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ORIGINAL RESEARCH - CLINICAL

Transfusion-Related Renal Dysfunction After Cardiac Surgery

The Role of Myeloid-Related Protein_14 in Neutrophil-Mediated Tubular Damage

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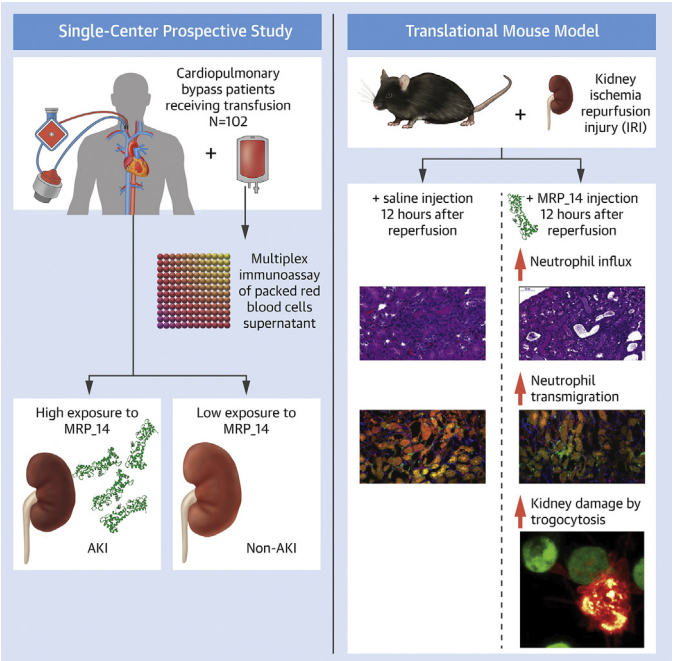
VISUAL ABSTRACT



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HIGHLIGHTS

- **Following cardiac surgery, 20% of patients will present with AKI, which is associated with increased mortality, and transfusion increases the risk of AKI.**
- **The main objective was to determine whether the composition of transfusion was associated with AKI.**
- **In this study, AKI patients received higher amount of MRP_14 through transfusion vs non-AKI.**
- **MRP_14 has been reported to activate and enhance neutrophil transmigration into damaged tissues. In a murine model of ischemia-reperfusion, MRP_14 increased renal damage and enhanced neutrophil influx into the kidney. MRP_14 also increased neutrophilic-trogocytosis toward tubular cells.**
- **The sex of the donor and the method of preparation of the blood determined the concentration of MRP_14 in packed red blood cells.**

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ABBREVIATIONS AND ACRONYMS

AKI = acute kidney injury

CPB = cardiopulmonary bypass

IRI = ischemia-reperfusion injury

MPO = myeloperoxidase

MRP_14 = myeloid-related protein 14

PRBC = packed red blood cells

SUMMARY

Transfusion is a specific cause of acute kidney injury (AKI) after cardiac surgery. Whether there is an association between the composition of blood products and the onset of AKI is unknown. The present study suggests that the transfusion of packed red blood cells containing a high amount of myeloid-related protein 14 (MRP_14) could increase the incidence of AKI after cardiac surgery. In a mouse model, MRP_14 increased the influx of neutrophils in the kidney after ischemia-reperfusion and their ability to damage tubular cells. Higher concentrations of MRP_14 were found in packed red blood cells from female donors or prepared by whole blood filtration. (J Am Coll Cardiol Basic Trans Science 2022;7:627–638)

During open-heart surgery, extracorporeal circulation is required to establish cardiopulmonary bypass (CPB) and ensure organ oxygenation. CPB is often described as an experimental model of kidney ischemia-reperfusion injury (IRI).[1] Consequently, up to 20% of patients will present with acute kidney injury (AKI) following cardiac surgery which is associated with increased mortality.[2]

Although transfusion is a paramount treatment to overcome blood loss, the appropriate transfusion threshold is still undetermined, and each additional packed red blood cell (PRBC) could increase the risk of AKI by 15%.[3] However, the mechanisms underlying this association remain unclear. Interestingly, using mass spectrometry-based proteomic analysis of the urine, Ho et al[4] showed that all CPB patients experienced an "initiation phase" of renal damage related to kidney IRI.[4] This phase could be spontaneously resolutive or could progress toward AKI in case of protracted inflammatory activity in the kidney.

The storage of PRBC has been reported to impair the survival of red blood cells, leading to hemolysis and accumulation of proinflammatory molecules,[5] including danger associated molecular patterns. In this respect, we reasoned that the transfusion of inflammatory proteins could synergize with CPB-induced kidney IRI and lead to AKI. To test this hypothesis, the association between the inflammatory contents of PRBC and the onset of postoperative renal failure was analyzed. The findings were then transposed to a mouse model of kidney IRI to determine the biological relevance of the results (NCT02763410).

METHODS

STUDY SETTING AND ETHICS. The TRANSNEPHRON study was a monocenter prospective study. Patients were recruited in Nantes University Hospital from September 2016 to September 2018 (NCT02763410). An institutional board reviewed the protocol; patients received oral and written information and gave their written consent (Ethics Committee Ouest V, Rennes, #16/02-1000). The experimental model of kidney IRI was set up in the Peter Doherty Institute, University of Melbourne, Australia (approved by the Animal Ethics Committee, Melbourne University, Protocol #1814615).

PARTICIPANTS, STUDY DESIGN, AND COLLECTION OF BIOLOGICAL SAMPLES. Patients undergoing cardiac surgery with CPB were eligible. Patients who required a transfusion of 1-5 PRBC during surgery or the following 6 hours were included. Exclusion criteria were: transfusion of any blood products before surgery or in the prior 3 months, age under 18, pregnancy, protected adult, opposition of the patient on recording his/her data, ongoing infection, myocardial infarction in the previous 15 days, inotropic or vasopressor agents before surgery,

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The authors attest they are in compliance with human studies committees and animal welfare regulations of the authors' institutions and Food and Drug Administration guidelines, including patient consent where appropriate. For more information, visit the Author Center.

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immunosuppressive treatment, estimated glomerular filtration rate below 40 mL/min/m^2, or positive irregular red cell antibodies (ie, irregular agglutinin).

A sample of each PRBC received by the participants was drawn (1 milliliter), and the supernatant was collected after centrifugation (500 × *g*, 10 minutes) for conservation (−80 °C).

