
**DEVELOPING NANOFIBROUS SHEETS TO SCAVENGE STORAGE
LESION TO ENHANCE THE QUALITY AND SHELF-LIFE OF STORED
RED BLOOD CELLS AND PREVENT TRANSFUSION-RELATED
COMPLICATIONS**

**A THESIS TO BE SUBMITTED TO
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DOCTOR OF PHILOSOPHY**

BY

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DECLARATION BY THE CANDIDATE

I declare that this thesis entitled "Developing nanofibrous sheets to scavenge storage lesion to enhance the quality and shelf-life of stored Red Blood Cells and prevent transfusion-related complications" submitted for the award of Doctor of Philosophy to THE UNIVERSITY OF TRANS-DISCIPLINARY HEALTH SCIENCES AND TECHNOLOGY, Bengaluru, is my original work, conducted under the supervision of my guide Dr. Praveen Kumar Vemula. I also wish to inform that no part of the research has been submitted for a degree or examination at any university. References, help and material obtained from other sources have been duly acknowledged

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CERTIFICATE

This is to certify that the work incorporated in this thesis "**Developing nanofibrous sheets to scavenge storage lesion to enhance the quality and shelf-life of stored Red Blood Cells and prevent transfusion-related complications**" submitted by Subhashini Pandey was carried out under my supervision. No part of this thesis has been submitted for a degree or examination at any university. References, help and material obtained from other sources have been duly acknowledged. I hereby confirm the originality of the work and that there is no plagiarism in any part of the dissertation.

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In The Loving Memory Of

Shakti Singh Goap

(1992-2021)

Dedications

To my Dearest Mom and Dad

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Table of Contents

LIST OF TABLES	xii
LIST OF FIGURES	xiii
SYNOPSIS	xv
1. An Overview of Blood Storage Lesions and Current Technologies to Improve Blood Quality.....	1
1.1 Role of DAMPs.....	3
1.1.1 NETs (Neutrophil Extracellular Traps)	4
1.1.2 Cell-Free Hemoglobin (fHb).....	5
1.1.3 Microparticles.....	6
1.1.4 Bioactive Lipids.....	7
1.2 Current technologies to improve the quality and shelf-life of RBCs	8
1.2.1 Cryopreservation.....	8
1.2.2 Alternative additives or rejuvenation solutions.....	9
1.2.3 RBC washing.....	10
1.2.4 Nitric Oxide treatment.....	10
1.6 Anaerobic / Hypoxic Storage.....	10
1.6.1 Use of Hemopexin and Haptoglobin.....	11
1.6.2 Leuko-reduction.....	11
1.3 Objective and scope of the thesis	12
1.4 References.....	13
2. Intermittent scavenging of storage lesions from stored red blood cells by Electrospun nanofibrous sheets enhance their quality and shelf-life.....	23
2.1 Introduction	23
2.2 Materials and Methods	26
2.2.1 Synthesis of anionic and cationic scaffolds	27

2.2.2 Fabrication of electrospun nanofibrous sheets.....	29
2.2.3 Quantitation of mitochondrial membrane potential	29
2.2.4 Contact angle measurement.....	30
2.2.5 Hemolysis assay.....	30
2.2.6 Polymer leaching test.....	30
2.2.7 Packing of human RBC.....	31
2.2.8 Scavenging of DAMPs from stored human RBCs using Tau-NFS and Acr-NFS, in vitro.....	31
2.2.9 Quantification of free Hb using Cyanomethaemoglobin (Drabkin's) method	31
2.2.10 Quantification of free DNA by PicoGreen assay.....	31
2.2.11 Quantification of nucleosomes.....	31
2.2.12 Osmotic fragility test.....	32
2.2.13 Primary and secondary fixation of RBCs Primary and secondary fixation of RBCs for SEM.....	33
2.2.14 Bioactive lipid estimation by LCMS.....	33
2.2.15 In vivo experimental design.....	35
2.2.16 Packaging and storage of mice RBCs and their treatment with Tau-AcrNFS to scavenge DAMPs.....	35
2.2.17 Quantification of PS on RBCs by Annexin V (AV)	36
2.2.18 Quantification of pro-inflammatory cytokine levels in plasma.....	36
2.2.19 Transfusion of RBCs.....	36
2.2.20 Quantification of total iron in the spleen.....	38
2.2.22 Statistical analysis.....	38
2.3 Results	39
2.3.1 Charged electrospun nanofibrous sheets efficiently scavenge DAMPs.....	39
2.3.2 Intermittent scavenging of DAMPs by Tau-AcrNFS significantly	

enhances the quality of stored old RBCs.....	43
2.3.3 Transfusion quality of Tau-AcrNFS treated stored old RBCs is comparable to freshly collected RBCs.....	47
2.3.4 Intermittent scavenging of storage lesion with Tau-AcrNFS enhances the shelf- life of stored old RBCs	49
2.4 Discussion	53
2.5 Conclusion.....	54
2.6 Reference.....	55
3. Improving the quality of leukoreduced RBCs by scavenging erythrocytes-associated storage lesions by electrospun nanofibrous sheets.....	59
3.1 Introduction	59
3.2 Materials and Methods	61
3.2.1 Scavenging of DAMPs from stored human RBCs using Tau-NFS and Acr-NFS, in vitro.....	62
3.2.2 Quantification of free Hb using Cyanomethaemoglobin (Drabkin's) method.....	62
3.2.3 free DNA by PicoGreen assay.....	63
3.2.4 Quantification of nucleosomes.....	63
3.2.5 Osmotic fragility test.....	63
3.2.6 Bioactive lipid estimation by LCMS.....	63
3.2.7 In vivo experimental design.....	65
3.2.8 Packaging and storage of mice LR RBCs and their treatment with Tau-AcrNFS to scavenge DAMPs.....	65
3.2.9 Transfusion of RBCs.....	66
3.2.10 Statistical analysis.....	67
3.3 Results	68
3.3.1 Significant production of RBCs associated lesions in LR	

units during storage and the role of charged Tau-AcrNFS in scavenging them.....	68
3.3.2 Intermittent scavenging of RBCs associated DAMPs in stored LR units by Tau-AcrNFS significantly enhances their quality.....	71
3.3.3 Intermittent scavenging of storage lesions with Tau-AcrNFS increases the circulation time of transfused LR RBCs in mice.....	74
3.4 Discussion.....	77
3.5 Conclusion.....	78
3.6 Reference.....	79
4. Removal of DAMPs from stored RBCs units using nanofibre scaffolds to prevent transfusion related acute lung injury (TRALI)	84
4.1 Introduction.....	84
4.2 Materials and Methods.....	87
4.2.1 Experimental Mice.....	87
4.2.2 Packaging and Storage of Mice RBCs and their treatment with Tau-AcrNFS scavenge DAMPs.....	87
4.2.3 The non-antibody mediated two-event TRALI in vivo mode.....	88
4.2.4 Vasculature permeability through EBD (Evans Blue Dye) assay.....	89
4.2.5 Lung tissue histology and Scoring.....	89
4.2.6 MPO (Myeloperoxidase) assay.....	89
4.2.7 Bronchoalveolar lavage fluid (BALF) collection.....	89
4.2.8 Differential counting of Immune cells in BALF.....	90
4.2.9 Protein estimation in lung BALF.....	90
4.2.10 BALF DNA measurements.....	90
4.2.11 Cytokine Measurements in Plasma and BALF: Mice.....	91
4.2.12 Statistical analysis.....	91

4.3 Result.....	92
4.3.1 Intermittent treatment with Tau-AcrNFS on 5th and 10th day enhances quality and shelf life of stored old mouse RBCs.	92
4.3.2 Intermittent cleaning of stored mouse blood on 5th and 10th day with Tau-AcrNFS maintains vasculature integrity and prevent TRALI condition.....	95
4.3.3 Age of stored blood for transfusion determines the severity of TRALI	97
4.3.4 Intermittent treatments with scaffolds reduce circulating NET biomarkers and immune cells.....	100
4.3.5 Removal of DAMPs from stored blood units reduces the expression of inflammatory cytokines	103
4.4 Discussion.....	105
4.5 Conclusion.....	108
4.6 Reference.....	109
Conclusions &Future Directions.....	113
APPENDIX A: Permission for re-use of figures	114
APPENDIX B:Patent granted	115
APPENDIX C:Front Page of first Paper	116
APPENDIX D:Front Page of second Ppaer.....	117

List of Tables

Table 2.1. The ion Q1/Q3 transitions and other parameters to quantify PUFAs.....	35
Table 3.1 Histological analysis of stored blood transfusion with/without treatment induced ALI in LPS mice displayed different parameters.....	98

List of Figures

Figure 1.1 Illustration depicting the effects of storage lesion.....	2
Figure 1.2 Illustration depicting RBCs undergo hemolysis.....	5
Figure 2.1 Schematic representation of approach to scavenge Charged DAMPs.....	25
Figure 2.2 Scheme for the synthesis of charged polymers.....	27
Figure 2.3 Hemocompatible charged nanofibrous sheets scavenge storage lesions.....	40
Figure 2.4 Scavenging of DAMPs using charged nanofibrous sheets with varying surface area.....	41
Figure 2.5 Storage-induced production of DAMPs as a function of time.....	42
Figure 2.6. Intermittent treatment of stored RBCs with Tau-AcrNFS significantly reduced the accumulation of DNA.....	44
Figure 2.7. Intermittent removal of DAMPs reduced the damage of RBCs and enhanced the membrane integrity of stored old RBCs.....	46
Figure 2.8 Intermittent treatment has increased the quality of stored old RBCs equivalent to fresh RBCs.....	48
Figure 2. 9. During the storage, exposure of phosphatidylserine (PS) on the outer leaflet of the RBC membrane.....	50
Figure 2.10. Intermittent removal of DAMPs has decreased RBC damage, and enhanced the shelf-life of RBCs.....	52

Figure 3.1. Schematic representation of storage lesions presents in LR vs NLR blood units.....	60
Figure 3.2. Storage-induced production of DAMPs as a function of time in LR units.....	68
Figure 3.3 Scavenging of storage lesions by scaffolds in LR units.....	70
Figure 3.4. Intermittent treatment of stored RBCs with Tau-AcrNFS reduced the accumulation of storage lesions.....	72
Figure 3.5. Intermittent removal of DAMPs reduced the damage of RBCs and enhanced the membrane integrity of stored old RBCs.....	73
Figure 3.6. Intermittent scavenging suggesting increased quality of stored old RBCs after leukoreduction.....	75
Figure 3.7. Intermittent removal of DAMPs has decreased RBC damage, and enhanced quality of RBCs.....	76
Figure 4.1 The schematic representation of Two hit non-antibody mediated TRALI model.....	86
Figure 4.2. Schematics of mouse blood treatment with Tau-AcrNFS.....	88
Figure 4.3. Hypothermal storage of mouse RBCs induced DAMPs production.....	92
Figure 4.4. Incubation of DAMPs scavenged and reduced the concentration of DAMPs significantly.....	93
Figure 4.5 Representation of whole-body perfusion performed in after administering EBD to evaluate vasculature permeability.....	95
Figure 4.6 Histological evidence of lung morphology with and without scaffold treatment in TRALI mouse model.....	98

Figure 4.7 MPO activity in the plasma and BALF of mice.....	100
Figure4.8 Intermittent treatments with scaffolds decreases circulating NET and protein permeability in BALF.....	101
Figure4.9 Intermittent treatments with scaffolds reduce immune cells in BALF.....	102
Figure4.10 Reduction in acute phase inflammatory response in mice plasma after treatment.....	103
Figure4.11 Removal of DAMPs from stored blood units significantly reduces the expression of Inflammatory cytokines in BALF and plasma.....	104

List of Acronyms

RBCs	Red Blood Cells
FDA	Food and Drug Administration
SAGM	Saline, Adenine, Glucose, and Mannitol
NACO	National Aids Control Organization
DAMPs	Damage Associated Molecular Patterns
NETs	Neutrophils extracellular traps
HETE	Hydroxyeicostetranoic acids
ROS	Reactive oxygen species
TRALI	Transfusion-related acute lung injury
PUFAs	Polyunsaturated fatty acids
LR	Leuko-reduced
NLR	Non-Leuko-Reduced Blood
WBCs	White Blood Cells
MPO	Myeloperoxidase
CfDNA	Cell-free DNA
MtDNA	Mitochondrial DNA
IL-8	Interleukin-8

NO	Nitric Oxide
MPs	Microparticles
Hp	Haptoglobin
EVs	Extracellular-vesicles
TG	Thrombin generation
PS	Phosphotidyl serine
PLTs	Platelets
BRMs	Biologic response modifiers
Lyso-PCs	Lysophosphatidylcholine
ICU	Intensive care unit
Hb	Free-hemoglobin
pRBCs	Packed RBCs
NFS	Nanofibrous Scaffolds
APTMS	3-(Aminopropyl)trimethoxy silane)
THF	Tetrahydrofuran
RH	Relative humidity
CPD-A	Citrate-phosphate-dextrose with adenine
CXP	Cell exit potential
EP	Entrance potential
MRM	Multiple reaction monitoring

IAEC	Institutional Animal Ethics Committee
AV	Annexin V
Ar	Argon
In	Indium
PMVEMA	Poly (methyl vinyl ether-alt-maleic acid)
AA	Arachidonic acid
MCF	Mean cell fragility
RCSL	Red cell storage lesions
LOX	Lipoxygenase
CYPs	Cytochrome P450s
OFT	Osmotic fragility test
HLA	Human leukocyte antigens
HNA	Human neutrophil antigens
EBD	Evans Blue Dye
H&E	Hematoxylin and eosin
TE	Tris-EDTA
LPS	Lipopolysaccharides
ET	End Day Treatment
IT	Intermittent Treatment
AGE	Advanced glycation end products

MIP-2	Macrophage inflammatory protein
VEGF	Vascular endothelial growth factor
TNF	Tumour necrosis factor
BALF	Bronchoalveolar lavage fluid)
ELISA	Enzyme-linked immunosorbent assay
IAEC	Institutional Animal Ethics Committee
PMN	Polymorphonuclear neutrophils

Synopsis

Developing nanofibrous sheets to scavenge storage lesion to enhance the quality and shelf-life of stored red blood cells and prevent transfusion-related complications

Blood transfusion is a pivotal practice performed frequently in critically ill patients with anaemia (7-8g/dl hemoglobin)¹. According to the data published by the National Aids Control Organization (NACO) in 2017, 6.57 lakh liters of blood (roughly 53 water tankers) were discarded in the last five years. One of the main reasons that can be cited for dumping this enormous volume of collected blood was the deterioration of blood quality during the storage time of 35-42 day². Previous investigations in cardiac research have reported an increase in severe complications and mortality when RBCs stored for > 2 weeks were used for transfusion³. These increased complications are attributed to the accumulation of a wide range of storage lesions, also known as Damage Associated Molecular Patterns (DAMPs) in stored blood over time, limiting the shelf life of stored RBCs. Transfusion of old stored whole blood consists of a pool of stored lesions that are primary mediators in the pathophysiology of fatal diseases like Transfusion-Related Acute Lung Injury (TRALI), deep vein thrombosis, and organ dysfunction^{4,5}. It has been long established that leucocytes associated with DAMPs in donor blood are potential immune elicitors in recipients. To reduce the accumulation of leucocytes-associated DAMPs like NETs, RBC units are treated with leukoreduction filters prior to storage⁶. However, this technology does not profess to eliminate all DAMPs, especially those contributed by erythrocytes. Past investigations suggest that adding preservatives, storing RBCs in an alkaline hypotonic solution with antioxidants, or rendering anaerobic storage conditions improves storage time up to 10-15 days⁷⁻¹⁰. Still, these efforts do not exclusively focus on removing RBC and WBCs associated lesions and improving blood quality or shelf life. Since no practical approach is listed in the field that claims to remove all classes of storage lesions, there is a need to develop a technology to solve this unmet clinical need. ***Therefore, the aim of the work is to improve the quality of NLR (Non-leukoreduced) & LR (Leuko reduced) stored RBCs and increase their shelf life by scavenging the wide range of DAMPs by using a biomaterial platform. This may also efficiently reduce transfusion-related complications such as TRALI.***

This thesis contains four chapters, and below we have described the contents of each chapter.

Chapter 1: Introduction - An overview of existing strategies and technologies to enhance the quality of stored blood

In Chapter 1, we reviewed all the strategies and technologies that were developed to enhance the quality of stored blood. We have comprehensively reviewed all technologies developed in the field. Furthermore, existing state-of-art technologies, and the potential limitations of those technologies have been discussed in detail.

