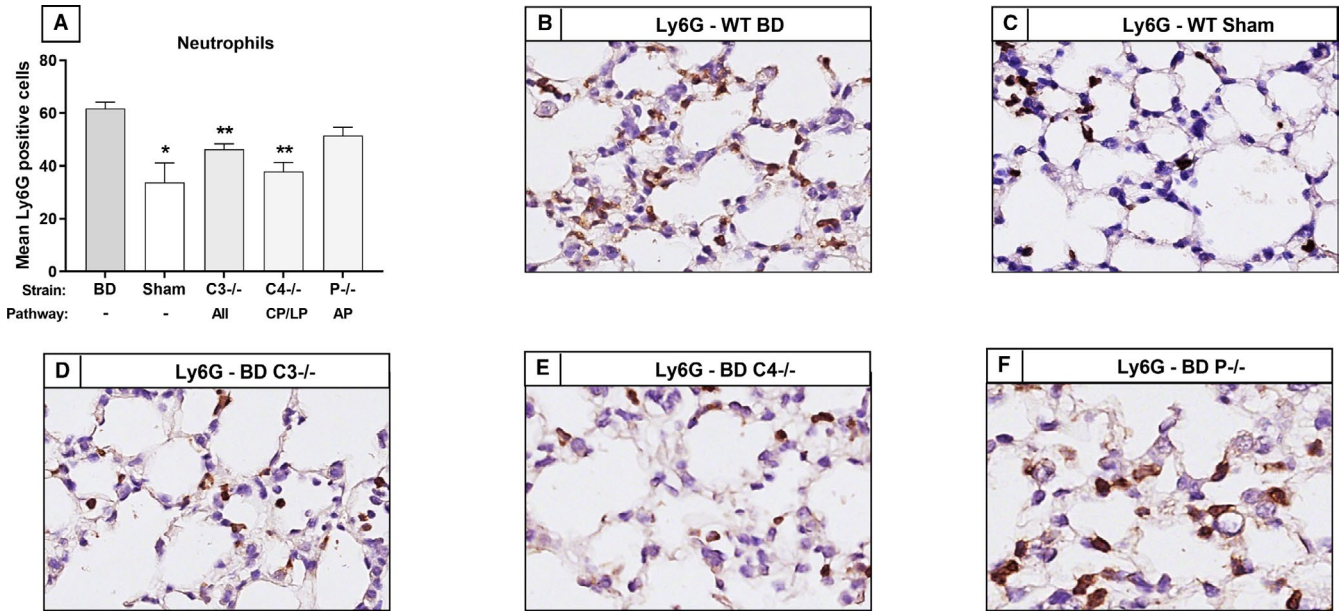

FIGURE 4 Brain death (BD)-induced neutrophil influx is reduced in absence of a functional classical/lectin and alternative pathway. BD was induced in wild-type (WT) mice, central complement $C3^{-/-}$ mice, $C4^{-/-}$ mice, and $P^{-/-}$ mice. $C3^{-/-}$ mice represented total complement deficiency and $C4^{-/-}$ mice and $P^{-/-}$ mice, respectively, represented deficiency of the classical/lectin and alternative complement activation pathway. Sham-operated mice served as controls. A, Quantification of neutrophils as depicted by Ly6G staining. B-F, Representative Ly6G-stained lung slides of WT brain-dead mice, WT sham-operated controls and brain-dead complement deficient mice. Data are presented as mean ± SD. *$P < .05$, **$P < .01$. Asterisks indicate significance relative to WT brain-dead mice