OBJECTIVES AND ENDPOINTS. The main objective of the study was to determine whether the composition of PRBC was associated with AKI after cardiac surgery. The primary outcome was the association between the onset of AKI in the first 48 hours postoperatively and patient exposure to the inflammatory contents of PRBC. According to the RIFLE classification,[6] AKI was defined as a decrease of at least 25% of the estimated glomerular filtration rate (according to the Modification of Diet in Renal Disease equation) or an increase in the creatinine level of at least 1.5-fold compared with the preoperative period. Our findings were then transposed to a mouse model of kidney IRI to determine the biological relevance of the results. Finally, we investigated whether PRBC composition could be anticipated according to donor characteristics and preparation methods.

PATIENT EXPOSURE. To determine patient exposure, the inflammatory content of each PRBC was analyzed by multiplex immunoassay (Luminex technology, see Supplemental Methods) for a panel of proteins. The amount of protein in PRBC #1 (ie, QT_{M1} for protein "M") was calculated by the product of the concentration of "M" [C_M] and the volume of the PRBC #1. Patient exposure to "M" was defined as the total amount of "M" received during transfusion and was determined by adding the QT_M ($QT_{M1} + QT_{M2} + QT_{M3} + \ldots + QT_{MX}$) (Supplemental Figure S1).

KIDNEY IRI MODEL. Kidney IRI was performed as previously described.[7] Briefly, mice were anesthetized with isoflurane 1%. After midline abdominal incision, blood flow was interrupted with a microclamp on the left renal pedicle. After 30 minutes, the clamp was removed and the restoration of renal blood flow was controlled, as demonstrated by a return to its original color. Sham-operated mice were submitted to the same surgery (namely, same duration of anesthesia, abdominal incision, and dissection of the renal pedicle without clamping). Phosphate-buffered saline (PBS) or 5 μg of MRP_14 (recombinant mouse S100A9, carrier-free, Biolegend, Cat # 765406) in 150 μL was injected intravenously 12 hours after reperfusion. Mice were sacrificed 14 hours and 2 and 7 days after surgery to harvest the left kidney.

ORGAN COLLECTION, TISSUE DISSOCIATION, AND CELL ISOLATION. After euthanasia, 30 mL of phosphate-buffered saline 0.5% heparin was perfused via the left ventricle until the left kidney was totally pale, to remove circulating blood cells from the kidney. The left kidney was then removed and digested in type IV collagenase DNAse (75 minutes, 37 °C).[8] Suspension was depleted of erythrocytes with NH_4Cl lysis buffer. Debris were removed by 45%/90% percoll (Sigma P1644-1L) gradient (500 × *g*, 25 minutes, room temperature).

For trogocytosis assay, neutrophils were isolated from the bone marrow of naive C57BL/6 male and LysM-eGFP mice. Neutrophils were purified using untouched immunomagnetic negative isolation kit (Miltenyi Biotec) routinely yielded cell population with purity of 92% to 96%.

PRIMARY PROXIMAL TUBULAR CELL ISOLATION. Primary proximal tubular cells were generated as previously described[9] from a UbiTomato mouse (see Supplemental Methods) expressing tdTomato Fluorescent Protein in most cells, including in the membrane of tubular cells. Briefly, CD133^{pos} cells were isolated from the kidney single-cell suspension by immunomagnetic positive selection and resuspended in complete K1 medium (see Supplemental Methods) until epithelial colonies. After a 10-day culture, cells were trypsinized, and purity was enhanced by cytometry cell sorting (gating on CD45.2^{neg}/CD133^{pos} alive cells, yielded cell population with purity of >96%) before trogocytosis assay.

TROGOCYTOSIS ASSAY. As previously described,[10] sorted neutrophils (50,000 per well in 100 μL) were cocultured with tdTomato tubular cells (ratio 1 neutrophil to 5 tubular cells) in a 96-well plate in a final volume of 200 μL vol/vol RPMI/HBSS, with or without MRP_14 stimulation (2.5 μg/mL). For CD18 blocking, neutrophils were incubated (30 minutes, 37 °C) with anti-CD18 mAb (30 μg/mL) or its isotype control and washed (10 minutes, 500 × *g*) before the assay.

IN VIVO CD45.2 STAINING FOR CYTOMETRY AND CONFOCAL MICROSCOPY. At 3 minutes before euthanasia, each mouse was intravenously injected with 3 μg of anti-CD45.2-PE mAb to stain either circulating leukocytes or leukocytes recruited onto the endothelium apical surface (CD45-PE^{pos}, further referred as "marginated"),[11] and to leave unstained leukocytes, which were located in the kidney interstitium (CD45-PE^{neg}, further referred as "interstitial") including neutrophils. Then, kidneys were harvested for cytometry analysis or fixed for confocal imaging (see Supplemental Methods). For cytometry analysis, after in vivo staining, the single-cell suspension was stained with anti-CD45 Brilliant Violet (BV785)-conjugated mAb and anti-Ly6G Peridinin Chlorophyll

TABLE 1 Baseline Characteristics

	Non-AKI (n = 87)	AKI (n = 15)	*P* Value
Male	29 (33)	9 (60)	**0.049**
Age, y	73.6 ± 7.4	71.6 ± 13.2	0.91
BMI, kg/m^2	26.4 ± 5.1	25.9 ± 4.1	0.95
Theoretical cardiac output, L/min/m^2	4.2 (3.8, 4.5)	4.2 (4.0, 4.7)	0.48
Preoperative medical history			
Active tobacco	21 (24)	3 (20)	0.99
Diabetes	23 (26)	3 (20)	0.75
Chronic obstructive pulmonary disease	7 (8)	1 (7)	0.99
Chronic heart failure (NYHA functional class III or IV)	37 (42)	6 (40)	0.85
Peripheral artery disease	11 (13)	5 (33)	0.056
Atrial fibrillation	15 (17)	4 (26)	0.47
Left ventricular ejection fraction, %	60.3 ± 9.3	59.3 ± 7.3	0.46
Medication before surgery			
Antiplatelet therapy	62 (71)	11 (73)	0.99
Anticoagulant	17 (20)	6 (40)	0.098
Beta-Blocker	52 (60)	12 (80)	0.13
Angiotensin-converting enzyme inhibitor	37 (43)	7 (47)	0.77
Calcium channel blocker	23 (26)	5 (33)	0.55
Biology before surgery			
Hemoglobin, g/dL	12.2 ± 1.3	11.6 ± 1.2	0.061
Platelet count, × 10^9/L	256 ± 71	265 ± 114	0.98
Blood creatinine, μmol/L	83.9 ± 22.9	83.5 ± 23.9	0.92
eGFR, MDRD, mL/min/m^2	70.5 (57.7, 87.9)	77.3 (64.2, 95.1)	0.31
Surgery			
Type of surgery			0.44
Coronary artery bypass	28 (32)	4 (27)	
Valve replacement	25 (29)	3 (20)	
Combined surgery	34 (39)	8 (53)	
Duration of extracorporeal circulation, min	106 ± 41.4	133 ± 27.8	**0.003**
No. of PRBC transfusions during surgery and the following 6 h	2.0 (1.0, 2.0)	2.0 (1.0, 3.0)	0.097
Postoperative outcome			
Hospital-acquired infection	7 (8)	3 (20)	0.21
Surgical site infection	1 (1)	2 (13)	0.20
Duration of vasopressive/inotropic support, h			
Norepinephrine	22.7 ± 51.1	52.6 ± 65.7	0.006
Dobutamine	16.0 ± 35.7	38.1 ± 33.0	0.002
Time on ventilator, h	16 ± 52.9	55 ± 91.5	**<0.001**
Duration of stay in the ICU, days	2.2 ± 3.8	10.3 ± 16.4	**<0.001**
Duration of hospital stay, days	15 ± 6.4	22 ± 11.5	**0.022**
Death at day 90	3 (4)	1 (7)	0.48