Chapter 2: Intermittent scavenging of storage lesions from stored red blood cells by electrospun nanofibrous sheets enhances the quality and shelf-life of stored RBCs

Transfusion of healthy RBCs is a lifesaving process. However, upon storing RBCs, a wide range of DAMPs, such as cell-free DNA, nucleosomes, free-hemoglobin, and poly-unsaturated-fatty-acids (PUFAs) are generated. DAMPs can further damage RBCs. Thus, the quality of stored RBCs declines during storage and limits their shelf-life. These DAMPs contain either positive or negatively charged species (**Figure 1a**). Hence, we developed taurine and acridine containing electrospun-nanofibrous sheets (*Tau-AcrNFS*), consisting of anionic and cationic charges on their surfaces.

Here, we show in **Figure 1b** that *Tau-AcrNFS* are efficient in scavenging DAMPs from stored human and mice RBCs, *ex vivo*. Furthermore, intermittent scavenging of DAMPs by *Tau-AcrNFS* during the storage, prevented the loss of RBC membrane integrity and reduced discocytes-to-spherocytosis transformation in stored-old-RBCs. Additionally, RBC-transfusion studies in mice reveal that intermittent removal

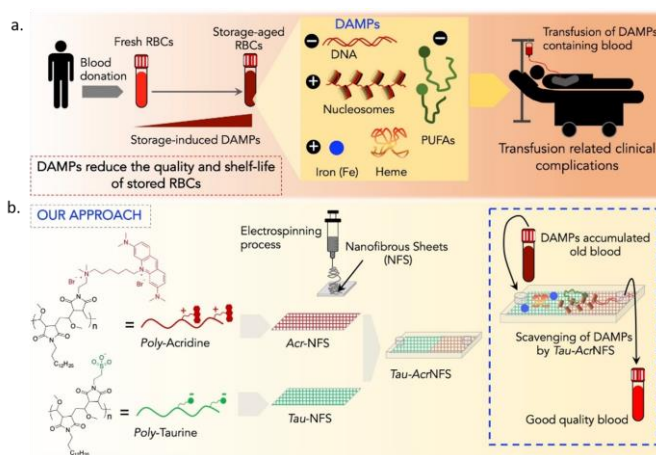


Figure 1: a) Schematic representation of the presence of DAMPs progressively reduces the quality of stored RBCs and limits their shelf-life. All DAMPs contain either positive or negatively charged entities. b) Using polymers, the charge-bearing electrospun nanofibrous sheets (*Tau-NFS* and *Acr-NFS*) were prepared to scavenge charged DAMPs from stored RBCs, which remarkably enhanced the quality and shelf-life of stored RBCs.

of DAMPs enhanced the quality of stored-old-RBCs equivalent to freshly collected RBCs and increased their shelf-life by ~22%.

In summary, our study implicates in situ generated DAMPs in causing accelerated deterioration of RBCs. Furthermore, we established a novel approach to slow down the damage of stored RBCs through intermittent scavenging of the storage lesion.

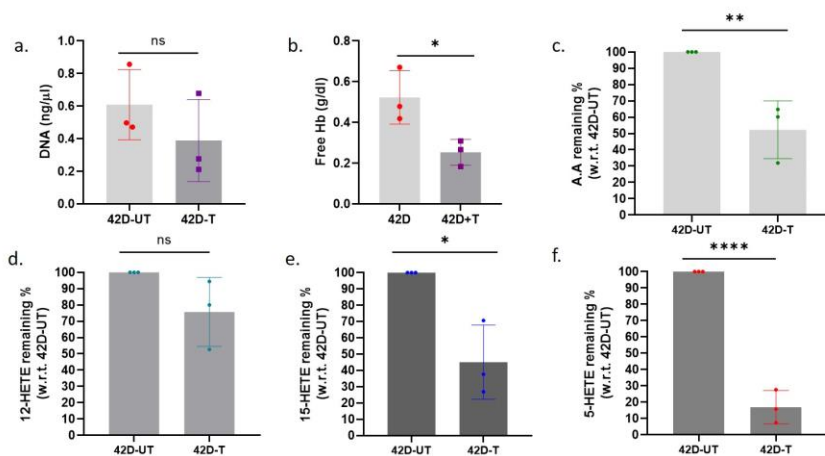


Figure 2: *Tau-AcrNFS significantly eliminates RBCs associated DAMPs:* **a)** There was no significant change in the concentration of free DNA in 42nd day LR unit post treatment since the production of free DNA was relatively low in 42nd Day LR units. **b-f)** A remarkable drop of Free Hemoglobin (Hb), Arachidonic acid (AA), 5 & 15-HETE value after scaffolds treatment provides a method to purify LR blood on 42nd day that contains soluble bioactive lipids generated from RBCs.

Chapter 3: Improving the quality of leukoreduced RBCs by scavenging erythrocytes-associated storage lesions by electrospun nanofibrous sheets

Our study was not restricted to the NLR(Non-leukoreduced) RBCs unit. The kinetic study of DAMPs production in Leukoreduced (LR) units was also investigated. Although the Leucocytes-associated DAMPs production rate was not significant as NLR units, the release of free hemoglobin and bioactive lipids raised our concern.

Similar to intermittent treatment of NLR blood, LR blood was also intermittently treated (21st /28th day) and DAMPs production were quantified on 42nd day. The production of extracellular DNA in LR blood units was low, as expected by the end of 42nd Day due to the removal of leukocytes (**Figure 2a**). A critical trend has been witnessed in free hemoglobin (Hb) production, which is increased up to 10 folds giving an idea that leukofilters had no control over the accumulation of

RBC-associated DAMPs. Thus, cleaning LR units with *Tau-AcrNFS* before transfusion could significantly reduce the formation of RBCs associated lesions. The data suggest that free Hb was significantly reduced to 50% post-treatment (**Figure 2b**). Additionally, significant production of AA and its metabolites was observed, however, scaffold treatment reduced its concentration up to 40-50% (**Figure 2c-f**). Herein, the concept of intermittent treatment worked quite efficiently where the free Hb and bioactive lipids concentration was significantly reduced when treated on the 21st/28th day of storage.

In summary, a remarkable drop of erythrocytes associated DAMPs after scaffold treatment provides a novel method to purify and maintain the quality of LR blood even after leukoreduction.

Chapter 4: Selective alleviation of DAMPs from stored blood products using nanofibre scaffolds to rescue transfusion related acute lung injury (TRALI)

Transfusion Related Acute Lung Injury (TRALI) is a rare complication of blood transfusion that occurs during or within 6 hours of transfusion of stored blood component¹¹. Herein, we developed a "Two-Hit non-antibody-mediated TRALI model" using 17th Day stored mice blood after the first hit of LPS (**Figure 3**). The rationale for storing mice blood up to 17days is extrapolated from our previous

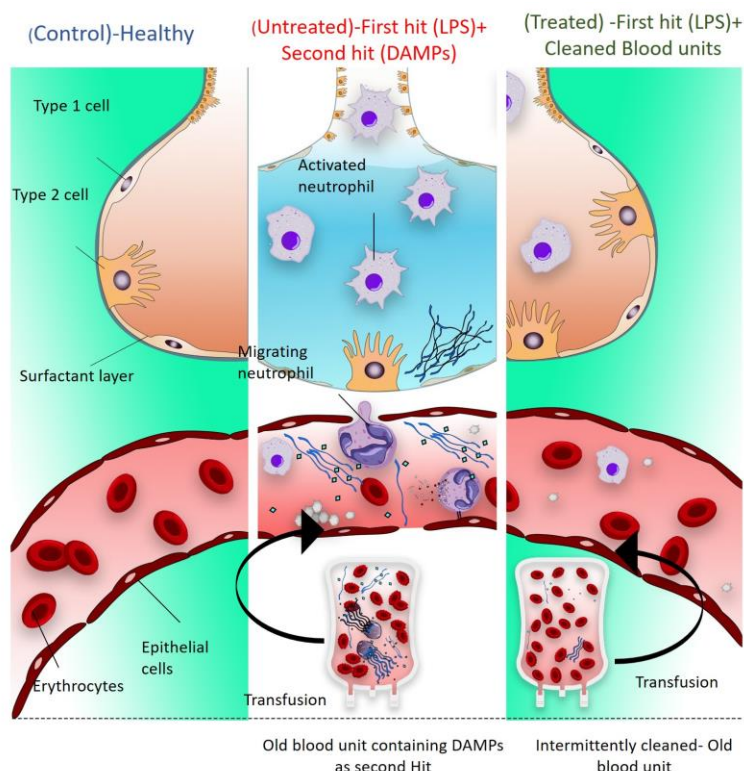


Figure 3: The control group shows the normal condition of lung and blood vessels, The untreated group comprises the two-hit non-antibody-mediated model of transfusion-related acute lung injury (TRALI) with hypothesized pathophysiology. The treated group describes the concept of treatment with *Tau-AcrNFS* improves the quality of 17D old mice blood and when transfused in LPS challenged mice did not lead to TRALI.

study, where we have shown that the twice periodic cleaning of DAMPs with *Tau-AcrNFS* can enhance the shelf life of stored mice RBCs from 14 Days to 17 Days. So, revisiting the same concept and aggravating the incidence of TRALI events, we have transfused 17th Day untreated (17D-UT), 17th Day old blood end day treatment (17D-ET), and intermittently treated 17th Day old blood (17D-IT) in LPS challenged mice.

Our data suggested that transfusing 17D(IT) blood does not stimulate the second event of TRALI.

The concept of removing DAMPs also explores that the stored blood needs to pass through scaffolds before transfusion (bedside purification) to reduce TRALI incidence.

In summary, the model's and treatment validity are confirmed based on evaluating specific clinically relevant endpoints. The periodic removal of DAMPs from old blood and their transfusion shows no histological evidence of lung injury, endothelial barrier permeability, and inflammation in mice compared to untreated TRALI mice.

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List of Publications

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Chapter-1

An Overview of Blood Storage Lesions and Current Technologies to Improve Blood Quality

Blood transfusion is one of the frequent therapeutic practices performed in patients suffering from severe blood loss or anemia due to trauma injury, surgery, or hematological disorder¹. In such a critical condition, transfusion of RBCs is essential to carry oxygen and other nutrients throughout the body, especially to vital organs like the brain. However, if the blood flow falls below a certain threshold, the body's tissues are deprived of oxygen, which can cause organ and tissue damage leading to death. The US-FDA (Food and Drug Administration) regulations currently allow the storage of human RBC units for up to 42 days at 4-6°C in a preservative solution that containing SAGM (Saline, Adenine, Glucose, and Mannitol)². In India, according to NACO (National Aids Control Organization), we require 8.5–9.0 million units of blood annually for transfusion. However, 6.57 lakh liters of blood were discarded in the last five years due to the deterioration of stored blood quality during a storage time of 35–42 days³. Moreover, in conditions like Sickle cell anemia or post-cardiac surgery, the average storage duration for units transfused per therapy is ~two weeks⁴. Similarly, previous investigations in cardiac research have reported a significant increase in severe complications and mortality when RBCs stored for > 2 weeks were used for transfusion⁵.

The deterioration in RBCs during storage is mainly contributed by progressive biochemical and morphological changes, referred to as storage lesions or DAMPs (Damage Associated Molecular Patterns) mention in **Fig1.1**. Residual leukocytes contribute to these DAMPs in RBC units, which are linked to an increased risk of organ dysfunction. During the storage period, bioactive lipids generated by RBCs activate neutrophils that release chromatin fibers to form extracellular traps (NETs), which are associated with histones⁶. As the storage time progresses, there is a change to the RBC cytoskeleton resulting in RBC shape change and increasing cell stiffness. The presence of NETs in stored blood has been reported to induce lung injury when infused into animals⁷⁻⁸. Other than the damage caused by residual leukocytes, there are various other DAMPs are produced in the storage medium that progressively degrades the quality of blood, such as accumulation of pro-inflammatory mediators like soluble

CD40 ligand, lipid breakdown by-products such as arachidonic acid and 5-, 12-, 15-hydroxyeicostetranoic acids (HETE), microparticles shed by RBCs and production of free Heme through hemolysis. Many changes occur due to oxidative stress, leading to reactive oxygen species (ROS) production followed by altered proteins and lipids⁹. These cellular products exponentially increase as the blood units are stored for longer. Thus, the old stored NLR blood unit poses a severe risk of transfusion-related complications such as transfusion-related acute lung injury (TRALI), deep vein thrombosis, and organ failure.

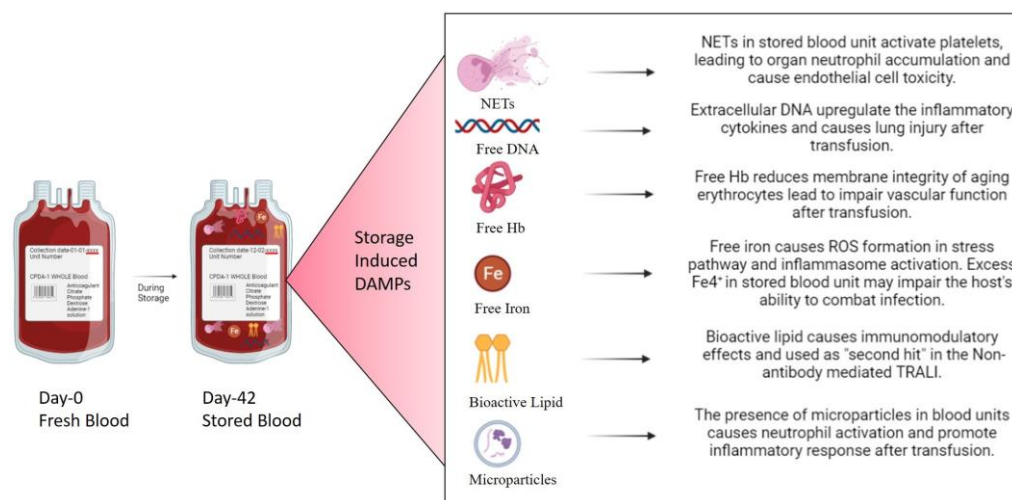


Fig 1.1 Fresh blood undergoes various biochemical changes producing storage lesion or damage-associated molecular patterns (DAMPs) such as DNA, nucleosomes, heme (Hb), iron, and polyunsaturated fatty acids (PUFAs). The following DAMPs are listed that shows various detrimental effect if present in excess and causes transfusion-related complications, including systemic inflammation and organ injury. The presence of DAMPs progressively reduces the quality of stored RBCs and limits their shelf-life (The figure is made by the author, Subhashini Pandey).

These changes are more evident in the cells stored in the storage solution for extended periods, classifying the stored RBCs into young and old blood cells. The young blood cells are between 14 and 21 days, whereas the old blood cells are those stored for longer than 21 days. Thus, the period of 2 weeks could be safe for blood storage and transfusion, without compromising the efficacy of erythrocytes¹⁰. Until now, leukoreduction has been the only technique employed to reduce the concentration of

NETs and histones by filtering out leukocytes from whole blood. Patients with cancer, organ transplant surgery, and immune suppression receive leuko-reduced (LR) blood⁵. Apart from these surgeries, non-Leuko-reduced Blood (NLR) is used for other major surgeries. The LR technique reduces the leukocytes count from 5×10^9 to 5×10^6 per unit, but residual white blood cells (WBCs) in packed blood units are also linked to an increased risk of infection and organ dysfunction. Therefore, among transfusion of blood or blood components, the non-leukoreduced (NLR) blood transfusion is most common in medical practice in India and developing countries in general.

Though the use of leukoreduction filters before transfusion has reduced the formation of leucocyte-associated DAMPs, this technology does not remove the RBC-associated DAMPs where stored blood cannot be used after 42 days of phlebotomy¹¹. Moreover, leukoreduction enhances the cost for each blood unit (~\$100). The estimated cost for implementing universal leukodepletion in the USA is \$600 million⁸. The extra cost is the major hurdle for using universal leukodepletion of blood and blood products in developing countries like India. Thus, there is a need for an alternative technology to the leukoreduction method, which can remove storage lesions (NETs and histones) from old stored NLR blood efficiently. The quality and shelf-life of the stored blood are essential factors that can maximize the effectiveness of transfused blood. There is a greater risk of the patient developing critical complications if low-quality blood is transfused. This introduction chapter describes the role of each DAMPs in deteriorating the quality and shelf life of stored RBCs, the available technologies to prevent blood wastage and transfusion-related complications, and their shortcomings.

1. Role of DAMPs in degrading the quality of blood and causing post transfusion complexities.

1.1.1 NETs (Neutrophil Extracellular Traps): The formation of NETs (NETosis) requires unwinding chromatin, which is induced by reactive oxygen species (ROS), neutrophil elastase, and myeloperoxidase (MPO)¹², followed by histone hypercitrullination by peptidylargininedeiminase¹³. The liberation of NETs from neutrophils after plasma membrane lysis¹⁴ does not require cell death¹⁵. Previous studies have shown that NETs might mediate non-leukoreduced (NLR) blood-induced transfusion reactions. Extracellular DNA in association with neutrophil enzymes can

be found in the plasma of patients with deep vein thrombosis¹⁶ and systemic thrombotic microangiopathies¹⁷, autoimmunity¹⁸, and transfusion-related acute lung injury (TRALI)¹⁹. Since ROS are rapidly cell-permeable, adding exogenous sources of ROS can prevent deficiencies in NADPH oxidase²⁰. It is observed that in the presence of some neutrophil stimuli, ROS formation may not be needed to form NETs²¹. In NLR-stored blood, it was observed that levels of cfDNA (cell-free DNA) and associated histones increase with time²². It is shown that cfDNA triggers coagulation via the contact pathway²³. Histones activate platelets, leading to organ neutrophil accumulation and causing endothelial cell toxicity²⁴. They are released in the form of NETs in the occurrence of microbial or inflammatory stimuli²⁵.