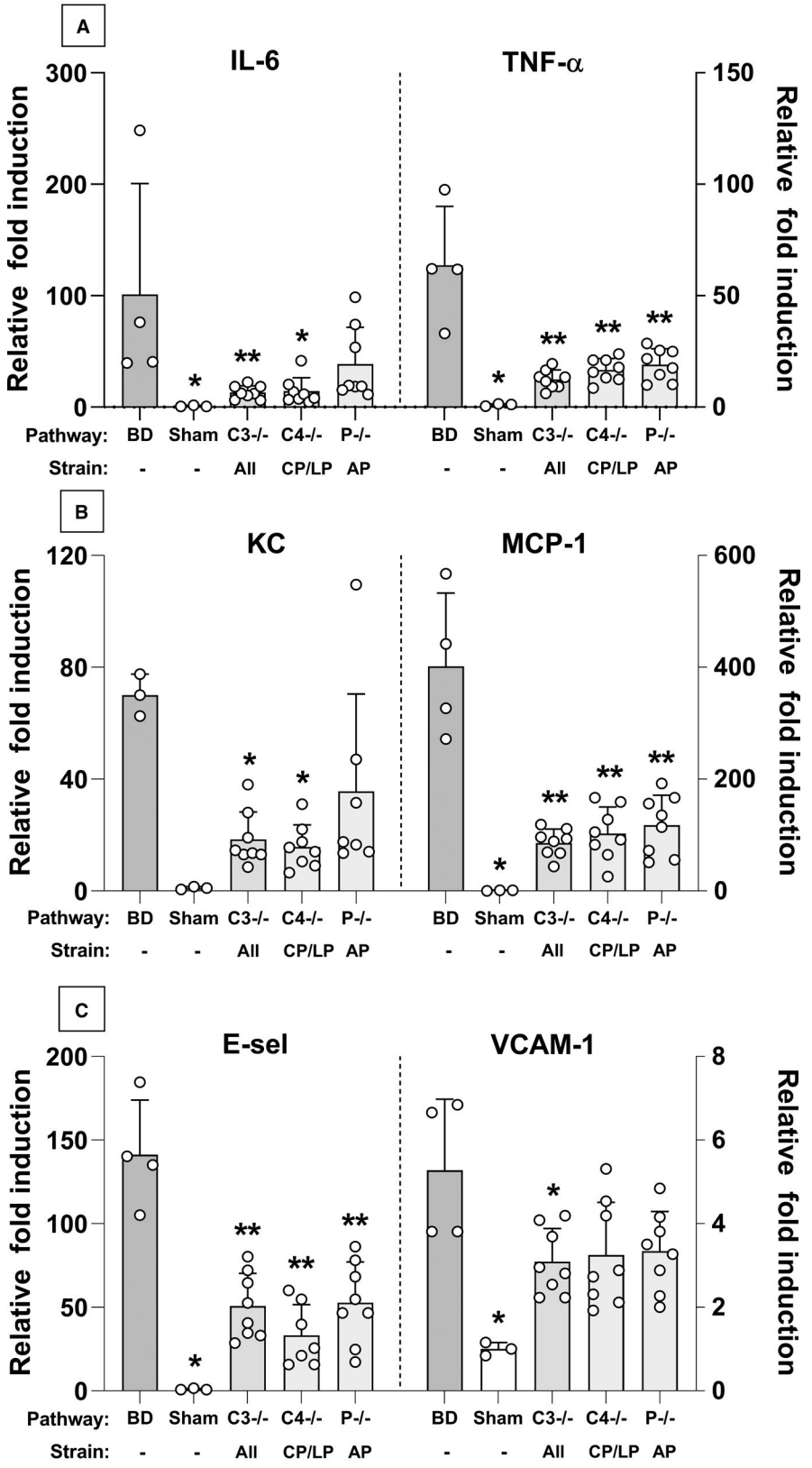

FIGURE 5 Brain death (BD) -induced proinflammatory gene expression is mainly attenuated in absence of a functional classical/lectin and alternative pathway. BD was induced in wild-type (WT) mice, central complement $C3^{-/-}$ mice, $C4^{-/-}$ mice, and $P^{-/-}$ mice. $C3^{-/-}$ mice represented total complement deficiency and $C4^{-/-}$ mice and $P^{-/-}$ mice, respectively, represented deficiency of the classical/lectin and alternative complement activation pathway. Sham-operated mice served as controls. A, mRNA gene expressions of cytokines IL-6 and TNF-α. B, mRNA gene expressions of chemokines KC and MCP-1. C, mRNA gene expressions of adhesion molecules E-selectin and VCAM-1. Data are shown as expression relative to β-actin. Values of sham-operated mice are set at 1, the other values were calculated accordingly. Data are presented as mean ± SD. $^{*}P < .05$, $^{**}P < .01$. Asterisks indicate significance relative to WT brain-dead mice

previously been published on complement deficiency or blockade on a C3 level in lungs from brain-dead donors. However, Atkinson et al studied the effect of C3 deficiency in hearts from brain-dead donors and showed similar beneficial results in cardiac histology and inflammatory gene expression.[23]