Values are n (%), mean ± SD, or median (Q1, Q3). This table presents the baseline characteristics of the population. Modification of Diet in Renal Disease equation (MDRD) used to estimate the glomerular filtration rate (eGFR). Combined surgery stands for surgery which includes both coronary artery bypass and valve replacement.

AKI = acute kidney injury; BMI = body mass index.

Protein Complex-conjugated mAb. Interstitial neutrophils were defined as CD45-BV785^{pos}/Ly6G^{pos}/CD45-PE^{neg} and marginated neutrophils as CD45-BV785^{pos}/Ly6G^{pos}/CD45-PE^{pos}. As positive and negative controls, we ensured that 100% of the neutrophils within the blood were CD45-PE^{pos} and none of them were CD45-BV785^{pos}/CD45-PE^{neg}.

STATISTICAL ANALYSIS. Analyses were performed with GraphPad prism software. Baseline characteristics are reported as number (percentage) for qualitative variables and as mean ± SD or median (25th, 75th percentiles [Q1,Q3]), according to distribution, for quantitative variables. The normality of distributions was determined using the Kolmogorov-Smirnov test. The Kruskal-Wallis test was used to compare multiple groups (ie, blood groups). The Mann-Whitney *U* test was used to compare patient exposure between groups. A *P* value <0.05 was considered statistically significant. Patients who received transfusion of fresh frozen plasma or platelet concentrate before the diagnosis of AKI, or who developed AKI after surgical complications (ie, hemorrhagic or cardiogenic shock) were excluded a priori from the analysis. Multivariable logistic regression model was applied to assess the association between the occurrence of AKI in the first 48 hours after surgery and MRP_14 exposure. This model was adjusted for age, sex, duration of extracorporeal circulation, and baseline creatinine (ie, risk factor of AKI).

All additional methods (including multiplex immunoassay, ELISA assay, cytometric bead array, and immunohistochemistry), references for antibodies, reagents, creatinine level measurement, and the origin of mouse strains are detailed in Supplemental Table S1 and the Supplemental Methods.

RESULTS

Over the study period, 3,183 patients underwent cardiac surgery at the Nantes University Hospital, and 105 (3.3%) were included. A total of 3 patients were excluded from the final analysis: 2 with hemorrhagic shock and 1 with postcardiotomy cardiogenic shock (Supplemental Figure S2).

BASELINE CHARACTERISTIC OF THE PATIENTS. Patient characteristics, surgery, and postoperative follow-up are detailed in **Table 1**. The population included 38 (37.3%) men and 64 (62.7%) women with an overall mean age of 72 (8.4). Combined surgery (ie, coronary artery bypass with valve replacement) represented 41.1% of the inclusions. In the first 48 hours, 15 patients (14.7%) developed AKI and 87 (85.3%) did not. The median (Q1-Q3) number of PRBCs per patient was 2 (1.0-3.0) in the AKI group vs 2 (1.0-2.0) in the non-AKI group. AKI patients had longer mean duration of extracorporeal circulation: 133 ± 27.8 minutes vs 106 ± 41.4 minutes ($P = 0.003$); longer mean time on ventilator: 55 ± 91.5 hours vs 16 ± 52.9 hours ($P = 0.0002$); longer mean duration of stay in the ICU: 2.2 ± 3.8 days vs 10.3 ± 16.4 days

($P < 0.0001$); and longer duration of hospital stay: 22 ± 11.5 days vs 15 ± 6.4 days ($P = 0.02$) compared with non-AKI patients.

SELECTION OF THE PROTEIN PANEL. The exposure of the first 42 patients to the inflammatory contents of 74 PRBCs was assessed for a panel of 20 proteins in a first-step selection (Supplemental Table S2). Then, in a second step, the exposure of the following 18 patients was assessed for another panel of 16 proteins (43 PRBCs, Supplemental Table S3). To establish the final panel, proteins for which patient exposure were null in the AKI group in Supplemental Tables S2 and S3 were discarded.

ASSOCIATION BETWEEN EXPOSURE AND POSTOPERATIVE AKI. The exposure of 102 patients was obtained for 8 proteins in the final panel (184 PRBCs) (**Table 2**). Compared with non-AKI, AKI patients received significantly higher median (Q1-Q3) amounts of HSP_70 (3.9×10^7 [2.4×10^7 to 8.1×10^7] vs 2.5×10^7 [1.5×10^7 to 4.1×10^7] picograms; $P = 0.04$); RANTES (3.5×10^4 [1.9×10^4 to 5.5×10^4] vs 2.2×10^4 [0.7×10^4 to 3.4×10^4] picograms; $P = 0.02$); and MRP_14 (7.3×10^4 [5.4×10^4 to 11.7×10^4] vs 4.5×10^4 [2.6×10^4 to 7.1×10^4] picograms; $P = 0.008$). Multivariable analysis (see Supplemental Table S4) suggested that MRP_14 (ie, showing the lowest *P* value in **Table 2** with area under the curve = 0.72, 95% CI: 0.58-0.86) was independently associated with the occurrence of postoperative AKI (OR: 5.13 [95% CI: 1.25-21.30]; $P = 0.023$). Interestingly, patients with the highest exposure to MRP_14 (ie, >50th percentile) had longer time on ventilator as well as longer stays in the ICU (see Supplemental Table S5). To determine whether these results corresponded to a relevant biological effect, we transposed our findings to a mouse model of kidney IRI. We hypothesized that MRP_14 administration after IRI could increase renal damage and recapitulate the renal effect of transfusion in cardiac surgery patients.