Studies have shown that there is a release of cfDNA from mitochondria as mitochondrial DNA (mtDNA), which is not associated with histones. However, mtDNA has similar platelet-stimulating potential characteristics to nuclear cfDNA but has distinct pro-inflammatory properties²⁶⁻²⁸. In humans, circulating cfDNA levels are highly linked with the increased mortality rate in sepsis condition²⁹ and thrombosis in cancer patients³⁰. Past studies have shown that the storage of both LR and NLR units is associated with the accumulation of free hemoglobin over time, and they are known to be potent inducers of NETs³¹. The studies have also shown the correlation between hemoglobin, cfDNA and citrullinated histone H3. Though the studies have shown the increased hemoglobin level in both LR units and NLR units, there was no significant NETosis in LR blood. Another plausible mechanism of NET generation is its induction by interleukin-8 (IL-8) in stored NLR blood but does not induce in LR blood³². In animal models, various methods such as DNA-digesting enzymes or anti-histone antibodies are employed to improve the survival in an animal model of transfusion-associated lung injury³³. In another case, neutralizing histones with antibodies can rescue mice from lethal sepsis³⁴. Thus, cfDNA released from WBC is potentially harmful after transfusion in a critical clinical scenario. To combat the risk of transfusion-related complexities, pre-storage leukoreduction is currently used to eliminate the risk of acute transfusion reactions. However, it does not eliminate the production of RBCs associated DAMPs such as bioactive lipids (Arachidonic acid and its metabolites)³⁵ and free heme³⁶, which is observed in blood units with the time that limits the quality and shelf life of stored LR blood.

1.1.2 Cell-Free Hemoglobin (fHb): Increasing hemolysis and release of fHb are well studied as a function of time during prolonged RBC storage. Intracellular and extracellular Hb undergoes a continuous autoxidation process³⁷. In stored blood units, Fe^{2+} (Ferrous state) is oxidized to a ferric (Fe^{3+}) state, whereas a higher oxidation ferryl heme (Fe^{4+}) is formed under stress conditions³⁸. Fe^{3+} stays as a non-functional Hb form in such conditions, and Fe^{4+} is highly reactive toward other biological molecules due to the higher oxidation state³⁸. In normal conditions, RBCs keep their reductive machinery effective so that Hb stays functional with little oxidation during their life span. It is

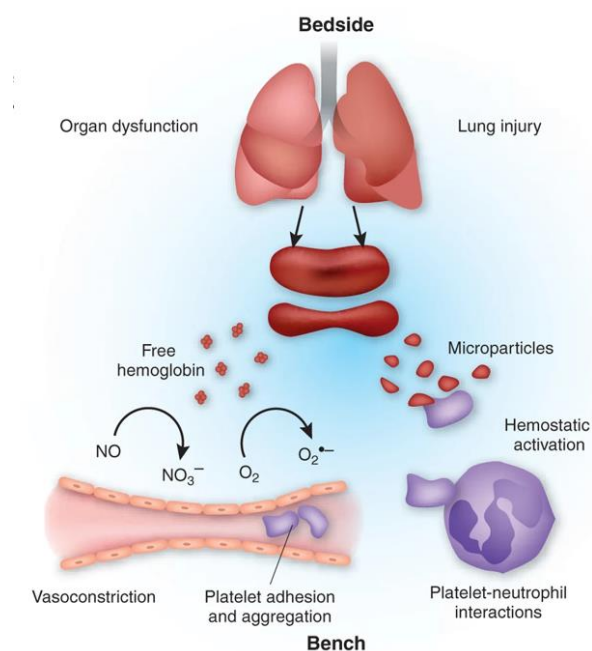


Fig 1.2 RBCs undergo hemolysis and microparticle formation during storage. Free Hb and microparticle hemoglobin scavenge NO, resulting in the loss of vasodilation and generation of ROS. Microparticles with exposed phosphatidylserine may promote haemostatic activation and platelet neutrophil aggregation.

Lee *et al.*, Nature Medicine, 2010, (License No. 5454921293472 to use this image)

reported that redox transformation of Hb occurs to a lesser extent in young RBCs.

However, higher redox transformation in old RBCs has detrimental effects on their membrane integrity during *ex vivo* storage³⁹.

During storage, various time-dependent oxidative challenges such as ROS formation, binding of denatured Hb to membrane phospholipid, and formation of Hb-contained macrovesicles are well studied in the literature⁴⁰.

It is observed that reduced integrity of the aging erythrocyte membrane during

storage leads to impair vascular function after transfusion. It is well-reported that fHb is a potent scavenger of nitric oxide (NO), the most crucial endogenous vasodilator⁴¹. Severe deregulation in the NO system is a significant cause of sepsis-induced microvascular perfusion failure. When stored, blood loaded with fHb and microparticles is transfused, leading to further hemolysis and breakdown of RBCs. Intravascular red cell hemolysis impairs nitric oxide (NO)–redox homeostasis, producing endothelial dysfunction, platelet activation, and vasculopathy⁴² (**Fig1.2**). In stored blood, due to hemolysis, free hemoglobin rich in iron may cause progressive oxidative stress to stored RBCs. Various studies show that Fe³⁺ accelerates Hb-mediated oxidation, which could be inhibited by iron-chelating agents⁴³. In sickle cell diseases, hemolysis and vaso-occlusion are their hallmarks. In sickle cell patients, pain episodes are highly associated with increased plasma hemoglobin⁴⁴. Various studies have been performed in animals to evaluate the effect of Hb toxicity. In one study, guinea pigs were transfused with old stored pig blood (the equivalent of >10 units)⁴⁵. Within 24 hr, post-transfusion, there was an increase in plasma Hb. The observation was further cemented by increased hemoglobinuria, acute renal failure, hypertension, and vascular injury⁴⁶. These observations are supported by several extensive studies on animal models suggesting that old blood transfusion is associated with increased blood pressure, NO depletion, and increased plasma Hb concentrations⁴⁷. To combat the adverse renal and vascular effects after old blood transfusion, the co-infusion of Hp (Haptoglobin) with older storage has been widely performed in animal models⁴⁷.

1.1.3 Microparticles: During the storage of RBCs, the loss of membrane integrity led to microparticles (MPs) formation. However, the detailed clinical impact of the RBCs storage lesion is not investigated. However, the formation of RBCs-MPs is related to reduced post-transfusion survival/efficacy⁴⁸. The transfusion of microparticle-loaded blood units also has a higher risk of adverse reactions in the recipients. The membrane loss of RBCs in the form of MPs is majorly contributed by structural transformation from discocytes to spherocytes. Firstly, as reported by Rumsby and colleagues in 1977, most MPs formed in the supernatant of RBCs units originated from RBCs and contained Hb. It is observed that their production increases with storage time. The same phenomena were observed in pre-storage leukoreduction of whole blood or packed RBCs units⁴⁹. It is shown in studies that blood products comprised a mixed population of EVs (Extracellular-vesicles), including exosomes and microvesicles. Based on the existing

knowledge, EVs in stored blood leads to immunomodulatory events such as neutrophil activation and promote an inflammatory response in the recipients of older blood⁵¹⁻⁵². Thus, the critical role of EVs in deteriorating blood quality, *in vitro* and *in vivo*, suggests further investigations. Previous studies have demonstrated that RBC-MPs intensify tissue factor-initiated thrombin generation (TG) in plasma⁵³. MPs in the supernatant lead to higher PS (Phosphatidyl serine) exposure (PS located in out leaflet of the membrane) in stored RBCs. Indeed, the concentration of PS-positive MPs increases progressively in packed RBCs over time⁵⁴ in old stored blood units⁵⁵ and may alter PLTs(platelets)-leukocytes, PLTs-endothelial cell, and RBCs-leukocytes interactions in transfused patients⁵⁶.

Previous studies showed that MPs collected from old stored mice blood showed neutrophil priming, activation, and hemorrhagic shock when injected into a new set of mice⁵⁷. In the other case, thrombin generation suggests a heterogenous population of MPs (RBCs®-MPs &Platelets(P)-MPs) in the NLR units of packed RBCs. The heterogeneous population variably contributes to the pro-inflammatory and pro-coagulant potential of stored blood products as a function of storage time⁵⁸. It is observed that the presence of P-MPs leads to the activation of PLTs during the storage, causing leukocyte apoptosis and increased interaction of platelets with leukocytes⁵⁹. To prevent the accumulation of microparticles, the leukoreduction method is widely used⁶⁰. However, it does not eliminate R-MPs present in plasma; nor suppress the membrane vesiculation of stored RBCs. As a result, leukoreduction can only alleviate the putative MPs associated with Lymphocytes to prevent the adverse effects of transfusions.

1.1.4 Bioactive Lipids:

During storage, several biochemical changes occur in the RBCs composition, causing protein and lipid oxidation, and exposure of PS in the outer leaflet of the RBCs membrane⁶¹. It is well reported that increased exposure to PS and change in membrane protein are related to the binding of hemoglobin to the membrane causing the removal of stored RBC from the circulation⁶². In addition, controversial information is available about the relative amount of cholesterol in the bilayer, which suggests that an increase in cholesterol leads to lipid loss. However, this notion has been challenged. The RBC membrane is composed of phospholipids, and during storage, there is an oxidation and enzymatic processing of lipids that leads to arachidonic acid production. Arachidonic acid can be converted into prostaglandins and/or leukotrienes (the latter are called

eicosanoids)⁶³. Eicosanoids are reported to have widespread effects on inflammation, vascular tone, vascular permeability, and platelet activation. Previous studies have documented that bioactive lipids produced in stored RBC units may cause inflammatory complications after transfusion⁶⁴. Accumulation of some bioactive lipids, such as lysophosphatidylcholine, is limited to an extent by leukoreduction. However, various poly-unsaturated fatty acids, including arachidonic acid, linoleic acid, docosahexaenoic acid, and their metabolites, accumulate in RBC units despite leukoreduction⁶⁵. These bioactive lipids are known to have immunomodulatory effects depending on the clinical situation of the transfusion recipient⁶⁶.

The role of bioactive lipids as a second hit in the LPS-primed rats is widely studied in the two-hit model of non-antibody-mediated TRALI⁶⁶. The presence of bioactive lipids with neutrophil priming activity in the plasma of TRALI patients shows the correlation between bioactive lipids and non-antibody-mediated TRALI condition. However, the extent to which bioactive lipids may contribute to systemic immune modulation outside of the TRALI condition still requires extensive research. It is well reported that WBCs and platelets (PLTs) are majorly responsible for most bioactive lipids production, and leukoreduction is the only solution. However, studies have shown a significant product of arachidonic acid and its metabolites in LR units⁶⁷.

A certain class of bioactive lipids was lowered in LR units. However, many PUFAs (Poly-Unsaturated Fatty Acid) and oxidized products still produced similarly to that observed in NLR units⁶⁸⁻⁶⁹. The data suggest that bioactive lipids are still a concern for transfused RBCs, and leukoreduction does not eliminate this concern⁷⁰⁻⁷².

1.2 Current technologies to improve the quality and shelf-life of RBCs

It is well-studied that the DAMPs in stored blood induce progressive biochemical and biomechanical changes affecting red cell viability, deformability, oxygen-carrying capacity, and microcirculatory flow. Despite the contradictory data reported, there is strong evidence from the systematic analysis that supports the notion that the deleterious effect of RBC storage has not only a direct link with the low efficacy of transfusion but also has adverse clinical consequences, especially in specific recipients⁷³. Therefore, an inadequate transfusion strategy will have a negative clinical impact and significant economic consequences. Various efforts are made to improve the shelf-life and quality of the RBC units through unbiased approaches to the characterization of the RBC unit during storage. Some of the current alternatives might have a specific application and are cost-effective.

1.2.1 Cryopreservation

Storage of RBCs at ultra-low temperatures halts the cellular metabolism, preventing the progressive deterioration responsible for the DAMPs generation. This technique also helps in the preservation of RBC units for prolonged periods. The first successful cryopreservation of human RBCs in glycerol was reported by Smith et al. in 1950, and later, the first transfusion of cryopreserved human RBCs was performed. Since then, several thousand units have been frozen, thawed, and transfused. The complex freezing process elevated cost, and the limited shelf-life of thawed products have limited the use of cryopreservation. The recent development of more sophisticated post-thaw washing and additive solutions has made cryopreserved RBCs better suited for clinical practice⁷⁴. There are several freezing/thawing/deglycerolisation method procedures. It was observed that the deglycerolisation washing process helps in removing detrimental substances, such as bioactive lipids, cytokines, potassium, and free hemoglobin. Additionally, it removes residual leukocytes after buffy-coat repletion⁷⁵. Cryopreserved washed RBCs contain hardly any biologically active substance in the supernatant.

Currently, cryopreserved RBCs are primarily used in different settings where the RBC availability is limited or unpredictable. For example, 1) military practice/battlefield, 2) to store RBCs from rare blood groups, 3) to bridge over supplies during short-term RBC deficits (as occurs during natural or civil disasters), 4) for autologous RBC transfusion when RBC count recovery is required prior to surgery, and 5) for patients with immunoglobulin A deficiency⁷⁶. Another limitation related to cryopreservation includes a reduction in ATP and 2,3-DPG levels post-thawing and hampering in the RBC agglutination profile⁷⁷. On the other hand, cryopreservation does not cause the deformability of RBCs, which is beneficial for retaining the quality of stored RBCs.

1.2.2 Alternative additives or rejuvenation solutions

Many groups have proposed alternatives to restore redox imbalance by supplementing storage solutions with antioxidants, such as vitamin C (ascorbic acid)⁷⁸ and serotonin⁷⁹. Other studies employ a novel mechanism for improving the quality of stored blood by inhibiting acid sphingomyelinase with amitriptyline, and thus stabilizing the erythrocyte membrane. Transfusion of stored RBCs treated with amitriptyline resulted in a dose-dependent reduction in acid sphingomyelinase activity, ultimately preventing lung inflammation in mice⁸⁰. Alternatively, novel studies in mice have demonstrated the

therapeutic benefit of co-infusion with hemopexin or haptoglobin in the recovery of hemorrhagic shock with stored RBCs⁸¹. Therefore, it increases the survival rate and decreases tissue inflammation. Interestingly, only co-infusion of haptoglobin with RBCs prevented hemoglobinuria and kidney injury⁸². There are certain studies showed a significant decrease in G6PD activity when RBCs were stored in a mannitol-containing additive solution (i.e., SAGM, AS-1, or AS-5). The decrease in G6PD activity leads to lipid and protein peroxidation, thereby damaging RBCs and leading to extra- and intravascular haemolysis. Therefore, rejuvenation solutions haven't completely addressed the issue related⁸³⁻⁸⁶.

1.2.3 RBC washing

RBCs washing is typically performed by normal saline (0.9% NaCl) in either an open or a closed system⁸⁷. The method involves the removal of ~95%–99% of the RBC supernatant, which contains, in addition to the additive solution, plasma proteins, electrolytes, some WBCs, platelets, microparticles, and cellular debris⁸⁸. RBCs washed in an open system are subjected to use within 24 hours post-washing to avoid the risk of bacterial contamination and maintain cell viability⁸⁹. Washing RBCs in a closed system has an expiration time of 14 days post washing⁹⁰. The washing process also involves reducing extracellular potassium, preventing hyperkalemia condition in the patients. Simultaneously increases the RBC membrane osmotic fragility. However, any error in RBC washing may also increase the osmotic fragility, leading to increased hemolysis following transfusion⁹¹.

1.2.4 Nitric Oxide treatment

Under normal conditions, Nitric Oxide (NO) is produced due to the interaction between the inner linings of the blood-carrying vesicles and that of the flowing blood⁹². This production is often hindered during the blood transfusion process due to the transfused blood cells containing abnormalities caused due to storage of the cells. Various methods can do restoration of the NO level in the body. The most straightforward approach is inhaling NO during the transfusion process. Other methods can also be employed, such as introducing nanoparticles containing NO and the blood used for transfusion⁹³⁻⁹⁴.