We studied the contribution of complement activation pathways in BD-induced lung injury in $C4^{-/-}$ and $P^{-/-}$ mice, which represented, respectively, the CP/LP and the AP. Improved histology, attenuated neutrophil infiltration, and reduced cytokine expression in $C4^{-/-}$ mice strongly suggest CP and/or LP involvement in BD-induced lung injury. $P^{-/-}$ mice showed less pronounced results based on unaltered numbers of infiltrated neutrophils and unaffected gene expression levels of IL-6 and KC, which suggests that the AP is to a lesser extent involved in BD-induced lung injury. Besides that, reduced C9 deposition in $C4^{-/-}$ mice but unaffected C9 deposits in $P^{-/-}$ mice, implies that BD-induced MAC formation runs mainly through the CP and/or LP. Based on these results, we speculate that the AP serves more as an amplifier than an initiator in complement activation upon BD, a role that has been described for the AP before.[24] To our knowledge, no previous studies dissected the contribution of the classical/lectin and alternative complement activation pathways in BD-induced lung injury. Though in hearts from brain-dead mice, Atkinson et al showed IgM deposition, which can form antigen-antibody complexes and activate the CP.[23] Moreover, they showed C4d deposition in hearts from human brain-dead donors, which further supports involvement of the CP.[25] Nevertheless, it should be noted that dissimilarities between physiology of organs might lead to different ways of complement activation.[26] Therefore, it remains important to study contribution of different complement components in the organ of interest.

Complement-targeted therapies in the donor may reduce BD-induced lung injury, which potentially improves transplantation outcomes in recipients. While inhibition on the level of C3 seems a promising target, central complement inhibition might increase susceptibility to infections.[27] Especially in lungs, given their function as a first line barrier defense to microorganisms. With regard to activation pathways, we speculate that the CP and/or LP are potential targets to treat BD-induced lung injury, which leaves the AP functional for complement activation. A functional AP is important, since aspergillus infection, a common complication in lung transplantation recipients, is known to be eliminated via the AP.[28]

This study serves as a first step in the identification of promising complement targets in BD-induced lung injury. However, one limitation of our study is that the contribution of the CP vs the LP was not further dissected, which should be considered in future studies. Furthermore, it should be noted that complement deficiency might show different results than complement inhibition of the same protein. To enable a more accurate translation to therapeutic options, the effect of complement inhibitors on BD-induced lung injury needs to be investigated. Topics that herein require attention are the identification of cells responsible for complement activation, and the most effective administration route of complement-targeted therapeutics. In this study, we identified BD-induced complement activation on both a systemic and local level, as corroborated by systemic iC3b levels and local C9 deposition. C9 deposition reflects MAC formation, the final step in the complement activation cascade. However, it should be noted that absence of C9 does not rule out the presence of upstream chemotactic split products such as C3a and C5a, which on itself might provoke influx and activation of inflammatory cells.[5,6] From the results of this study, it is suggested that both systemic and local therapies might be beneficial to attenuate BD-induced lung injury, such as intravenous or inhaled therapeutics. A possible benefit of systemic treatment in the organ donor, is the ability to simultaneously treat all potential donor organs, damaged by the BD process. However, it should be noted that not all organs might share the same target to inhibit BD-induced complement activation, thus favoring local treatment modalities.[26] Earlier studies described pulmonary alveolar type II epithelial cells as capable to secrete complement proteins C2, C3, C4, C5, and factor B.[29] Furthermore, bronchiolar epithelial cells seem able to generate C3.[30] However, besides resident lung cells, circulating immune cells recruited to the proinflammatory environment of the lung, might contribute to complement activation. The pathophysiology of BD is described to alter the hemostatic status of organ donors, in which amongst others, activation of blood platelets occurs.[31] The link between complement activation and thrombosis has been widely described in literature.[32] Recently, it has been shown that complement proteins can be expressed on the surface of blood platelets, in which both the CP and AP may be involved.[33,34] We consider the identification of complement producing cells and their contribution to BD-induced complement activation an important factor in the search for complement therapeutics in the brain-dead organ donor. Last, addition of a transplantation model might enhance translatability to the human transplant setting in future studies. This study was designed to focus on BD-induced lung injury alone. Therefore, we did not address the effect of complement inhibition on lung functionality after transplant.

We consider this study to be of importance for both scientists and clinicians, since we provide a foundation in understanding the role of complement activation in BD-induced lung injury. In this study, we demonstrated that BD-induced lung injury is complement dependent, with a primary role for the CP and/or LP activation pathway.

DISCLOSURE

The authors of this manuscript have no conflicts of interest to disclose as described by the *American Journal of Transplantation*.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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