MRP_14 INCREASES RENAL DAMAGE. To mimic kidney IRI during CPB, we used a mouse model of 30-minute left renal artery clamping (Supplemental Figure S3A). First, we investigated whether MRP_14 could increase histological damage when administered 12 hours after IRI compared with IRI alone. The 2 control groups were sham-operated mice with or without MRP_14 administration. Over a 7-day period, the weight of the mice did not differ between experimental groups (Supplemental Figure S3B). As previously described,[7] unilateral renal artery clamping did not affect the blood levels of creatinine regardless of MRP_14 administration (Supplemental Figure S3C). Histological analysis of the left kidney on day 7 after surgery showed that sham surgery with or without MRP_14 injection did not lead to renal damage (**Figures 1A and 1B**), whereas MRP_14 administration 12 hours after IRI increased tubular injury compared with IRI alone (**Figures 1C and 1D**). At 48 hours as well as 7 days after IRI, the proportion of necrotic tubules was increased in MRP_14-treated mice (**Figure 1E**) compared with IRI alone, without difference for tubular casts (**Figure 1F**). At 48 hours after IRI, compared with untreated mice, the increase of monocyte chemotactic protein-1 activity in the left kidney of MRP_14-treated mice supports the histological observations (Supplemental Figure S3D).[12] In contrast, neutrophil gelatinase-associated lipocalin (NGAL) activity increased after MRP_14 administration regardless of ischemia (Supplemental Figure S3E).

MRP_14 INCREASES THE INFLUX OF LEUCOCYTES IN THE KIDNEY. To determine how MRP_14 could worsen renal damage after IRI, left kidney was harvested 2 hours after MRP_14 injection. In IRI conditions, the number (**Figure 2A**) and the percentage (**Figure 2B,** Supplemental Figure S4A) of leucocytes was higher in MRP_14-treated mice compared with IRI alone. Conversely, MRP_14 did not increase the number or percentage of leucocytes after sham surgery compared with sham surgery alone. After IRI, MRP_14 significantly increased the percentage

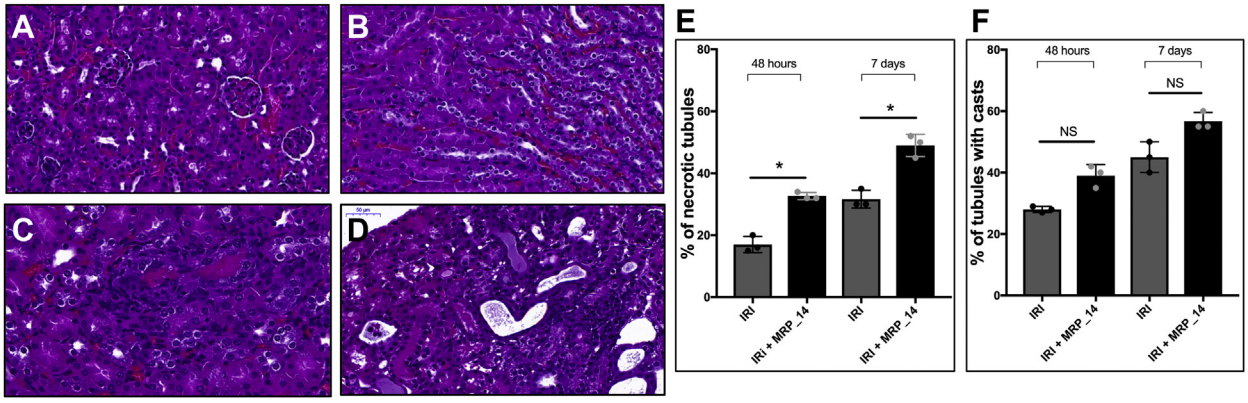
TABLE 2 Exposure of the Participants to a Panel of 8 Proteins

Exposure	Non-AKI (n = 87)	AKI (n = 15)	*P* Value
HMGB_1	25.1×10^4 (2.4×10^4; 19.5×10^5) [0; 9.2×10^6]	12.0×10^4 (3.5×10^4; 28.3×10^4) [0.4×10^4; 33.1×10^4]	0.48
HSP_70	2.5×10^7 (1.5×10^7; 4.1×10^7) [0.3×10^7; 11.3×10^7]	3.9×10^7 (2.4×10^7; 8.1×10^7) [0.9×10^7; 18.1×10^7]	**0.043**
PD_L2	1.2×10^5 (0.9×10^5; 2.1×10^5) [0.6×10^5; 5.6×10^5]	1.7×10^5 (1.1×10^5; 3.1×10^5) [0.2×10^5; 11.7×10^5]	0.15
RANTES	2.2×10^4 (0.7×10^4; 3.4×10^4) [0; 13.2×10^4]	3.5×10^4 (1.9×10^4; 5.5×10^4) [0.1×10^4; 11.4×10^4]	**0.023**
RBP_4	1.1×10^8 (0.6×10^8; 2.1×10^8) [0.1×10^8; 5.5×10^8]	1.2×10^8 (0.6×10^8; 1.8×10^8) [0.3×10^8; 3.2×10^8]	0.73
MRP_14	4.5×10^4 (2.6×10^4; 7.1×10^4) [0; 17.7×10^4]	7.3×10^4 (5.4×10^4; 11.7×10^4) [1.7×10^4; 19.4×10^4]	**0.008**
SDF_1α	1.3×10^4 (0; 2.9×10^4) [0; 27.3×10^4]	2.5×10^4 (1.4×10^4; 3.9×10^4) [0; 38.8×10^4]	0.089
TIMP_1	2.5×10^6 (1.3×10^6; 4.2×10^6) [0.38×10^6; 13.2×10^6]	2.7×10^6 (1.9×10^6; 4.9×10^6) [0.5×10^6; 34.6×10^6]	0.41

Values are median (25th; 75th percentiles) [min-max], picograms. This table presents the final protein panel established after a 2-step preselection (see Supplemental Tables S2 and S3 for first- and second-step selection). A total of 184 packed red blood cells (PRBCs) were analyzed to determine the exposure of the participants (ie, total amount of each of the 8 proteins in picograms received during transfusion). The strategies to determine the exposure of each patient are described in the Methods section and Supplemental Figure S1.

HMGB_1 = high-mobility group box 1; HSP_70 = heat shock protein_70; MRP = myeloid-related protein; PDL = programmed cell death ligand; RANTES = regulated upon activation, normal T cell expressed and presumably secreted; RBP_4 = retinol-binding protein_4; SDF_1α = stromal cell-derived factor_1 alpha; TIMP_1 = tissue inhibitor matrix metalloproteinase_1.