1.2.5 Anaerobic / Hypoxic Storage

Even though the blood is stored in storage solutions containing different preservatives, it is still vulnerable to oxidative damage. Decreased survivability and morphological changes of the cells are two of the primary harms that result from this. Even "young" cells that are 7 to 14 days old might show signs of harm⁹⁵. Instead of the widely used aerobic storage, switching to anaerobic means of storage time can be enhanced. As a result, the oxygen concentration must be less than 4%, which must be achieved before the samples are refrigerated and maintained throughout the storage period⁹⁶. It has been noted that it not only exhibits fewer cellular lesions and fewer malformed cells, but it also exhibits improved oxygen transport efficiency to the body's tissues. It has been noted that eliminating CO₂ throughout the procedure is beneficial, along with the depletion of oxygen⁹⁷. The elimination of CO₂ raises the pH to an alkaline state but also aids in preserving the levels of 2,3-DPG, which are pH-sensitive and break down at acidic pH⁹⁸.

1.2.6 Use of Hemopexin and Haptoglobin

As previously mentioned, stored blood is prone to DAMPs production, and when transfused into the body, the free Heme can cause several medical conditions. These include systemic inflammation, a loss of endothelial function, and damage to various tissues and organs⁹⁹. In order to prevent potential oxidative damage, hemopexin and hemoglobin are used to bind to free Heme. Blood infused with proteins like hemopexin and haptoglobin has shown positive results. Recent mouse studies have demonstrated that co-infusion of blood with the aforementioned proteins has increased the survival rate of blood cells and reduced tissue inflammation¹⁰⁰. Also, haptoglobin helps prevent organ damage in critically ill patients, such as Kidney dysfunction and hemoglobinuria¹⁰¹.

1.2.7 Leuko-reduction

Leucocyte removal by filtration is widely performed prestorage in the processing laboratory for better quality assurance. As per the FDA recommendation, the filtered unit of blood contains less than 5×10^6 per unit white blood cells (WBC) and a retention of approximately 85% of the original RBC¹⁰². Pre-storage leukoreduction (LR) of whole blood (WB) prevents white blood cells (WBC) and platelet contamination. This reduces the risk of febrile non-hemolytic transfusion reactions, alloimmunization, and the accumulation of biologic response modifiers (BRMs), including soluble CD40 ligand, cytokines, chemokines, and lysophosphatidylcholine (lyso-PCs)¹⁰³. These components

have been implicated in developing transfusion-related acute lung injury (TRALI). Three primary LR methods are used worldwide. Buffy coat removal (BCR), in-line filtration, and the combination of both approaches¹⁰⁴. BCR separates cells based on their differences in specific gravity. BCR is the most commonly used method for leukoreduction in developing countries due to its lower cost. In-line filtration is based on charged-based adhesion of negatively charged leukocytes to remove WBCs from blood products and is used predominately in the United States¹⁰⁵. The combination approach employs buffy coat removal followed by filtration before storing the red blood cell units and is predominately employed in Canada and Western Europe. However, the leukoreduction process is expensive; hence, most blood banks in developing countries like India do not use leukoreduction processes routinely¹⁰⁶. The cost implicated in universal leukoreduction policy is not practically feasible, especially in developing countries and other under-resourced nations. It is also observed that leukoreduced blood units have lesser WBCs associated lesions like extracellular DNA and free histone by the end of the storage period¹⁰⁷. However, several poly-unsaturated fatty acids (PUFAs) and oxidized products are still accumulated in leukoreduced blood units akin to non-Leukoreduced blood units. Indeed, PUFAs and their oxidized products are known to cause transfusion-related acute lung injury (TRALI)¹⁰⁸.

1.3 Research Gap and Objective of the thesis

Despite insufficient blood donation, millions of blood units are discarded due to declining quality during storage. Although multiple methods have been found to increase the shelf life of stored blood, they have limited success. So far, studies have focussed on improving storage conditions through additives, rejuvenation solutions, and cryopreservation protocols. However, none of these approaches address the cause of the problem-the presence of extracellular components in the blood units.

The use of leukoreduction filters and other technologies before blood storage has reduced the formation of DAMPs contributed by leukocytes. However, they do not serve the purpose of removing the whole spectrum of DAMPs or preventing the deterioration of RBC's quality. Hence, preventing the storage lesions before transfusion to patients is imperative to reduce mortality and increase the storage time to minimize blood.

In this thesis, we propose a strategy for eliminating a wide spectrum of DAMPs by developing charged-nanofiber scaffolds for selectively scavenging free extracellular

DNA, histones, Heme protein, and bioactive lipids from the old stored RBC units (both NLR&LR blood units) before transfusion. We are further proposing that removing DAMPs in stored blood units may help prevent the risk of TRALI. Due the lack of efficient technologies to increase the quality and shelf life of stored blood, my thesis focuses on developing technology to enhance the quality of both non-leukoreduced and leukoreduced blood. Subsequently, my thesis also focuses on demonstrating the efficacy of new technology to prevent TRALI.

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Chapter-2

Intermittent scavenging of storage lesion from stored red blood cells by electrospun nanofibrous sheets enhances their quality and shelf-life

2.1 Introduction:

Blood transfusion is often a lifesaving practice for patients in the intensive care unit (ICU), where ~50-70% of ICU patients are transfused with blood units during their stay¹. Typical indications for blood transfusion include sickle cell crisis, anaemia, and severe blood loss². The highest transfused blood components are red blood cells (RBCs), with >85 million RBC units are transfused annually worldwide^{2,3}. While RBC transfusion has therapeutic benefits, transfusion of 45 storage-aged RBCs may cause deleterious effects on the recipients. These deleterious effects have been attributed to the storage lesion consisting of damage-associated molecular patterns (DAMPs) generated due to biochemical, morphological, and structural changes in RBCs during the storage-aging process^{4,5}. The most studied DAMPs in this context are; i) extracellular free iron and free-hemoglobin (Hb) generated due to lysis of RBCs, ii) bioactive lipids such as polyunsaturated fatty acids (PUFAs), iii) extracellular DNA, and iv) nucleosomes generated by neutrophils. These DAMPs are a potential source of sequelae in vulnerable patients⁶. Based on storage-time-dependent accumulation of DAMPs, stored human RBC units are classified as young RBCs or fresh RBCs (RBCs of <14-21 days of storage) and old RBCs (stored between 21 to 42 days)⁷. Storage of RBCs induces a sequence of biochemical and biomechanical changes in a progressive manner that affect deformability, cell viability, microcirculatory flow, and oxygen carrying capability of RBCs⁸, hence, the detrimental effect of RBC storage can negatively impact the transfusion outcomes. Therefore, developing strategies and technologies to enhance the quality and shelf-life of stored RBCs to reduce the incidence of transfusion-related complications will significantly impact healthcare. The United States Foods and Drug Administration (US-FDA) regulation states that human packed RBCs (pRBCs) can be stored up to 42 days before transfusion⁹. Post 42 days, the increased DAMPs (extracellular DNA, nucleosomes, Hb, and bioactive lipids) pose a fatal risk to the patient by eliciting an immunomodulatory response¹⁰. Leukocytes associated DAMPs such as extracellular DNA and nucleosomes are potential immune elicitors in recipients and implicated in transfusion-related complications¹¹.

Leukodepletion of packed RBCs using specialized filters mitigates risk to a large extent, putatively by reducing the load of extracellular DNA and nucleosomes in blood units¹². However, leukodepletion does not remove other critical RBCs generated DAMPs such as free-hemoglobin and bioactive lipids, which are also significant mediators of transfusion-related complications. Therefore, in alternative to the leukoreduction process, studies thus far have focused on improving storage conditions by alternative cryopreservation protocols^{13,14}, anaerobic storage^{15,16}, and usage of additives/rejuvenation solutions¹⁷⁻¹⁹. Additionally, washing of stored RBCs with saline (0.9% NaCl) to remove accumulated bioactive factors is another established strategy approved to use in transfusion medicine^{20,21}. Past investigations suggest that adding preservatives, storing RBCs in an alkaline hypotonic solution with antioxidants, or rendering anaerobic storage conditions improve storage time up to 10-15 days¹⁷⁻¹⁹. Although these approaches have shown encouraging results, efficient technologies to scavenge the spectrum of DAMPs and further reduce their formation in stored RBCs remains an unmet need. Additionally, the leukoreduction process is expensive. Hence, most blood banks in India and developing countries do not routinely use leukoreduction processes. Interestingly, prior to initiating this work, our discussions with blood banks in India revealed that only <10% of blood banks use the leukoreduction process. It is used only in cases where recipients are immunosuppressed/immuno-compromised. Therefore, an important driver for developing new technology is the desire to develop an affordable intervention that could be used in developing and under-resourced countries where non-leukoreduced blood products are the standard inventory. Here we develop a new approach, demonstrating that intermittent scavenging of DAMPs using electrospun charged nanofibrous sheets slows the deterioration of stored RBCs, increasing their quality and shelf-life. Critical DAMPs such as extracellular DNA, nucleosomes, free hemoglobin (Hb), and PUFAs are charged molecules with anionic or cationic nature. Therefore, we hypothesized that *charged nanofibrous sheets* made with cationic and anionic polymers may scavenge DAMPs through ionic interactions (**Fig.2.1a**). We have synthesized novel polymers with anionic and cationic nature by introducing poly taurine and poly acridine, respectively. An efficient process to fabricate electrospun nanofibrous sheets with anionic (*Tau-NFS*) and cationic (*Acr-NFS*) charges has been generated. Additionally, acridine group in *Acr-NFS* may act as a DNA intercalator. We have demonstrated that a combination of *Tau-NFS* and *Acr-*

NFS, hereafter *Tau-AcrNFS*, is efficient in scavenging storage lesion such as free extracellular DNA, nucleosomes, Hb, and PUFAs from stored human and mice RBC units, *ex vivo* (**Fig 2.1b**).

Two major phenomena were demonstrated using *Tau-AcrNFS*; i) after storage lesion formed in human old RBCs (stored for 42 days), *Tau-AcrNFS* could efficiently

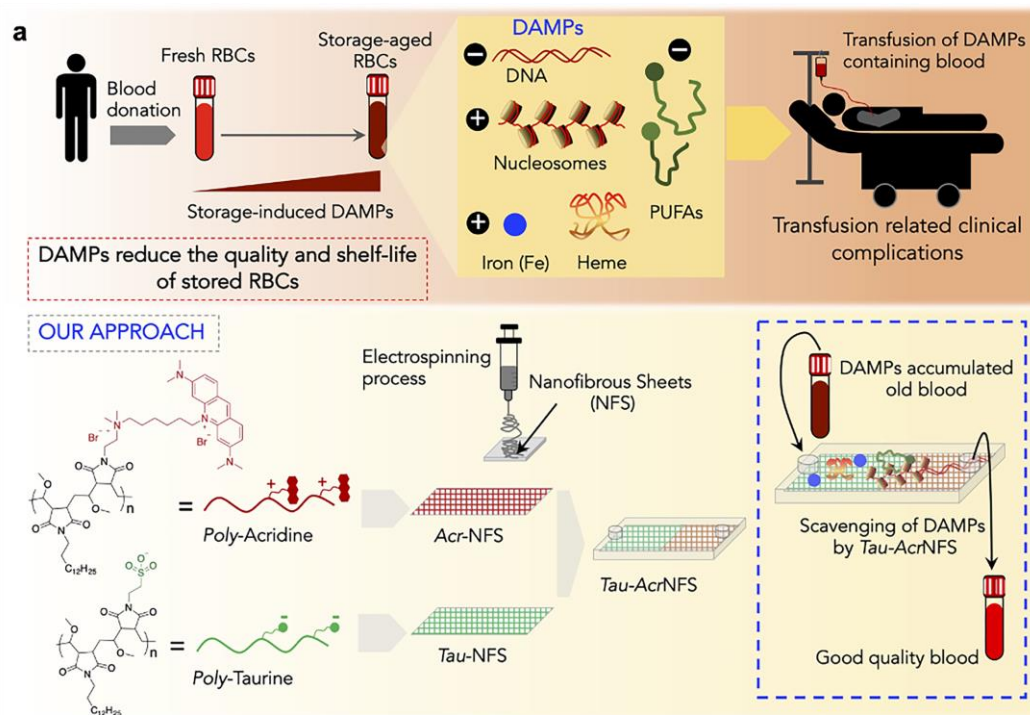


Fig 2.1 a) Stored RBCs produce storage lesion or damage-associated molecular patterns (DAMPs) such as DNA, nucleosomes, heme (Hb), iron, and polyunsaturated fatty acids (PUFAs). Transfusion of DAMPs containing RBCs could lead to transfusion-related complications, including systemic inflammation and organ injury. The presence of DAMPs progressively reduces the quality of stored RBCs and limits their shelf-life. All DAMPs consist of positive or negative charge entities. b.) Therefore, in our approach, we designed hemocompatible polymers poly-acridine and poly-taurine composed of complementary cationic and anionic charges, respectively. Using these polymers, charge-bearing electrospun nanofibrous sheets (Tau-NFS and Acr-NFS) were prepared to scavenge charged DAMPs from stored RBCs, which remarkably enhanced the quality and shelf-life of stored RBCs

scavenge and remove accumulated DAMPs, and ii) the intermittent scavenging of DAMPs using *Tau-AcrNFS* either at 21st or 28th day resulted in significantly less production of DAMPs at maximum storage time (on 42nd day), and reduced the loss of RBC membrane integrity. We have demonstrated that intermittent removal of DAMPs either on 21st or 28th day by *Tau-AcrNFS* enhanced the quality of the blood and increased the shelf-life by ~22%.

Additionally, the RBCs transfusion studies suggested that the quality of intermittently treated and stored for a maximum allowed 14 days (old RBCs) is equivalent to the quality of freshly collected RBCs without storage. These results are remarkable, and we aim to generate either novel blood bags or an insert based medical device using *Tau-AcrNFS*, which may have an enormous impact on improving the quality and shelf-life of stored blood.

2.2. Method

Ethics

Our research involved in preclinical study and human volunteers for blood collection complies with all relevant ethical guidelines. Human blood was collected according to the approved protocols from the Institutional Human Ethical Committee (inStem/IEC-10/003) of the Institute for Stem Cell Science and Regenerative Medicine (inStem). Informed consent has been obtained from the participants. Blood donors are both male (N = 3, age 26–30 years) and female (N = 3, age 25–28 years). All mouse studies strictly adhered to institutional and national guidelines for humane animal use. The experimental protocols were approved by the Institutional Animal Ethics Committee (IAEC) at the Institute for Stem Cell Science and Regenerative Medicine (INS-IAE-2020/16(R1)).

Materials

Poly (methoxy vinyl ether-alt-maleic anhydride) average Mw-330,000 (PMVEMA), was used in all synthesis procedures. Tetrahydrofuran (THF), tetradecyl amine, taurine, sodium bicarbonate, ethyl acetate, 3,6-bis(dimethylamino) acridine hemi (zinc chloride) salt, benzene, methanol, and ammonium hydroxide, N,N-dimethyl ethylene diamine, 1,6-dibromohexane, sodium Sulfate, toluene, dimethylformamide (DMF), triton X-100, chamber slides(HI Media), blood collection tubes (BD Vacutainer), citric

acid monohydrate, trisodium citrate dihydrate, sodium dihydrogen phosphate, dextrose, sodium chloride, glucose anhydrous, mannitol adenine, APTMS (3-(Aminopropyl)trimethoxy silane), glutaraldehyde, cacodylate buffer, osmium tetroxide, hexamethyldisilane, ethanol, double distilled water and deionized water were used. Unless mentioned otherwise, all chemicals were procured from Sigma-Aldrich.

2.2.1 Synthesis of anionic and cationic scaffolds

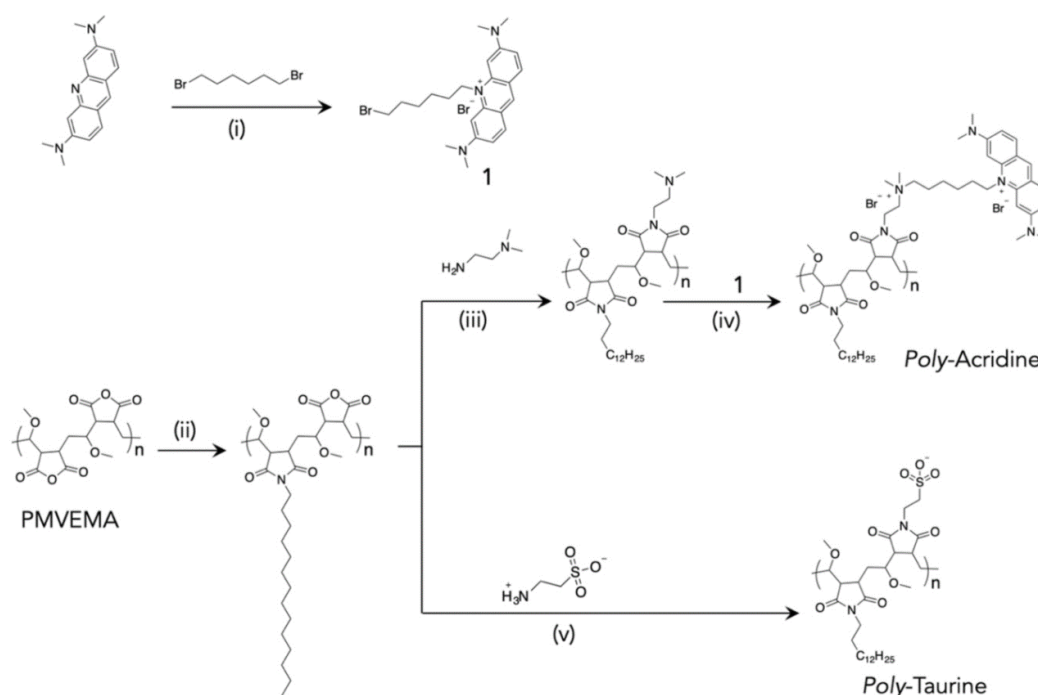


Fig 2.2. Scheme for the synthesis of charged polymers, poly-acridine and poly-taurine. Poly-acridine and poly-taurine polymers were synthesized using solvent phase synthesis. A detailed procedure has been described in Methods. The reaction conditions were as following; (i) anhydrous toluene, 120 °C, 12 hours; (ii) tetradecyl amine, anhydrous tetrahydrofuran, 80 °C, 3 hours; (iii) anhydrous tetrahydrofuran, 80 °C, 12 hours; iv) anhydrous dimethylformamide, 50 °C, 16 hours; and (v) anhydrous tetrahydrofuran, 50°C,16 hours.