FIGURE 1 Effect of MRP_14 on Tubular Damage After Kidney Ischemia-Reperfusion



Left kidney section after hematoxylin and eosin staining (magnification 40×) 7 days after surgery in the 4 experimental groups: **(A)** sham surgery, **(B)** sham surgery with MRP_14 treatment 12 hours after surgery, **(C)** ischemia-reperfusion (IRI), and **(D)** ischemia-reperfusion with MRP_14 treatment 12 hours after reperfusion (IRI + MRP_14). Percentage of necrotic tubules **(E)** and percentage of tubules with cast **(F)** in the left kidney 48 hours and 7 days after surgery based on histological analysis. Sham groups are not represented in **E and F** because of the lack of histological damage in these groups. Data are shown as median with 25th and 75th percentiles; *$P < 0.050$. Analysis was performed on 12 mice (3 mice per group). NS = nonsignificant difference.

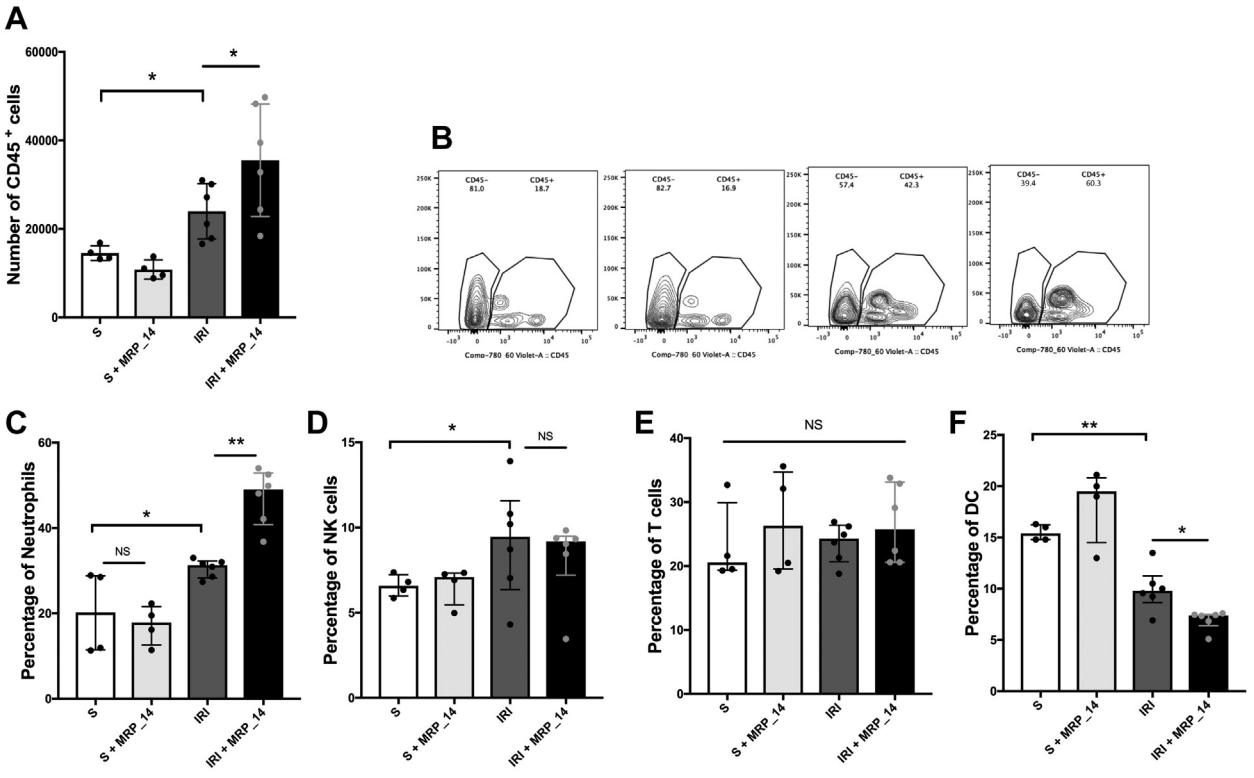
(**Figure 2C**) and the number (Supplemental Figure S4B) of neutrophils compared with IRI alone, contrary to natural killer cells (**Figure 2D,** Supplemental Figure S4C) or T cells (**Figure 2E,** Supplemental Figure S4D). After IRI, MRP_14 decreased the percentage of dendritic cells (**Figure 2F**) without altering their total number (Supplemental Figure S4E) compared with IRI alone (Gating strategy in Supplemental Figure S4F). Further analyses therefore focused on neutrophils.

MRP_14 INCREASES THE TRANSMIGRATION OF NEUTROPHILS. Neutrophil influx in the interstitium of the kidney was reported to be deleterious for renal function.[13] We reasoned that if MRP_14 increased the number of interstitial neutrophils after IRI, this could explain the increased renal damage in **Figure 1D** and our findings in **Table 2**. To test this hypothesis, we analyzed the compartmentalization of neutrophils in the kidney by in vivo anti-CD45-PE staining (**Figure 3A**).[11] After sham surgery, <2% of the neutrophils were identified as interstitial, regardless of MRP_14 stimulation. After IRI, MRP_14 increased significantly the percentage and the number of interstitial neutrophils, compared with IRI alone (**Figures 3B and 3C**). The percentage of marginated neutrophils showed the opposite trends (**Figure 3D**). Interestingly, MRP_14 also increased the total number of marginated neutrophils after IRI (**Figure 3E**). After in vivo staining, confocal microscopy confirmed the following: 1) the absence of interstitial neutrophils after sham surgery regardless of MRP_14 (**Figures 3F and 3G**); and 2) the increased proportion of interstitial neutrophils after MRP_14 in IRI condition compared with IRI alone (**Figures 3H to 3J**). This demonstrated that MRP_14 enhanced the transmigration of neutrophils into the interstitium provided a preliminary IRI.

These results were in line with previous data showing that MRP_8/14 could activate CD18 (β2 integrin), which is part of CD11a/CD18, CD11b/CD18, or CD11c/CD18,[14] all involved in neutrophil adhesion and transmigration. However, the effect of MRP_14 on CD18 activation status was not studied because of the lack of available antibody in mice.[14] After IRI, MRP_14 did not increase the expression of adhesion molecules either on interstitial neutrophils (ie, CD18, CD11a, CD11b, CD11c, or CD44) (Supplemental Figures S5A to S5E) or on endothelial cells (ie, ICAM_1 and VCAM_1) (Supplemental Figures S5F to S5G) compared with IRI alone.

EFFECT OF MRP_14 ON THE FUNCTIONS OF NEUTROPHILS. To explain the increased renal damage after MRP_14 stimulation, we first investigated its effect on neutrophil functions, including reactive oxygen species, myeloperoxidase (MPO), and tumor necrosis factor (TNF)-α productions (ie, all involved in renal damage during IRI[15,16]). Following IRI, the production of reactive oxygen species by interstitial neutrophils was not altered by MRP_14 compared with IRI alone (**Figure 4A**). Compared with sham surgery, IRI increased MPO and TNF-α activities measured in kidney lysate. Following IRI, MRP_14 increased TNF-α

FIGURE 2 **Effect of MRP_14 on Leukocytes Influx After Kidney Ischemia-Reperfusion**



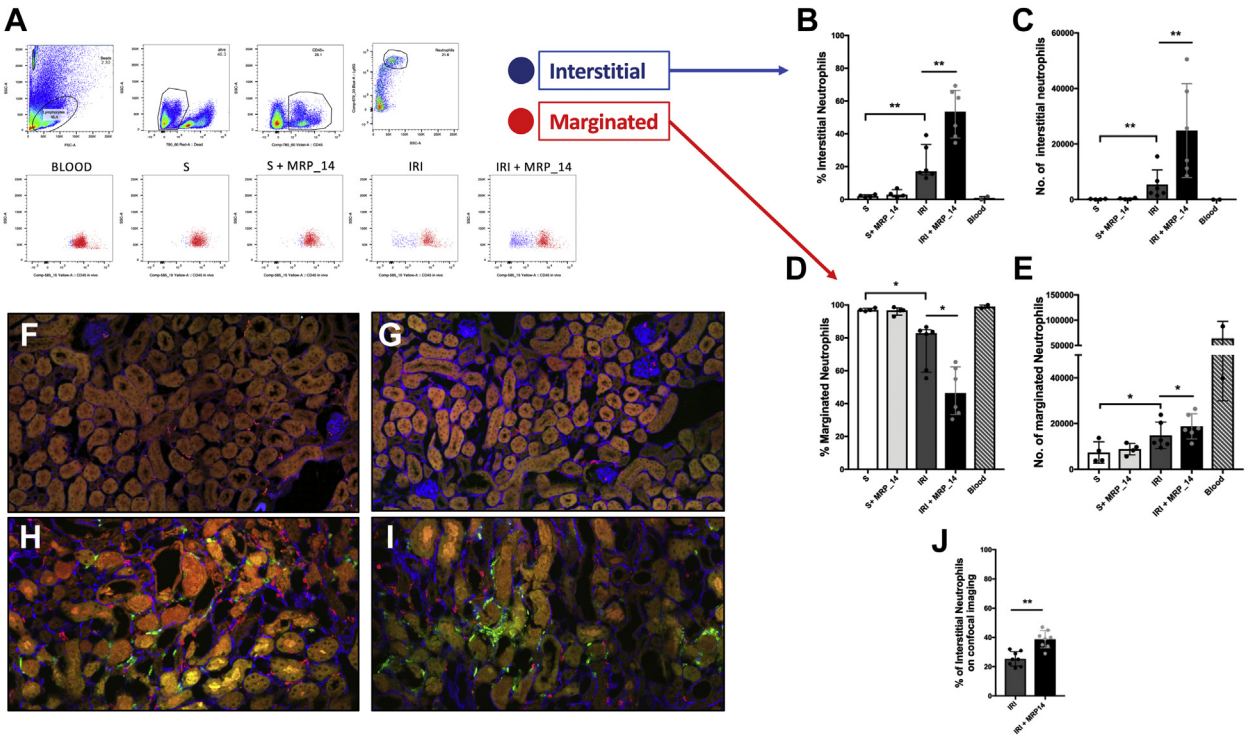
The kidney single-cell suspension was analyzed by flow cytometry in the 4 groups: S = sham surgery; S + MRP_14 = sham surgery with MRP_14 treatment; IRI = ischemia-reperfusion; IRI + MRP_14 = ischemia-reperfusion with MRP_14 treatment. **(A)** Total number of leukocytes was determined by $CD45^{pos}$ gating. **(B)** In the leukocyte gate, CD3, NK1.1, Ly6G, CD11c, and MHC class II allowed to determine the percentage of neutrophils ($Ly6G^{pos}$, **C**), NK cells ($CD3^{neg}/NK1.1^{pos}$, **D**) and T cells ($CD3^{pos}/NK1.1^{neg}$, **E**). In non-T, non-NK cell population, dendritic cells (DC) were defined as $CD11c^{pos}$/MHC Class II^{pos} cells **(F)** (see Supplemental Figure S4, **F** for gating strategy). *$P < 0.050$; **$P < 0.010$; NS = nonsignificant difference; NK = natural killer. Data are shown as median with 25th and 75th percentiles of 2 distinct experiments for a total of 4 S, 4 S + MRP_14, 6 IRI, and 6 IRI + MRP_14.

but reduced MPO activity compared with IRI alone (**Figures 4B and 4C**).

Aside from adhesion and transmigration, neutrophils can kill targets during cell-to-cell contact by trogocytosis (ie, removal of membrane fragments leading to the loss of integrity and death of the target cell).[17] This function depends on CD18, which is part of the complement-receptor 3 (CR3, CD11b/CD18). We reasoned that once they reach the interstitium of the kidney, neutrophils could therefore damage tubular cells. This hypothesis was strengthened by the ability of tubular cells to synthetize and to present the complement-component 3.[18] Tubular cells from Ubi-Tomato mice[19] were cocultured with sorted neutrophils (Supplemental Figures S6A and S6B for purity check). Trogocytosis activity was determined by the rate of neutrophils presenting fluorescent positivity for tdTomato protein ($tdTomato^{pos}$) after 12-hour coculture (Gating strategy Supplemental Figure S6C).[10] MRP_14 significantly increased neutrophilic-trogocytosis compared with unstimulated condition. Compared with isotype control, anti-CD18 blocking mAb (αCD18) reduced trogocytosis, including in MRP_14 stimulated condition (**Figures 4D and 4E**). This result suggested that the increase of trogocytosis after MRP_14 stimulation was CD18-dependent. Confocal microscopy after 12-hour coculture confirmed that neutrophils could acquire tdTomato membrane fragments (**Figure 4F**) as well as confocal live imaging recorded during the first 4 hours of coculture between sorted LysM-eGFP neutrophils and tdTomato tubular cells (Video 1).