PMVEMA -C₁₄

The PMVEMA (500 mg, 3.2 mmol) was added to tetrahydrofuran (THF) (10 ml) in a pressure tube and stirred at 80 °C until dissolution. Subsequently, tetradecyl amine

(345 mg, 1.6 mmol) in THF (10 ml) was added to the previous mixture. The reaction mixture was heated at 80 °C for 3 h. The solvent was finally removed using a rotavapor to get the polymer in dry powder form (800 mg, ~90% yield). H-NMR (CD₃OD, 600 MHz), δ : 3.0-3.51 (Br, 9H, -OCH₃, -O-CH, PMVEMA and -NH-CH alkyl protons), 1.47 (Br, 2H, alkyl protons), 1.22 (Br, 22H, alkyl protons), 0.845 (t, 3H, alkyl protons). As per NMR data, ~50% of PMVEMA polymer was substituted with tetradecyl amine. FTIR (cm): 3354, 2926, 1858, 1782, 1733, 1697

Poly-Taurine: PMVEMA-C₁₄-Tau

A solution of taurine (100 mg, 0.8 mmol) and sodium bicarbonate was prepared (84 mg, 1.0 mmol) in deionized water (1 ml). This solution was added dropwise to the solution of PMVEMA-C (50%) (800 mg) in THF (20 ml) and left for stirring at 80 °C for 16 h. The reaction mixture was subjected to rota vapour to concentrate. Subsequently, it was sequentially triturated with ethyl acetate and deionized water to remove the residual tetradecyl amine & taurine. The final polymer was obtained in a gel form, which was lyophilized to a white powder (720 mg, ~75% yield). H-NMR (CD OD, 800 MHz), δ : 3.9-4.1 (Br, 1H, taurine), 2.7-3.5 (Br, 5H, -OCH₃, -O-CH, PMVEMA and -NH-CH alkyl protons), 1.95 (Br, 2H, PMVEMA protons), 1.4 (Br, 1H, alkyl protons), 1.1-1.2 (Br, 11.5H, alkyl protons), 0.8 (t, 1.5H, alkyl protons). As per the NMR data, PMVEMA was approximately substituted by 50% of C H -NH and ~25% of taurine. FTIR (cm): 3209, 3046, 2946, 1722, 1616, 1037.

Acridine hexyl bromide

3,6-bis(dimethylamino) acridine hemi (zinc chloride) salt (10 g, 27.02 mmol) was dissolved in methanol (50 ml), and excess ammonium hydroxide (50 ml) was added. Acridine orange (AO) base was extracted from benzene and dried over sodium Sulfate; the AO base (5 g) was recovered from benzene using rotavapor. The AO base (2.65 g, 10 mmol) was dissolved in anhydrous toluene (100 ml), and 1,6-dibromohexane (7.7 ml, 50 mmol) was added to the solution and refluxed at 120 °C overnight. The bright red precipitate in the reaction mixture was filtered and dried (yield- 2.1 g, 41.2%). H NMR (CDCl₃, 600 MHz) δ : 8.7 (s, 1H), 7(d, 2H), 7.08 (m, 2H), 6.75 (s, 2H), 5.0 (t, 2H), 3.4 (t, 2H), 3.3 (s, 12H), 2.048 (m, 2H), 2.029 (m, 2H), 1.96 (m, 2H), 1.94 (m, 2H).

Poly-Acridine

The solution of N, N-dimethyl ethylene diamine (87 μ l, 0.8 mmol) in THF (10 ml) was added dropwise to the solution of PMVEMA-C₁₄ (50%) (800 mg) in THF (20 ml) in a pressure tube. The mixture was allowed to stir at 80 °C for 12h. After its dissolution, THF in the reaction mixture was reduced until a viscous solution appeared using rota vapour. Dry DMF (10 ml) was added to this viscous solution, followed by the addition of Acridine-hexyl bromide (243 mg, 0.48 mmol) in DMF (5ml). The resulting mixture was stirred at 50 °C for 16 h. The polymer was triturated thoroughly with deionized water to remove unreacted Acr-Hexyl-Bromide and DMF. Further, the polymer was lyophilized to an orange powder (800 mg, ~80%) H-NMR (CDCl₃, 800 MHz), δ : 6.5–8.6 (Br, 0.77H, acridine protons), 3.0–3.5 (Br, 4H, PMVEMA protons), 0.98–1.7 (Br, 9.5H, alkyl protons), 0.87 (Br, 3H, alkyl protons). As per the NMR data, approximately 50% of tetradecyl amine (C₁₄H₂₉-NH₂) and ~11% of acridine hexyl bromide were substituted on PMVEMA polymer. The complete reaction scheme is mentioned in **Fig 2.2**

2.2.2 Fabrication of electrospun nanofibrous sheets

The electrospinning solutions were prepared by dissolving *poly*-Taurine (20%, w/v) and *poly*-Acridine (15%, w/v) in dry DMF at room temperature (RT). These solutions were stirred overnight before electrospinning. During the electrospun procedure using the Espin device (ES2, Espin nanotech, India), a 2-mL plastic syringe (Dispo Van, India), and a blunt 22 G needle (Dispo Van, India) were used. The solutions were pumped via a syringe pump at a constant flow rate of 0.5 ml/h (*poly*-Taurine) and 0.3 ml/h (*poly*Acridine). The electrospun nanofibrous sheets (*Tau*-NFS and *Acr*-NFS) were collected on an aluminum foil placed 15 cm away from the needle (where 15 kV voltage was applied). All experiments were carried out at 25 °C and less than 55% relative humidity (RH). The electrospun mats with a length and width of approximately 10 cm \times 10 cm were stored in a desiccator until further use.

2.2.3 Field emission scanning electron microscopy

The morphology of the *Tau*-NFS and *Acr*-NFS was investigated by FESEM (Carl Zeiss MERLIN VP compact). Dried electrospun mats were mounted on aluminum

stubs and sputter-coated with gold (Pelco® SC-7 Auto sputter coater). The gold-coated mats were imaged at a voltage of 2 kV. The scaffolds treated with pRBCs were imaged to investigate the adherence of blood cells.

2.2.4 Contact angle measurement

The wettability of *Tau*-NFS and *Acr*-NFS was studied using the sessile drop method. The scaffolds were cut uniformly and placed on a glass slide before the measurement. Water droplets (5 μ L) \sim 2 mm in diameter were dropped on the electrospun NFS, and the images were acquired. The images were processed using ImageJ software (V1.53).

2.2.5 Hemolysis assay

A hemolysis assay was performed to evaluate the haemolytic nature of *Tau-Acr*NFS. The blood was collected in a tube containing anticoagulant sodium citrate. The collected blood was centrifuged (500 G, 10 min), and the supernatant was discarded. These washed RBCs were used to prepare 2% hematocrit in saline and added to *Tau-Acr*NFS fixed in a chamber slide (16 cm²) prewashed with double distilled water. The RBCs were further resuspended in saline and pelleted down. This setup was kept on an incubator shaker (37 °C and 100 rpm) for 1 hour. After 1 hour, the hematocrit was transferred into a 2 ml centrifuge tube and centrifuged at 500 G for 10 min at RT. A 100% lysis sample of the untreated RBC specimen was prepared with 0.1% Triton-X100 in 2% hematocrit. The concentration of Hb in a 100% lysed RBCs sample was determined by Cyanomethaemoglobin (Drabkin's) method, which is considered as 100%. Afterward, a series of dilutions was prepared from lysed sample to generate a standard curve. The level of hemolysis of the *Tau-Acr*NFS-treated RBCs is then derived from the standard curve using Drabkin's method. For Drabkin's method, the absorbance was recorded at 540 nm using Varioskan LUX Multimode Microplate Reader (Thermofisher, Massachusetts, USA).

2.2.6 Polymer leaching test

Acr-NFS (8 cm²) was fixed on the chamber slide and incubated in Optisol (AS-5) solution [composition: sodium chloride (8.77 mg/mL), glucose anhydrous (8.18 mg/mL), mannitol (0.01% w/v) and adenine (0.03 mg/mL)]. The sheets were incubated for 2 h. The Optisol solution was removed after both incubations and lyophilized to recover the leached *poly*-Acridine from the sheets. The lyophilized

poly-Acridine was dissolved in DMF and estimated using a fluoro spectrophotometer (Horiba Fluro log QM, France). Fluorescence intensities were recorded as a function of time with excitation at 502 nm and emission at 535 nm. The Leaching (%) was determined via a pure *poly*-Acridine polymer standard curve prepared at the excitation and emission wavelengths mentioned above.

2.2.7 Packing of human RBC

Blood was collected according to the approved protocols from the Institutional Human Ethical Committee (inStem/IEC-10/003) of the Institute for Stem Cell Science and Regenerative Medicine (inStem). Informed consent has been obtained from the participants. RBC units were prepared from human whole blood (both male and female, $n = 3$) of different blood groups collected in 20 ml vacutainers containing citrate-phosphate-dextrose with adenine (CPD-A) that contains citric acid monohydrate (3.577 mg/mL), trisodium citrate dihydrate (29.972 mg/mL), sodium dihydrogen phosphate (2.496 mg/mL) and dextrose (25.5 mg/mL).

The whole blood was centrifuged for 20 min at 500g, and the supernatant was removed. The removal of supernatant was followed by the addition of Optisol (AS-5, preservative) that contains sodium chloride (8.77 mg/mL), glucose anhydrous (8.18 mg/mL), mannitol (0.01% w/v) and adenine (0.03 mg/mL) to obtain a hematocrit of ~65%, which was transferred into standard PVC bags, and stored at 4 °C in the dark.

2.2.8 Scavenging of DAMPs from stored human RBCs using *Tau*-NFS and *Acr*-NFS, *in vitro*

On different days of, stored RBCs (0, 14, 21, 28, and 42) were incubated with *Tau*-NFS and *Acr*-NFS individually and in combination for 5 min in total to scavenge DAMPs. In both treatment conditions, the nanofibrous sheets were prewetted with distilled water for 30 min before the addition of RBCs. After 30 mins, distilled water was removed and the scavenging experiments were performed on ice under the aseptic condition. *Tau-Acr*NFS was mounted on the chamber slides, and the stored RBCs samples were added. It was made sure that the RBCs sample should cover the entire surface area and be in contact with nanofibers. The total surface area of the scaffold was kept at 24 cm² for each experiment. Scaffold treatment of RBCs was done at 4 °C for 5 min to keep the cold chain storage.

2.2.9 Quantification of free Hb using Cyanomethaemoglobin (Drabkin's) method

Drabkin's reagent lyses RBCs and oxidizes all forms of Hb, except for the minimally present sulfhaemoglobin, to the stable HiCN²². The supernatant of the stored RBCs unit (scaffold-treated or untreated) was diluted (1:10) with Drabkin's reagent (Sigma, St. Louis, MO, USA). Human Hb diluted with Drabkin's reagent (1:10) (Sigma Aldrich, USA) was used to prepare the standards (0–40 mg/dl) and the calibration curve. The samples and standards were incubated in the dark at RT for 15 min. Absorbance was recorded at 550 nm using a Varioskan LUX Multimode Microplate Reader (Thermofisher, Massachusetts) USA). The Hb concentration of each sample was calculated from the human Hb calibration curve.

2.2.10 Quantification of free DNA by PicoGreen assay

RBC supernatants from scaffold-treated and untreated groups were thawed on ice. Supernatants were diluted (1:50) with PicoGreen® reagent and Tris-EDTA (TE) buffer (Quant-iT™ PicoGreen™ dsDNA Assay Kit, Invitrogen, USA), followed by 5min incubation in the dark. Free-DNA estimation was performed using a fluoro spectrophotometer (Horiba Fluoro log QM, France)²³.

2.2.11 Quantification of nucleosomes

Stored RBC supernatant was diluted in PBS (1:5). The Anti-Histone-Biotin-Monoclonal antibody (cloneH11-4) and Anti-DNAPOD-Monoclonal antibody from mouse (clone MCA-33) was used for quantifying nucleosomes by ELISA (Catalog No.11920685001, Cell Death Detection kit, Roche, Indianapolis, IN), according to manufacturer's instructions.

2.2.12 Osmotic fragility test

The stored RBC units (treated and untreated group) were added to 9 tubes containing saline concentration series ranging from 0% (distilled water) (Tube 1) to 0.9% (Tube 9). The tubes were gently mixed and incubated at room temperature for 30 min. The incubation was followed by centrifugation at 500g for 20 min, and supernatants were collected. The optical density of the supernatant was measured by Varioskan LUX Multimode Microplate Reader (Thermofisher, Massachusetts, USA) at 540 nm.

Hemolysis in each tube was expressed in percentage. The maximum absorbance value of haemolyzed RBCs was taken at 100% in the distilled water²⁴. The erythrocytes treated with normal saline were used as a negative control (0% hemolysis).

2.2.13 Primary and secondary fixation of RBCs for SEM

Glass coverslips were sonicated in acetone and cleaned. They were further dipped in 2% APTMS (3-(Aminopropyl)trimethoxy silane), and diluted in acetone to impart a coating of APTMS. RBCs were primarily fixed with 2% glutaraldehyde (GTA), incubated for 30 min in the dark, and washed with PBS. The primary fixed RBCs were incubated on APTMS-coated coverslips for 1 h. After 1 h, the coverslips were rinsed twice with PBS and incubated with 0.1 M cacodylate buffer for 10 min. Secondary fixation was achieved via 1% (w/v) Osmium tetroxide (OsO₄) in 16h. The secondary fixed coverslips were then rinsed with MilliQ water (10 min), followed by a dehydration step that involves a gradient ethanol wash, ranging from 40 to 100%. The dehydration step is followed by HMDS (Hexamethyldisilane) drying²⁵. Finally, dried samples were mounted on aluminum stubs, sputter-coated with gold, and imaged under FESEM (Carl Zeiss MERLIN VP compact).

2.2.14 Bioactive lipid estimation by LCMS

Chemicals and standards

Eicosanoids; 5(S)-HETE, 12(S)-HETE, 15(S)-HETE, and AA were purchased from Cayman Chemical Co. (Ann Arbor, Michigan, USA). 5(S)-HETE-d₈ (Cayman Chemical Co) was used as an internal standard (IS) for this study. Acetonitrile (Baker analyzed® LC-MS reagent), water (Baker analyzed® LC-MS reagent), formic acid, ethanol, ethyl acetate (EtOAc), and glacial acetic acid were used in sample preparations for LC–MS/MS measurements.

Preparation of stock solutions

5-(S)-HETE, 12(S)-HETE, 15(S)-HETE, AA, and IS 5(S)-HETE-d₈ were constituted in ethanol by the provider. 5, 12 & 15 (S)-HETE was further processed and serially diluted to obtain solutions ranging from 0.05 to 100 ng/ml. AA solutions ranged from 0.5 to 1000 ng/ml. The working concentration of the pure IS was 1000 ng/ml.

Sample preparation

Lipid extraction from stored RBCs supernatant was performed by gently mixing the samples with 0.5 mL of EtOAc containing 0.13% acetic acid (v/v). All samples were spiked with 5 μ l of the IS. After 10 min of vigorous shaking (1500 rpm), samples were centrifuged (10,000 rpm, 10 min, 4 °C), and 450 μ l of the organic layer was transferred to fresh tubes and evaporated in speed vac (Scan Vac, Labogene, Demark) at 4 °C. The dry residue was reconstituted in 25 μ L of nitrogen-purged EtOH and then directly injected (2 μ l) into the LC–MS/MS system.