PRBC CHARACTERISTICS AND MRP_14 CONCENTRATION. Our data suggested that MRP_14 could increase renal damage in patients undergoing CPB. The identification of PRBCs with a high concentration of MRP_14 could therefore prove beneficial to improving the safety of transfusion. MRP_14 concentration was

FIGURE 3 Effect of MRP_14 on Neutrophil Compartmentalization After Kidney Ischemia-Reperfusion



(A) Representative density plot illustrating neutrophil gate, **(B and C)** interstitial, and **(D and E)** marginated neutrophils (see the Methods section for gating strategy). Data are shown as median with 25th and 75th percentiles of 2 experiments: 4 S, 4 S + MRP_14, 6 IRI, 6 IRI + MRP_14. **(F to I)** Kidney confocal microscopy after anti-CD45 **(red)** in vivo staining followed by anti-Ly6G **(green),** and anti-CD31 **(blue)** staining of the cryosections in the 4 groups: S **(F)**, S + MRP_14 **(G),** IRI **(H),** IRI + MRP_14 **(I)**. Marginated **(green and red)** vs interstitial **(green only)** neutrophils were quantified manually as median with 25th and 75th percentiles **(J)** in 4 kidneys (2 IRI and 2 IRI + MRP_14) on 4 slides for each kidney. *$P < 0.050$; **$P < 0.010$. Abbreviations as in Figure 2.

analyzed according to the gender, ABO group, and Rh of the donor as well as past pregnancy, duration of storage at the blood bank (ie, time between blood donation and transfusion), and preparation methods. PRBCs from female donors showed higher mean concentration of MRP_14 than those of the male donors: 131 ± 67 pg/mL vs 86 ± 52 pg/mL; $P < 0.0001$ (**Table 3,** Supplemental Figure S7A). Prior pregnancy, ABO group, or Rh of the donor did not alter MRP_14 concentration. Interestingly, regarding the preparation methods of the PRBC, whole blood filtration led to a significantly higher mean concentration of MRP_14 compared with buffy coat removal ($P < 0.0001$) (Supplemental Figure S7B).

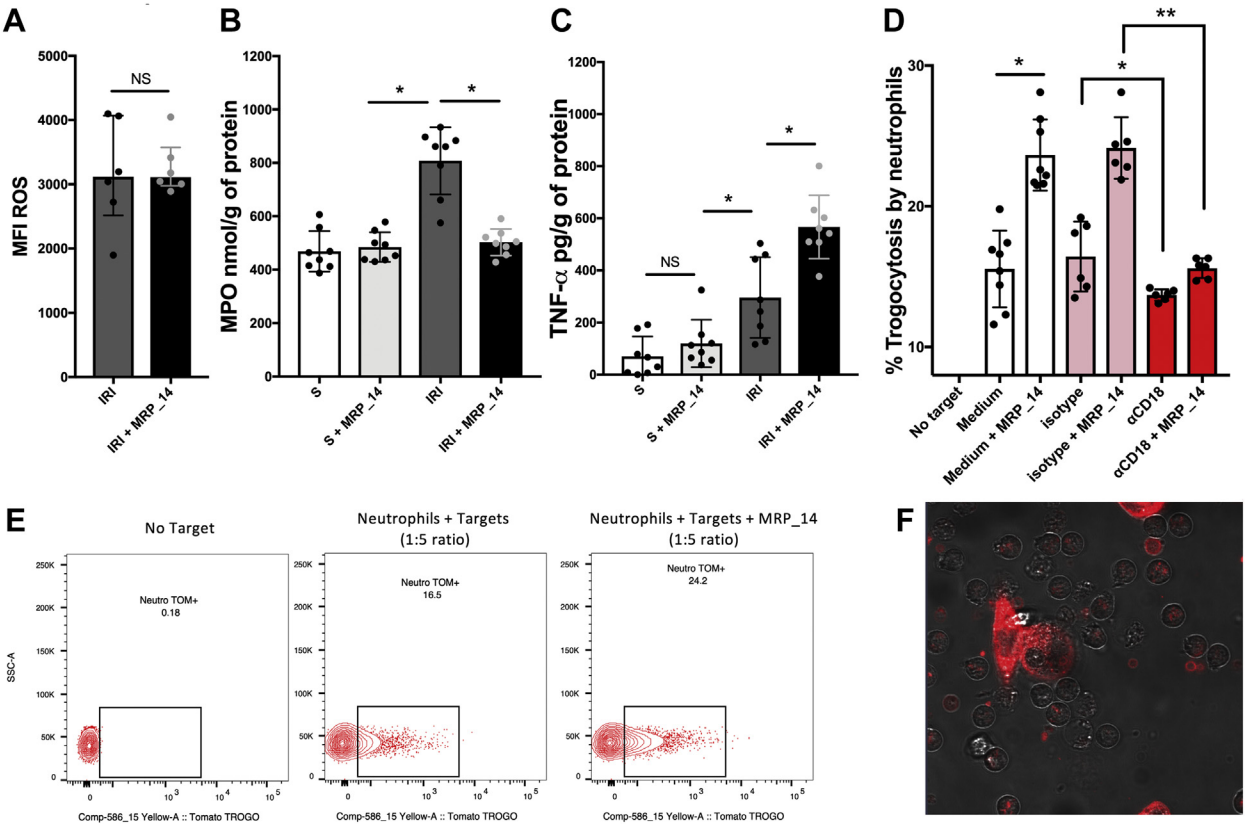
Although MRP_14 is often described as a heterodimer (MRP_8/14), MRP_14 elicits distinct functions from MRP_8.[20] In PRBC, there was a poor correlation between MRP_8 and MRP_14 concentrations (Supplemental Figure S7C), which suggested that homodimers or monomers of MRP_14 were the prevailing forms. Finally, there was also poor correlation between MRP_14 concentrations and the storage duration (Supplemental Figure S7D).

DISCUSSION

In this prospective single-center study, transfusion of PRBC with a high level of MRP_14 was associated with the onset of AKI after CPB. Experimental data suggested that MRP_14 could lead to renal damage by the following: 1) enhancing the transmigration of neutrophils into the kidney interstitium after IRI; and 2) increasing their ability to damage tubular cells by CD18-dependent trogocytosis. Interestingly, PRBCs from female donors or prepared by whole blood filtration method showed higher concentrations of MRP_14 than PRBCs from male donors or prepared by buffy coat removal, respectively.

These results suggested that the onset of transfusion-related AKI after cardiac surgery requires subsequently: an initiation phase of renal damage by CPB-related ischemia reperfusion[4]; and the exposure

FIGURE 4 Effect of MRP_14 on Neutrophil Functions



(A) Reactive oxygen species (ROS) production by interstitial neutrophils with (IRI + MRP_14) or without (IRI) MRP_14 after ischemia. **(B)** Myeloperoxidase (MPO) and **(C)** tumor necrosis factor (TNF_α) activities in the left kidney lysate 48 hours after surgery. Data are shown as median with 25th and 75th percentiles of 2 experiments, (6 **[A]** and 8 mice **[B and C]** per group). Trogocytosis assay **(D)** over 12 hours with or without MRP_14 or anti-CD18 blocking mAb (αCD18), and **(E)** corresponding contour plots illustrating tdTomato^{pos} neutrophils. Data are shown as median with 25th and 75th percentiles of 2 experiments: 8 mice per group for **white bars**, 6 mice per group for **pink bars** (isotype control) and **red bars** (αCD18); **(F)** confocal microscopy after 12-hour trogocytosis assay: tdTomato tubular cells (red channel) and neutrophils (transmitted light). *$P < 0.050$; **$P < 0.010$. Medium: vol/vol RPMI/HBSS. Abbreviations as in Figure 2.

to high concentration of inflammatory molecules (ie, MRP_14) leading to protracted renal inflammation. Storage-related hemolysis was reported to increase the formation of hemoglobin-laden microvesicles and the level of free hemoglobin in PRBCs, leading to renal inflammation and tubular toxicity, respectively.[21] Nevertheless, the storage duration of PRBCs was found to have no impact on patient outcome in randomized trials.[2] In the same line, the effect of transfusion with blood from ever-pregnant women and sex mismatch between donor and recipient are uncertain.[22,23] Although all participants received transfusion, only 15% developed AKI. This strengthens the idea that although the amount of transfusion may be a risk factor of AKI,[24] unexplored parameters of blood products including the inflammatory content of PRBC may affect patient outcome as well.

After cardiac surgery, the association between patient blood level of MRP_8/14 and the risk of AKI has already been reported.[25] MRP_8/14 is a danger-associated molecular pattern, agonist of the toll like receptor_4.[26] Neutrophils are among the main producers of MRP_8/14 and one of the first immune subset to be recruited in the kidney after ischemia-reperfusion.[27] The role of MRP_14/CD18 interaction was important to address for the understanding of AKI after cardiac surgery considering the following: 1) the expression of CD18 on circulating neutrophils increases after CPB[28]; 2) MRP_14 has been reported to activate CD18 which enhances neutrophil transmigration; and 3) blocking CD18 could prevent renal

TABLE 3 MRP_14 Concentration According to PRBC Characteristics

Characteristics of the 184 Donors	MRP_14 Concentration pg/mL, mean ± SD	*P* Value
Gender		**<0.001**
Male (n = 91)	86 ± 52	
Female (n = 93)	131 ± 67	
Blood group		0.25
A (n = 88)	106 ± 58	
B (n = 16)	79 ± 50	
AB (n = 5)	117 ± 58	
O (n = 75)	115 ± 72	
Rhesus D (Rh1)		0.42
Negative (n = 35)	117 ± 91	
Positive (n = 149)	107 ± 57	
Prior pregnancy		0.49
No (n = 28/93)	124 ± 38	
Yes (n = 65/93)	134 ± 76	
Preparation methods		**<0.001**
Whole blood filtration (n = 110)	125 ± 64	
Buffy coat removal (n = 74)	64 ± 64	

This table presents the concentration of MRP_14 in the supernatant of PRBC according to the characteristics of the donor and the preparation methods. In the "whole blood filtration" group (also called "top and top" method), the unseparated components (ie, whole blood of the donor) undergo in-line leucocyte filtration followed by centrifugation to separate plasma and red blood cells. In the "buffy coat removal" group (also called "top and bottom" method), the whole blood undergoes centrifugation to separate red blood cells, platelets, and plasma. Red blood cells undergo filtration afterwards.

damage during IRI.[29] MRP_14 has been supposed to originate from platelet fragments remaining in PRBC.[30] Accordingly, PRBC prepared by whole blood filtration, which were described to contain more platelet-derived extracellular vesicles, showed higher concentration of MRP_14 than their counterpart.[31]

Postoperative AKI is a patient-centered outcome considering that an increase of serum creatinine by 50% is associated with a 2- to 4-fold increase of mortality.[32] Patients in the AKI group consistently had longer duration of mechanical ventilation and longer hospital stay (See Supplemental Table S5 for postoperative outcomes according to MRP_14 exposure). Aside from possible renal effects, MRP_14 was reported to promote tumor growth and increase amyloid burden in Alzheimer's disease.[33]

STUDY LIMITATIONS. Establishing a causality link between 1 single parameter (ie, transfusion) and the onset of AKI after cardiac surgery is probably illusive because of multiple interconnected events. Nevertheless, multivariable analysis suggested that MRP_14 exposure was independently associated with the occurrence of postoperative AKI regardless of confounding factors such as baseline blood creatinine or the duration of CPB (see Supplemental Table S4). Whether these results can be generalized is questionable given the small number of patients with AKI as well as the high number of exclusion criteria (ie, inclusion of <5% of the patients). The selection of a short list of proteins for multiplex immunoassay was guided by the availability of commercial detection kits and could have therefore biased the analyses. Although the association between the blood level of MRP_14 and the onset of AKI after cardiac surgery has already been reported,[25] this data would have been interesting to confirm. Nevertheless, given the significant heterogeneity regarding the surgery duration and the timing of transfusion during and/or up to 6 hours after surgery, comparability for the MRP_14 level would have been uncertain based on a single blood sample. Moreover, other parameters were reported to alter the blood level of MRP_14 (ie, antiplatelet therapy or fluid loading during resuscitation).[34] As a translational model, the unilateral occlusion of the renal artery could have overemphasized the kidney injury compared with ischemia-reperfusion during CPB. However, it helped uncover complex biological effects that a model of CPB could have minimized. TNF_α and MPO levels, which are an incomplete view of the degranulation of neutrophils, were analyzed in whole kidney lysates, and thus only reflect the effects of MRP_14 on kidney inflammation. Finally, the causality link between neutrophilic-trogocytosis and kidney damage was not established.

CONCLUSIONS

The transfusion of a high amount of MRP_14 in cardiac surgery patients was associated with the onset of AKI in the first 48 hours postoperatively. In vitro, MRP_14 increased neutrophilic-trogocytosis toward tubular cells. After experimental IRI, MRP_14 increased neutrophil influx into the kidney interstitium as well as the magnitude of renal damage. PRBC from female donors or prepared by whole blood filtration showed the highest concentration of MRP_14. Altogether, these results advocate for better characterization of the determinants of PRBCs composition and development of new strategies to modulate the immune effects of transfusion.

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PERSPECTIVES

COMPETENCY IN MEDICAL KNOWLEDGE: The risk of transfusion-related AKI after cardiopulmonary bypass could depend on the inflammatory contents of packed red blood cells received during surgery.

TRANSLATIONAL OUTLOOK: The characterization and the close monitoring of the composition of blood products may prove beneficial to reducing transfusion-related organ dysfunction and improving the safety of transfusion.

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KEY WORDS acute kidney injury, cardiac surgery, neutrophils, packed red blood cells, transfusion, transfusion safety, trogocytosis

APPENDIX For an expanded Methods section as well as supplemental figures, tables, and a video, please see the online version of this paper.