LC–MS/MS conditions

HPLC analysis was done using a Shimadzu Prominence HPLC system. The instrument was equipped with a communication module (CBM-20A), degassing unit (DGU-20A5R), solvent delivery units (LC-30AD), column oven (CTO-20AC), and an autosampler (SIL-30AC). The temperature of the column oven was maintained at 40 °C while the autosampler was set at 4 °C. The lipids were separated using an Acquity UPLC® BEH C18 1.7 μ m column (2.1 \times 50 mm). The mobile phase comprised solvent A, i.e., water with 0.1% formic acid (FA) and acetonitrile with 0.1% FA as solvent B. The flow rate was maintained at 0.2 ml/min, and the injection volume was 2 μ l. The gradient for lipid elution (20.10 min) was set as 50% A and 50% B for one minute. This was followed by a linear gradient to 20% A and 80% B for 8 min to elute 5, 12, and 15 HETE. This was further followed by a linear gradient to 100% B for 2 min and kept for 5 min to elute AA²⁶. Finally, the column was equilibrated at 50% A and 50% B for 4 min before the next injection. LC-MS analysis was performed by QTRAP 5500 (Sciex, Framingham, Massachusetts, USA), a triple quadrupole mass spectrometer. LC–MS analysis was performed by MultiQuant™ 3.0.3 Software (SCIEX). The operating parameters for the mass spectrometer were as follows: declustering potential (DP): –209.8 V, entrance potential (EP): –5.7 V, collision cell exit potential (CXP): –16.9 V, ion spray voltage: –4500 V, source temperature: 500 °C, curtain gas: 35 psi, ion source gas 1: 40 psi and ion source gas 2: 50 psi. All the spectra of lipids were recorded and quantified in multiple reaction monitoring (MRM) mode. Negative electrospray ionization was employed for all the

lipids, and the deuterated IS. The ion Q1/Q3 transitions and other parameters can be seen in Table 2.1.

Table 2.1. The ion Q1/Q3 transitions and other parameters like (declustering potential (DP), entrance potential, collision cell exit potential (CXP), ion spray voltage, source temperature are used to quantify PUFAs.

Species	Q1 (m/z)	Q3 (m/z)	Ion Mode	DP (Volts)	EP (Volts)	CE (Volts)	CXP (Volts)
Arachidonic Acid (AA)	302.900	259.300	Negative	-209.800	-5.700	-16.200	-16.900
5-HETE	319.00	115.00	Negative	-127.00	-3.900	-18.00	-7.200
15-HETE	319.00	219.00	Negative	-158.400	-5.100	-17.200	-11.900
12-HETE	319.00	179.200	Negative	-135.500	-8.100	-19.00	-10.900
5-HETE-d ₈	327.200	116.00	Negative	-127.00	-3.900	-18.00	-7.200

2.2.15 *In vivo* experimental design

Mice

Both male and female C57BL/6J mice of age 8–12-weeks-old were used for the experiments. The animals were housed in the animal facility at the National Center for Biological Sciences, Bengaluru. Animals were caged (maximum, four per cage) during the experiment, food and water were provided ad libitum. Mice were housed in facility that was subjected to 12/12 h light/dark cycle, at a temperature of 20–25 °C and humidity of 35–65%. All mouse studies strictly adhered to institutional and national guidelines for humane animal use. The experimental protocols were approved by the Institutional Animal Ethics Committee (IAEC) at the Institute for Stem Cell Science and Regenerative Medicine (INS-IAE-2020/16(R1)).

2.2.16 Packaging and storage of mice RBCs and their treatment with Tau-AcrNFS to scavenge DAMPs

Blood collected in CPD was immediately transferred and centrifuged at 500g for 20 min at 4°C. Plasma was carefully aspirated, and the pRBCs were pooled together. Optisol (AS-5) was added to the pRBCs at 65% hematocrit. Totally 550 µL and 250

μ L blood units were finally aliquoted from these pRBCs and stored in sterile Eppendorf tubes (0.6 and 0.3 ml). Mouse pRBCs were treated with Tau- AcrNFS (24 cm^2) for 5 min on the 5th and 10th day of storage at 40 °C. After the scavenging procedure, the RBCs were again transferred into autoclaved 2 ml Eppendorf tubes, leaving a small residual space, and stored till the 17th day. Its supernatant was taken for extracellular Hb and nucleosome estimation.

2.2.17 Quantification of PS on RBCs by Annexin V (AV)

The number of erythrocytes expressing PS on their outer membrane was determined as described **elsewhere**³⁸. Briefly, labeling with AV was performed by adding 5 μ L of AV-Alexa Fluor™ 568 conjugate (Invitrogen, USA) in 10 erythrocytes in 100 μ L Annexin binding buffer. The binding buffer was prepared by adding 140 mM and 2.5 mM CaCl_2 in 10 mM HEPES Buffer (all from Sigma-Aldrich). After incubation on ice for 15 min, cells were diluted four times with binding buffer and analyzed on a BD LSR Fortessa cytometer (BD Biosciences). Data was collected with FACSDiva v8.02, and data analysis was performed FlowJo v10.0.8. The erythrocyte population with exposed PS labeled with AV-Alexa Fluor™ 568 conjugate was measured as a percentage of Annexin V–positive RBCs.

2.2.18 Quantification of pro-inflammatory cytokine levels in plasma: C57BL/6J mice (8–12 weeks, n = 3)

were transfused with either fresh RBCs or RBCs stored for 17 days (untreated or intermittently treated with Tau-AcrNFS). After 2 h of transfusion, blood was collected and plasma was separated and analyzed for pro-inflammatory markers at 1:10 dilution with array buffer. Cytokines/chemokines, including stromal cell-derived factor1 (CXCL12), macrophage-colony-stimulating factor (M-CSF), complement component 5 A(C5A), and keratinocyte-derived chemokine/CXCL1 (KC/CXCL1) were quantified using Mouse Cytokine Array Panel A (R&D Systems, Minnesota, USA, Serial No. 893560) following the manufacturer's instruction.

2.2.19 Transfusion of RBCs

Biotinylation of RBCs, *in vivo*

Briefly, Sulfo–NHS Biotin (Thermofisher Scientific, US) powder was dissolved in sterile PBS, vortexed, and passed through a 0.22 μ m filter. It was diluted to a final

concentration of 1 mg biotin–sulfo–NHS per 300 μ L 1 \times PBS. Mice were restrained, and the biotin reagent solution was administered through intravenous injection. After 48 h of the biotin administration, under mild anesthesia, around 1 ml of biotinylated blood was collected through a cardiac puncture in CPD-containing vials and immediately centrifuged at 500g for 20 min at 4 $^{\circ}$ C. Plasma was carefully aspirated, and pRBCs were pooled together. The biotin-labeled blood was processed and stored using Optisol as a preservative in sterile Eppendorf tubes (0.5 and 1 ml).

Treatment of Biotin-labeled RBCs with Tau-AcrNFS

Functionality tests Biotin-labeled RBCs were divided into treated and untreated groups. The treatment group (n = 3) pRBC units were treated with Tau-AcrNFS on the 5th and 10th days of storage and then packed until 18 days. The untreated group pRBCs units (n = 3) were not treated with sheets during the storage timeline. Both groups were analyzed for PS exposure during Evaluating the enhanced shelf-life of biotin-labeled RBCs by recovery experiment on different days of storage (0, 7, 14, 15, 16, 17, and 18 days), biotinylated pRBC units with and without treatment were diluted (1:1) with PBS. Totally, 200 μ L of diluted biotinylated pRBC was administered to the restrained mice via intravenous injection²⁴. At specific time points, 6 μ L of blood was collected via tail snip and stored in microfuge tubes containing 250 μ L of sterile PBS. To detect biotinylated RBCs through flow cytometry, approximately 10⁶–10⁷ RBCs (100 μ L of the diluted sample) were added to 0.125 μ g of streptavidin–APC-eFluor[®] 780 Conjugate (1:20 of 0.2 mg/mL; eBioscience[™]). Samples were incubated in the dark for 5 min, washed with 400 μ L PBS, and centrifuged at 1000 g for 4 min. The RBC pellet was resuspended in 200 μ L PBS. Samples were analyzed using BD LSR Fortessa cytometer (BD biosciences, USA). The red laser was used to excite the dye (λ emission = 780 nm) with a bandpass filter of 780/60. The following formula was used to calculate the post-transfusion survival (%).

$$\frac{\text{Post transfusion RBC recovery (\%)} \text{ at } t(t)}{\text{Post transfusion Rbc Recovery at } t(1h)} \times 100$$

where (t) stands for post-transfusion time point, i.e., 1, 24, 48, and 72 h. This formula is used for all pRBCs units stored for different days and then transfused in a different set of mice. The data was collected with FACSDiva v8.02 and data analysis was performed FlowJo v10.0.8.

2.2.20 Quantification of total iron in the spleen

Sample digestion and preparation:

Splenectomy was performed in mice after 2 h of transfusion. Spleen from all the groups was taken in a tared 2 ml Eppendorf, and the dry weight was recorded. Then, 1 ml protein precipitation solution and 1 ml (8%) nitric acid were added to the spleen tube and homogenized for 2 min. The protein precipitation solution was prepared with 0.53N HCl and 5.3% trichloroacetic acid in HPLC water. The solution containing a homogenized spleen with a closed lid was boiled at 200°C for 30 min. Cooled in RT for 2 min, and caps were opened to release air bubbles. Finally, the solution was centrifuged at full speed for 10 min. For total iron quantification, the supernatant from all the groups (n = 3) was taken.

ICP method: A peristaltic pump was used to deliver the samples (0.1 mL/min) to the nebulizer, converting the sample into a spray mist using argon (Ar) gas. Automated adjustments were made for torch alignment, detector voltage, and ion lens voltages for optimized resolution, sensitivity, and stability across a broad range of atomic masses. The doubly charged ion/charge ratio (Ce 69.95 to Ce 139.90) and oxide ratio (CeO 155.90 to Ce139.90) were also monitored and were maintained below 3% and 2.5%(intensity), respectively. The Prep Fast system was interfaced with the ICP–MS and auto-diluted the stock solutions to generate the calibration curves and QCs samples. The 30-ppb Indium (In) solution was also mixed in-line by the Prep-Fast during the analysis as an internal standard to all the analyzed samples and standards. The method was evaluated for its selectivity, sensitivity, linearity, precision, and accuracy.

2.2.21 Statistical analysis

In experiments with multiple groups, ordinary one-way ANOVA with Tukey's post hoc test was used. In experiments with multiple groups, which are time-course studies, repeated measures of one-way ANOVA were used. The two-tailed Student's t-test with Welch's corrections was used to compare two experimental groups. The probability value (P) < 0.05 was considered as a statistically significant difference. Statistical analysis and graphing were performed with GraphPad PRISM 9.

2.3 Results

2.3.1 Charged electrospun nanofibrous sheets efficiently scavenge DAMPs

To synthesize polymers with anionic/cationic charges to generate electrospun nanofibrous sheets, we have selected poly (methyl vinyl ether-*alt*-maleic acid) [PMVEMA, average Mw: 330,000], to which 50% of tetradecyl amine was functionalized to impart hydrophobic nature to the polymer. Subsequently, either 25% of taurine or 11% of hexyl acridine were functionalized to obtain anionic (poly-Tau) and cationic (poly-Acr) polymers, respectively. In addition to furnishing cationic charge, acridine can also bind DNA by intercalation, which could enhance the ability of *Acr*-NFS to scavenge extracellular DNA. Anionic (*Tau*-NFS) and cationic (*Acr*-NFS) electrospun nanofibrous sheets were generated through conventional electrospinning of *poly*-Taurine and *poly*-Acridine, respectively (Methods). *Tau*-NFS and *Acr*-NFS are comprised of nanofibers with 100–200 nm width and >50–100-micron length (**Fig. 2.3a**). Furthermore, contact angle measurements show that both NFS are hydrophobic (**Fig. 2.3b**), which is critical to prevent aqueous fluid absorption. Surface charge measurements confirmed the overall negative and positive charge of *Tau*-NFS and *Acr*-NFS, respectively (**Fig. 2.3c**). A 2-h incubation of *Tau*-NFS and *Acr*-NFS in Optisol (AS-5) solution that is used as a preservative for storing RBCs suggested that NFS are stable in preservative solution, and the quantification of leached polymers shows that neither polymer have any significant leaching (<0.2%). Scanning electron microscope images of *Tau*-NFS and *Acr*-NFS post-incubation with stored RBCs show that RBCs or neutrophils do not adhere to NFS (**Fig. 2.3d**); hence, there will not be any loss of RBC count due to the NFS treatment. Additionally, the hemolysis rate was measured upon incubation of RBCs with *Tau*-*Acr*NFS at 37 °C for 1 h. A <1% of hemolysis was observed for *Tau*-*Acr*-NFS (**Fig. 2.3e**). Cumulatively, these results suggest that *Tau*-*Acr*NFS are stable and do not cause significant hemolysis. However, it should be noted that to evaluate hemocompatibility quantitatively, highly sensitive chemical analyses, such as by untargeted liquid chromatography–mass spectrometry (LC–MS/MS), will be required to determine any biochemical changes following exposure of RBCs to *Tau*-*Acr*NFS treatment. These experiments will be done in the next study.

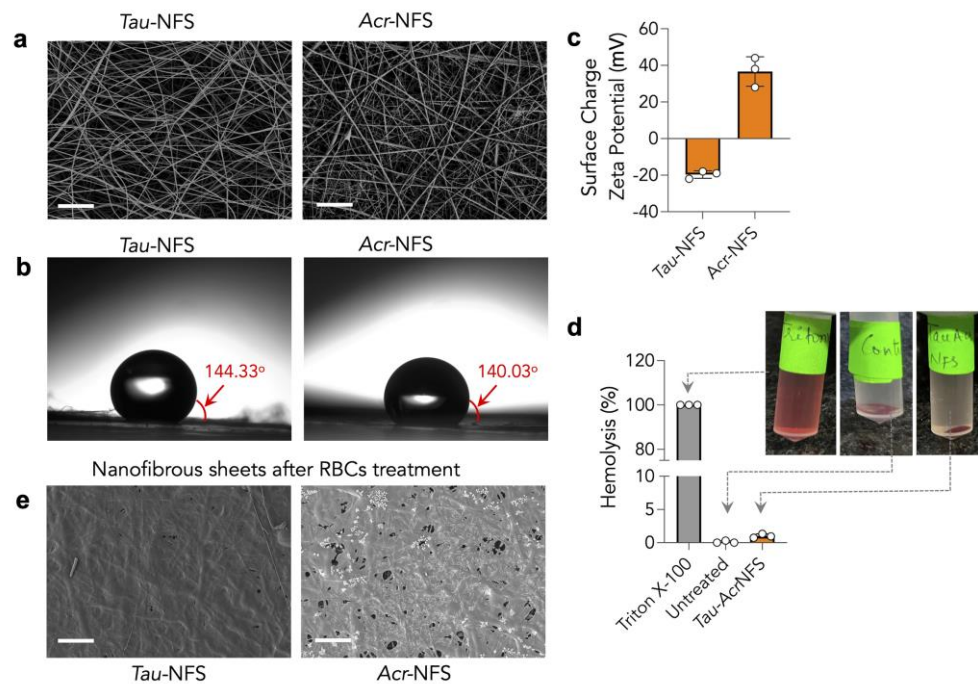


Fig 2.3 Hemocompatible charged nanofibrous sheets scavenge storage lesions to improve the quality of blood. a.) Scanning electron microscopy images of Tau-NFS and Acr-NFS reveal the presence of nanofibrous mesh with a high aspect ratio (scale bar = 2 μ m, representative images were selected from 15 images from three independent experiments). **c.)** Surface charge of Tau-NFS and Acr-NFS indicate that Tau-NFS has a net negative charge (-19.7 mV) and Acr-NFS has a net positive charge (+36.7 mV). **b.)** Contact angle measurements showed that surfaces of Tau-NFS and Acr-NFS are hydrophobic, which could reduce the absorption of aqueous components during the treatment of stored blood. **d.)** SEM images of Tau-NFS and Acr-NFS post-treatment with stored RBCs reveal that except for deposition of DAMPs, no cells have adhered to the NFS (scale bar = 10 μ m, representative images were selected from 15 images from three independent experiments). **e.)** Hemolysis assay revealed that <1% hemolysis was observed upon treatment with Tau-AcrNFS. Data are mean \pm s.d. (n = 3, from independent experiments).

Before testing the DAMPs-scavenging efficacy of NFS, we quantified the production of DAMPs (extracellular DNA, Hb, and PUFAs) in stored human non-

leukoreduced RBCs in a time-dependent manner for up to 42 days. Various analytical techniques such as PicoGreen assay, ELISA, Drabkin's assay, and LC-MS were used to quantify extracellular DNA, nucleosomes, Hb, and PUFAs, respectively (Methods). Quantitative elucidation of the storage lesion revealed that DAMPs, including extracellular DNA (Fig. 2.5a), Hb (Fig. 2.5b), and PUFAs such as arachidonic acid (AA, Fig. 2.5c) and hydroxy eicosatetraenoic acids (5-HETE, 12-HETE, and 15-HETE, Fig. 2.5d–f) increase over time. For example, on the 42nd day, extracellular DNA and Hb concentration increased by ~150-fold and 40-fold, respectively, compared to the 0th day. These observations are in agreement with previous reports²⁵⁻²⁸.

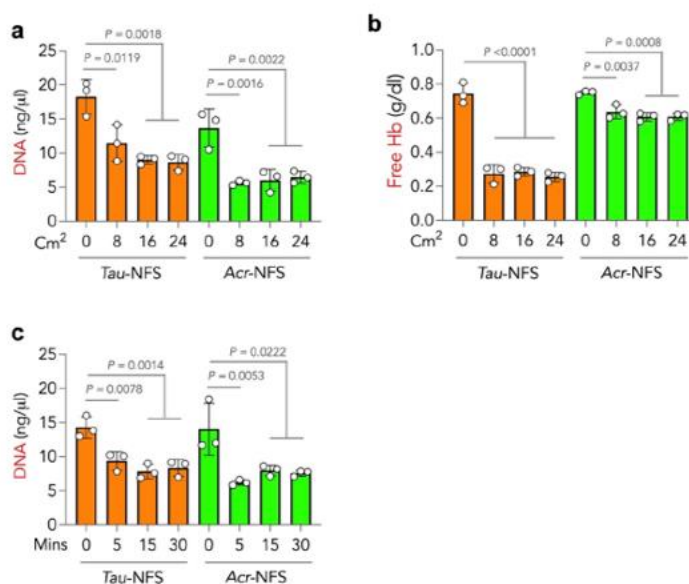


Figure 2.4. Scavenging of DAMPs using charged nanofibrous sheets of varying surface area & time of incubation. a, b.) 42 days-stored RBCs were incubated with either Tau-NFS or Acr-NFS with varying surface area (8, 16, and 24 cm²). **c.)** 42 days-stored RBCs were incubated with either Tau-NFS or Acr-NFS with varying incubation time (5, 15, and 30 mins).

At the onset, we have systematically optimized the required surface area of nanofibrous sheets and incubation time to scavenge DAMPs. One millilitre of RBCs (42 days stored) was added to the different surface areas of sheets (8, 16, and 24 cm²) and incubated for 30 min at 4 °C. Quantification of extracellular DNA suggested that both nanofibrous

sheets could scavenge DNA efficiently with an 8 cm² sheet, and above 8 cm² up to 24 cm² did not increase the scavenging capacity (**Fig. 2.4a**)

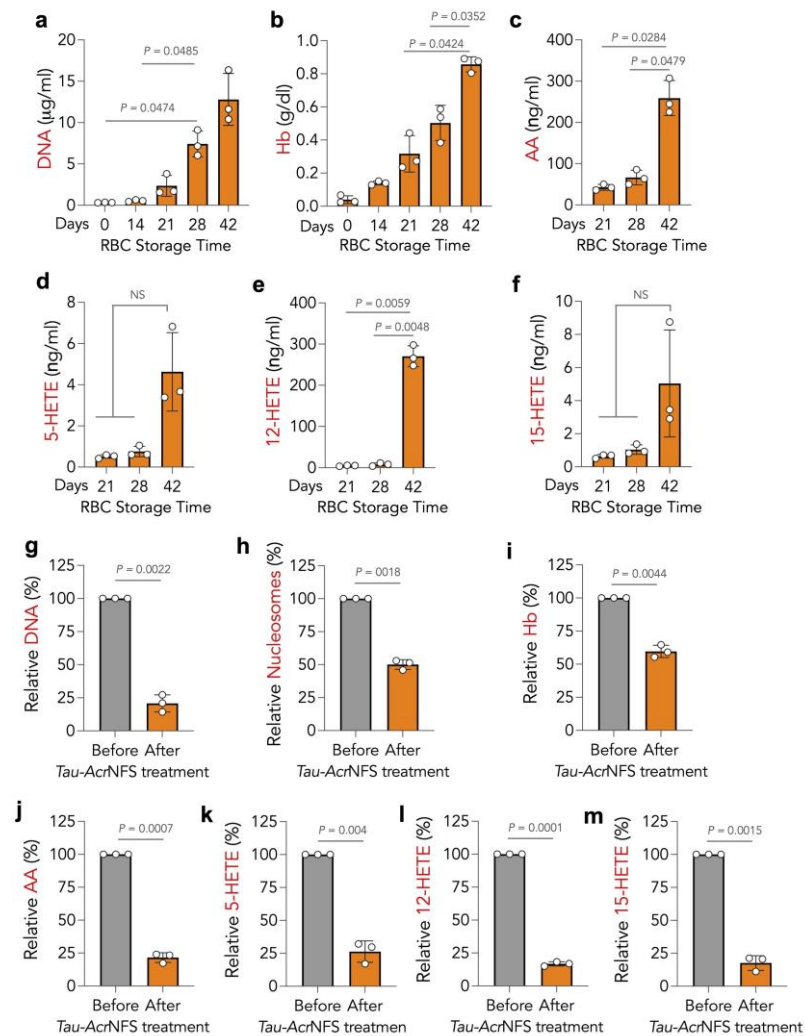


Fig 2.5: Storage-induced production of DAMPs as a function of time. RBCs stored at 4 °C progressively produce DNA a), free hemoglobin (Hb, b), and polyunsaturated fatty acids (PUFAs) such as AA (c), 5-HETE (d), 12-HETE (e), and 15-HETE (f). g–m.), Incubation of DAMPs accumulated 42 days-stored RBCs with anionic and cationic NFS, Tau-AcrNFS, for 5 min at 4 °C efficiently scavenged and reduced the concentration of DAMPs significantly. The relative concentration of DAMPs before and after Tau-AcrNFS treatment has significantly reduced accumulated DNA (g), nucleosomes (h), Hb (i), and PUFAs (j–m). Data are mean \pm s.d. (n = 3, from independent experiments).

Subsequently, RBCs were incubated on 8 cm² nanofibrous sheets for 5, 15, and 30 min to identify the optimum incubation time. Data suggests that Tau-NFS and Acr-NFS could scavenge the DNA within 5 min of incubation, and more prolonged incubation did not enhance scavenging capacity (Fig. 2.4b). To scavenge all DAMPs (extracellular DNA, nucleosomes, Hb, and PUFAs) from old RBCs, 1 ml of 42 days-stored RBCs were incubated with cationic/anionic nanofibrous sheets (Tau-AcrNFS) for 5 min at 4 °C. Data in **Fig. 2.5(g–m)** suggests that Tau-AcrNFS are efficient in scavenging DAMPs. Upon incubation, nanofibrous sheets scavenged 80% of DNA (**Fig. 2.5g**), 50% of nucleosomes (**Fig. 2.5h**), 45% of Hb (Fig. **2.5i**), and 75–80% of PUFAs including AA, 5-HETE, 12-HETE, and 15-HETE (**Fig. 2.5j–m**). These results suggest that Tau-AcrNFS can efficiently scavenge and significantly reduce the concentrations of DAMPs in 42 days of stored old RBCs. SEM images of Tau-NFS and Acr-NFS post-treatment with stored RBCs (**Fig. 2.3e**) shows a gummy layer on the surface, which could be due to the accumulation of sequestered proteins and lipids on the nanofibrous sheets.

2.3.2 Intermittent scavenging of DAMPs by Tau-AcrNFS significantly enhances the quality of stored old RBCs

The data in **Fig. 2.6a–f** suggest that DAMPs formation is minimal until 28 days of storage; subsequently, an exponential production of DAMPs occurs. It is reasoned that the accumulated DAMPs, until 28 days, might have a synergistic effect on accelerating the deterioration of stored human RBCs; hence, large quantities of DAMPs are produced within 42 days. Therefore, we envisaged that intermittent removal of DAMPs either on the 21st or 28th-day using Tau-AcrNFS would reduce further damage to RBCs and enhance the quality of 42 days-stored RBCs. To test this hypothesis, either on the 21st or 28th day, stored RBCs were incubated with Tau-AcrNFS for 5 min (**Fig. 2.6a**), stored back at 4 °C, and measured the DAMPs on the 42nd day. The data in **Fig. 2.6 (b–h)** suggest that intermittent removal of DAMPs either on the 21st or 28th day significantly reduces the number of DAMPs present on the 42nd day. Furthermore, intermittent treatment either on the 21st or 28th day has remarkably reduced the formation of cell-free DNA, nucleosomes, cell-free Hb, and PUFAs by 75, 50, 50, and 75–98%, respectively, compared to 42 days of stored untreated RBCs (**Fig. 2.6b–h**).

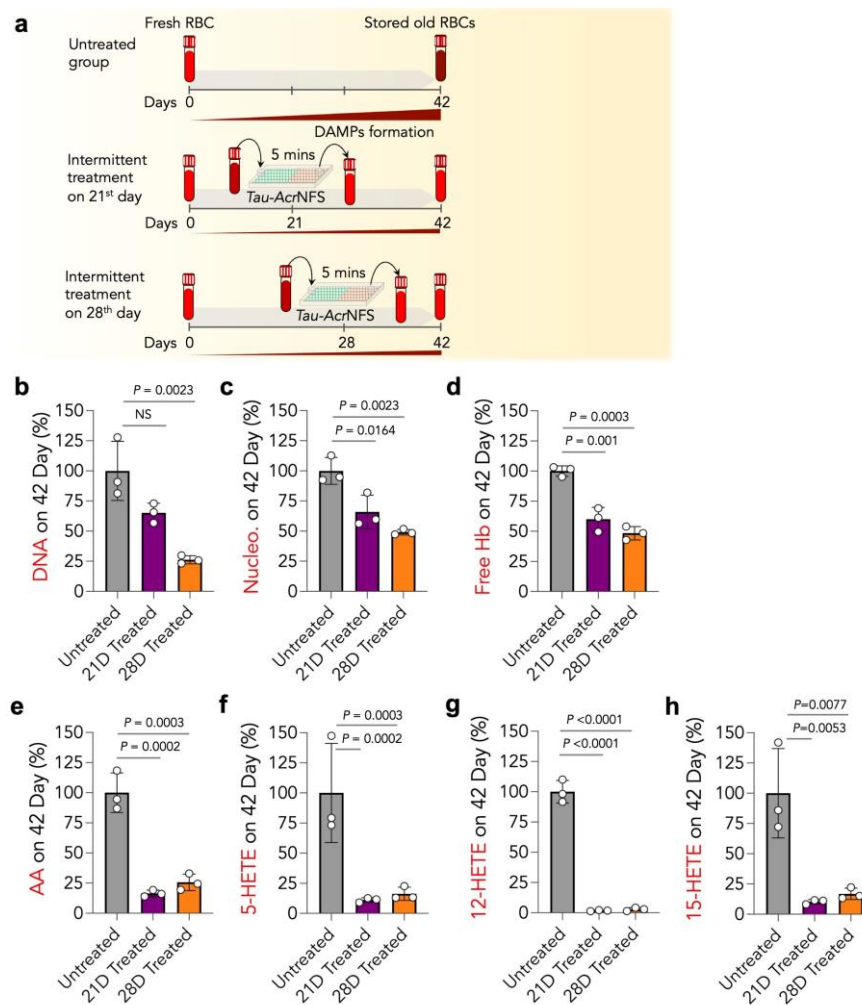


Fig 2.6 a.) A schematic of DAMPs production in untreated and Tau-AcrNFS treated stored RBCs. In the untreated group, RBCs were stored at 4 °C for 42 days, in treated groups, RBCs were incubated with Tau-AcrNFS for 5 min on either the 21st or 28th day, and stored at 4 °C for 42 days, and DAMPs were quantified on 42nd day for all groups (b–h). Intermittent treatment of stored RBCs with Tau-AcrNFS on either the 21st or 28th day significantly reduced the accumulation of DNA (b), nucleosomes (c), Hb (d), and PUFAs (e–h) on the 42nd day. Data are mean ± s.d. (n = 3, from independent experiments);

RBCs' shape and membrane rigidity play critical roles in their quality, function, and fate, such as gas transport capacity and RBC clearance from circulation^{29,30}. RBCs should stretch/elongate and undergo deformation to pass through capillaries. Hence to retain function, they should be mechanically stable and withstand extensive passive deformation²⁸. DAMPs are known to have a deleterious effect on RBCs' quality. The morphology of RBCs progressively deteriorates throughout hypothermic storage. While discocytes are considered healthy RBCs, upon storage, they progressively convert into sphero-echinocytes and echinocytes due to loss of the membrane and reduced surface area (**Fig. 2.7a**). Storage-aged RBCs have fewer discocytes and more sphero-echinocytes. Therefore, we investigated whether intermittent DAMPs scavenging either on the 21st or 28th day by Tau-AcrNFS can prevent/reduce the transformation of discocytes into sphero-echinocytes. Fresh RBCs and storage-aged RBCs (42nd day) were imaged through a field-emission scanning electron microscope and estimated the number of discocytes and sphero-echinocytes. The images revealed that most RBCs were transformed into either sphero-echinocytes or spherocytes, while healthy discocytes reduced significantly (**Fig. 2.7b**). The number of discocytes present in untreated 42 days of stored RBCs sample was considered 100% (counted from 10,000 cells, **Fig. 2.7c**). Similarly, the number of discocytes and sphero-echinocytes were counted in 42 days of stored RBCs samples that were untreated and intermittently treated with Tau-AcrNFS on either the 21st or 28th day (**Fig. 2.7b–d**). The intermittent treatment has increased total discocytes on the 42nd day by 250 and 350%, respectively (**Fig. 2.7c**). On the contrary, compared to untreated samples, intermittent treatment on the 21st or 28th day has decreased the total number of sphero-echinocytes on the 42nd day to 30 and 15%, respectively (**Fig. 2.7d**). These results suggest that intermittent removal of DAMPs using Tau-AcrNFS reduces the conversion of healthy discocytes into sphero-echinocytes, hence, improves the quality of stored RBCs. Furthermore, we have conducted the osmotic fragility test to characterize RBC membrane integrity by subjecting stored RBCs to a hypotonic solution (**Fig. 2.7e**). Mean cell fragility (MCF) is the concentration of NaCl at which 50% hemolysis occurs. The quality of the RBC membrane is inversely proportionate to the MCF value. We have observed 0 and 100% hemolysis in 0.9% (isotonic) and 0.1% (hypotonic) NaCl solutions, respectively. The MCF values for 0, 21, 28, and 42 days of stored RBCs were 0.36, 0.45, 0.5, and 0.64% NaCl, respectively (**Fig.2.7f**), suggesting that the membrane integrity of RBCs

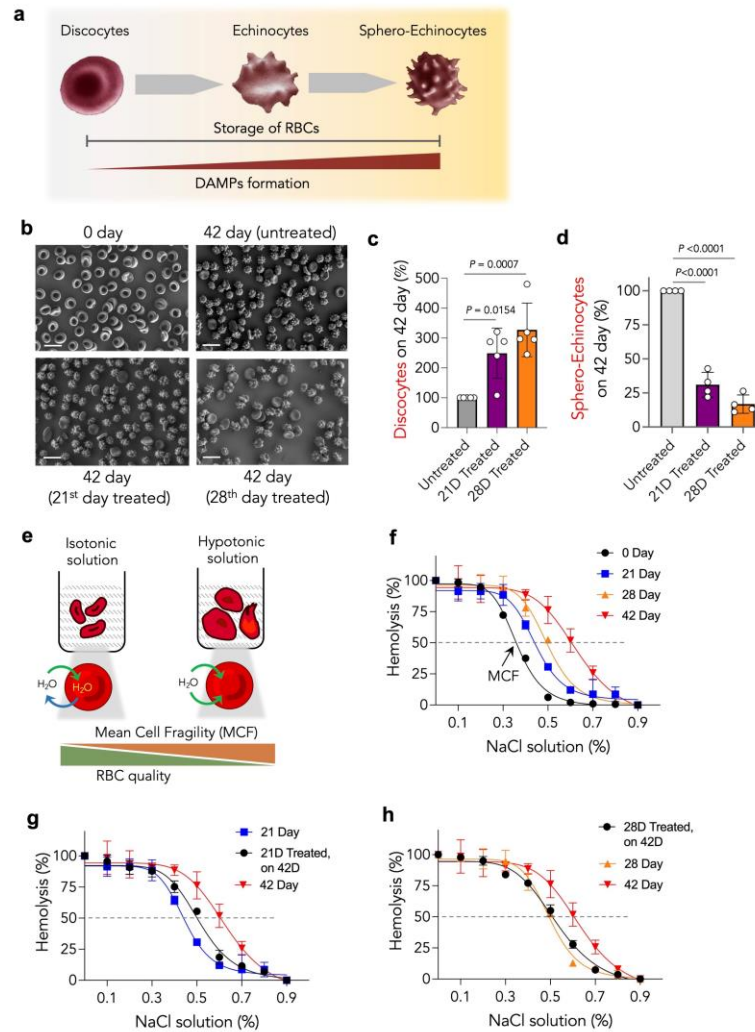


Fig 2.7: a.) a Schematic of storage-induced structural transformation of healthy discocytes into non-healthy echinocytes and sphero-echinocytes. b SEM images of freshly collected RBCs (0th day), and storage-aged RBCs on the 42nd day. (c) revealed that intermittent scavenging of DAMPs using Tau-AcrNFS has increased by 250% and 350% for the 21st and 28th day treated RBCs, respectively ($n = 5$, from independent experiments). (d) Intermittent treatment on the 21st or 28th day has decreased the total number of sphero-echinocytes on the 42nd day to 30 and 15%, respectively. Schematic of osmotic fragility test to measure the mean cell fragility (MCF) by placing RBCs in a hypotonic solution, where RBC swells and undergoes hemolysis (e). The lower MCF values are equivalent to higher membrane integrity. MCF values of stored RBCs progressively increased as a function of storage time (f), suggesting that loss of membrane integrity of RBCs during the storage. MCF values of 42 days of stored RBCs that were intermittently treated were lower than untreated stored old RBCs (f, g), suggesting that intermittent removal of DAMPs reduced the damage of RBCs and enhanced the

progressively deteriorates upon storage. Interestingly, MCF values for 42-day RBCs, which were intermittently treated with Tau-AcrNFS either on the 21st or 28th day, were 0.5 and 0.51%, respectively (**Fig. 2.7g, h**), suggesting that intermittent removal of DAMPs slows down the deterioration of RBC membrane quality. We have observed 0 and 100% hemolysis in 0.9% (isotonic) and 0.1% (hypotonic) NaCl solutions, respectively. The MCF values for 0, 21, 28, and 42 days of stored RBCs were 0.36, 0.45, 0.5, and 0.64% NaCl, respectively (**Fig. 2.7f**), suggesting that the membrane integrity of RBCs progressively deteriorates upon storage. Interestingly, MCF values for 42-day RBCs, which were intermittently treated with Tau-AcrNFS either on the 21st or 28th day, were 0.5 and 0.51%, respectively (**Fig. 2.7g, h**), suggesting that intermittent removal of DAMPs slows down the deterioration of RBC membrane quality.

2.3.3 Transfusion quality of Tau-AcrNFS treated stored old RBCs is comparable to freshly collected RBCs

Post RBCs transfusion, measuring the duration of transfused RBCs remaining in circulation (*in vivo* recovery) is a gold standard for quantifying the quality of stored RBCs³¹. A higher % of RBCs stay longer in circulation, which signifies the better quality of transfused RBCs. According to the FDA guidelines, transfusion of stored human RBCs is considered as successful when $\geq 75\%$ of transfused human RBCs survive in circulation after 24 h³². Hence, similar standards were adopted to quantify the efficiency of the transfusion of stored mouse RBCs into mice³³. Therefore, as a proof-of-concept, we measured the transfusion efficiency of Tau-AcrNFS treated stored mouse RBCs into mice, *in vivo*. The shelf life of C57BL/6J mice RBCs is reported to be 14 days, equivalent to 42 days stored human RBCs³⁴. First, we demonstrated that stored mouse RBCs generate DAMPs, such as cell-free Hb and cell-free nucleosomes, as a function of storage time (**Fig. 2.8a, b**). Subsequently, intermittent scavenging of DAMPs from stored mouse RBCs on the 5th and 10th days using Tau-AcrNFS reduced the production of DAMPs on the maximum allowed storage time, i.e., 14 days (**Fig. 2.8c, d**). To optimize intermittent treatment for RBC recovery experiments, mouse RBCs were intermittently treated with Tau-AcrNFS either once (on the 5th day or 10th day) or twice (on the 5th and 10th days), and DAMPs were measured on the 14th day (**Fig. 2.8 c, d**).

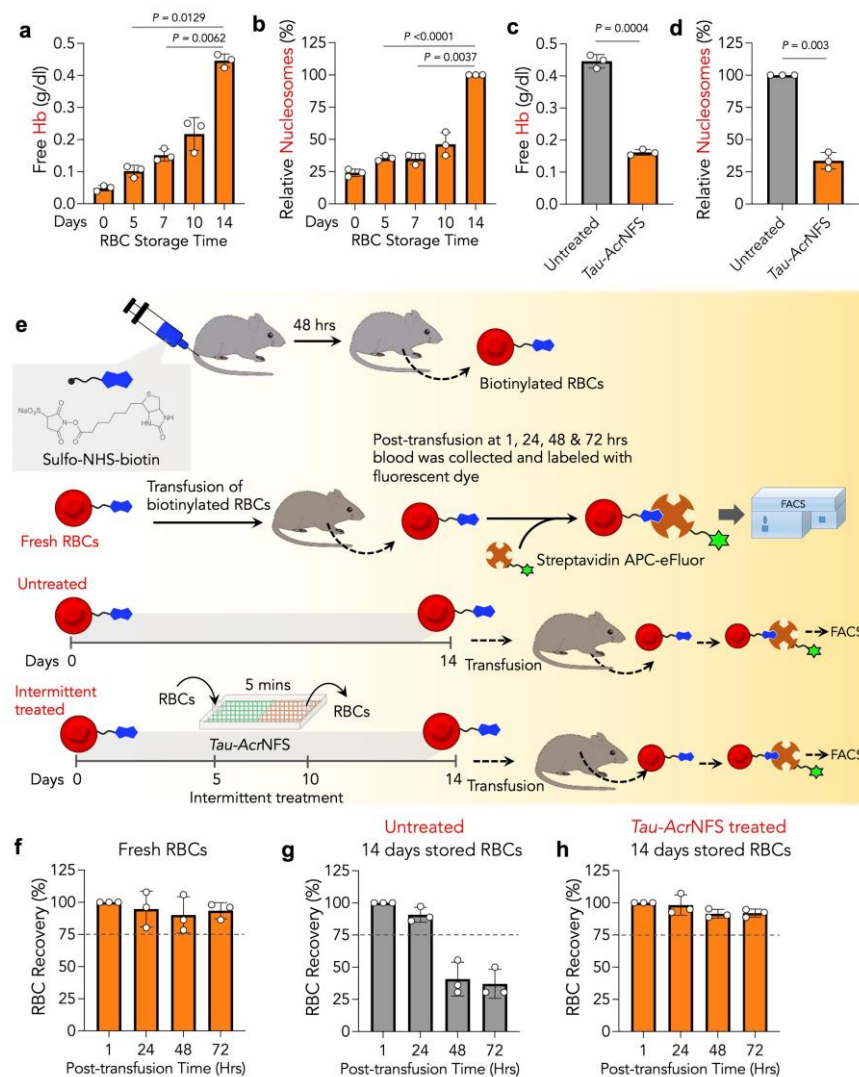


Fig 2.8 Mice RBCs generate DAMPs during their storage, and intermittent treatment with Tau-AcrNFS enhanced the quality of stored old RBCs equivalent to fresh RBCs *in vivo*: **a, b.)** Stored mice RBCs produced DAMPs such as free hemoglobin and nucleosomes. **c, d)** The relative concentration of DAMPs before and after Tau-AcrNFS treatment has significantly reduced accumulated Hb (c), and nucleosomes (d). **e–h** Biotinylation of RBCs, and post-transfusion recovery of RBCs, *in vivo*. Schematic of RBC recovery experiments, *in vivo*. In untreated stored RBCs, ~90% of RBCs were in circulation for 24 h but rapidly cleared thereafter (g). On the contrary, post-transfusion of intermittently treated RBCs, >90% of transfused-RBCs were found in circulation even at 72 hrs (h), suggesting that intermittent treatment has increased the quality of stored old RBCs equivalent to fresh RBCs. Data in a–d, and f–h are mean \pm s.d. (n = 3, from independent experiments).

For mouse RBC recovery experiments, stored mouse RBCs were intermittently treated on the 5th and 10th days. Cumulatively, these results suggest that intermittent scavenging of DAMPs might prevent progressive damage to RBCs and enhance transfusion efficiency.

In RBCs transfusion experiments, we labeled RBCs with biotin, *in vivo*. Intravenous administration of sulfo-NHS-biotin covalently binds and labels RBC membrane proteins with biotin³. After 48 h of post-administration of sulpho-NHS-biotin (1 mg/mouse), blood was collected from the same mouse. The biotinylated RBCs were transfused into a different mouse to study transfusion efficiency (**Fig. 2.8e**). Blood was collected at 1, 24, 48, and 72 h post-transfusion of biotin-labeled RBCs. The collected RBCs were complexed with Streptavidin APC-eFluor™ and quantified by flow cytometry to measure the % of labeled transfused RBCs that remain in circulation. Interestingly, ~95% of transfused fresh RBCs have remained in circulation for 72 h (**Fig. 2.8f**), suggesting that freshly collected RBCs have the highest quality. To test the effect of storage, the biotin-labeled mouse RBCs were stored for 14 days, either without treating or intermittently treating with Tau-AcrNFS on the 5th and 10th days (**Fig.2.8e**). After 14 days, biotinylated RBCs were transfused into mice, and tracked their circulation time. A total of 90% of untreated transfused RBCs were in circulation for 24 h, which was reduced to <40% at 48 h (**Fig.2.8g**), suggesting that the quality of stored old RBCs deteriorated significantly. On the contrary, Tau-AcrNFS treated stored old RBCs remain >90% in circulation even at 72 h, similar to fresh RBCs (**Fig.2.8h**), suggesting that intermittent treatment slows down stored RBC aging, and prevents their clearance from circulation by the spleen. Remarkably, these findings indicate that intermittent scavenging of DAMPs reduced the deterioration of stored RBCs, and the quality of 14 days-stored old RBCs is equivalent to freshly collected RBCs.

2.3.4 Intermittent scavenging of storage lesion with Tau-AcrNFS enhances the shelf-life of stored old RBCs

In situ-generated DAMPs deteriorate RBC quality during storage, limiting their maximum shelf-life to 42 and 14 days for human and mouse RBCs, respectively. Intermittent removal of DAMPs by Tau-AcrNFS treatment enhanced the quality of RBCs; therefore, we envisaged that enhanced quality of RBCs may increase the shelf-

life of stored old RBCs. Initially, the phospholipid architecture of RBCs was studied to understand their shelf-life. Storage-associated loss of phospholipid asymmetry results in phosphatidylserine (PS) exposure in the outer leaflet of the RBC membrane³⁶⁻³⁸. PS exposure serves as an ‘eat-me’ signal to phagocytes to clear the RBCs from circulation^{36,39} (**Fig. 2.9a**). PS exposure increases with storage and is linked with DAMPs production⁴⁰, hence, the low survivability of stored RBCs in circulation. Therefore, the number of RBCs expressing PS on the outer leaflet of their membrane was determined as described elsewhere⁴¹. In untreated stored RBCs, we found that 4, 20, and 40% of PS-exposed RBCs were present in 5, 14, and 17 days of stored mouse RBCs, respectively (**Fig. 2.9b**). On the contrary, intermittent scavenging of DAMPs by Tau-AcrNFS has significantly reduced PS-exposed RBCs on days 14 and 17 (**Fig. 2.9c**). Hence, the overall quality of RBCs is higher in the intermittently treated samples even on the 17th day, which is three days longer than the maximum allowed 14 days.

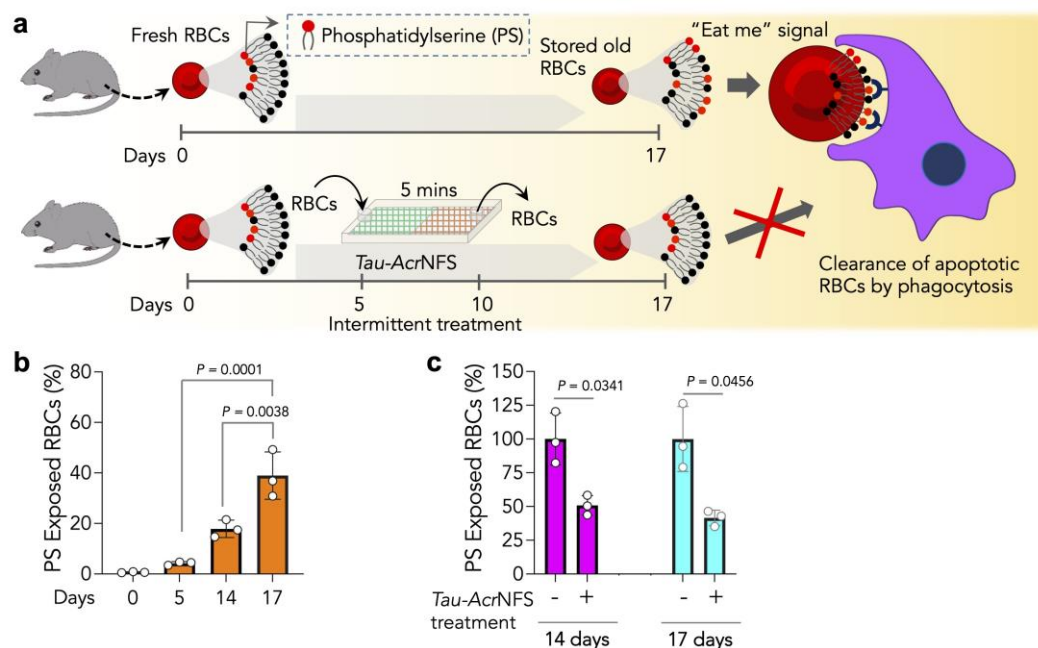


Fig 2.9: a–c.) During the storage, exposure of phosphatidylserine (PS) on the outer leaflet of the RBC membrane serves as an ‘eat-me’ signal, thus, RBCs will be eliminated from circulation by phagocytes. Intermittent scavenging of DAMPs by Tau-AcrNFS reduces PS exposure, therefore, keeping RBCs in circulation for longer periods (a). Time-dependent PS exposure on mice RBCs (b), intermittent treatment reduced PS exposure on RBCs (c). Data are mean \pm s.d. ($n = 3$, from independent experiments).

Furthermore, we have conducted RBC recovery experiments using long-term stored mouse RBCs. As described in the previous section, biotinylated murine RBCs were stored for up to 18 days either without treating or intermittently treated with Tau-AcrNFS on the 5th and 10th days. The different days of stored biotinylated RBCs (15–18 days) were transfused into mice, and measured the percentage of transfused RBCs in circulation (**Fig. 2.10 a–d**). As expected, when DAMPs were not scavenged, 15–17 days-stored RBCs could not remain longer in circulation, and the number of transfused RBCs reduced to <30% within 24 h, which is well below the accepted 75% range. On the contrary, 15–17 days-stored RBCs when they were intermittently treated with Tau-AcrNFS, post-transfusion, >95% of transfused RBCs remained in circulation after 24 h (**Fig. 2.10a–c**). However, whether treated or not, 18 days-stored RBCs could not remain in circulation for 24 h (**Fig. 2.10d**), suggesting that the upper limit for storing the intermittently treated RBCs is 17 days. Therefore, the maximum shelf life of murine RBCs was increased from 14 days to 17 days.

Post-blood transfusions, DAMPs are known to cause systemic immunomodulation. Hence, we tested the efficacy of Tau-AcrNFS treatment to prevent transfusion-induced immunomodulation. To test that, we have transfused fresh RBCs (0 day), untreated and intermittently treated 17 days-stored RBCs in C57BL/6J mice (8–12 weeks). Two hours post-transfusion, blood was collected, and cytokines/chemokines in plasma were quantified using Mouse Cytokine Array Panel A (Methods, **Fig. 2.10e**). Transfusion of untreated 17 days-stored RBCs has elevated expression of stromal cell-derived factor1 (CXCL12), macrophage-colony-stimulating factor (M-CSF), complement component 5A(C5A), and keratinocyte-derived chemokine/CXCL1 (KC/CXCL1). Transfusion of untreated 17 days-stored RBCs has led to 2–3 fold higher cytokine production levels than either fresh RBCs or intermittently treated 17-days-stored RBCs (**Fig. 2.10e**). Additionally, mice spleens were collected 2 h post-transfusion, and spleens were significantly darkened when transfused with untreated RBCs compared to treated RBCs (**Fig. 2.10f**). The darkening of the spleen is associated with iron deposition after transfusing poor quality RBCs, loaded with free Hb, which corroborated by Fe²⁺ quantification using ICP spectroscopy (**Fig. 2.10g**). These results suggest that intermittent scavenging of DAMPs using Tau-AcrNFS enhanced the quality of stored old RBCs equivalent to fresh RBCs and significantly enhanced their shelf-life.

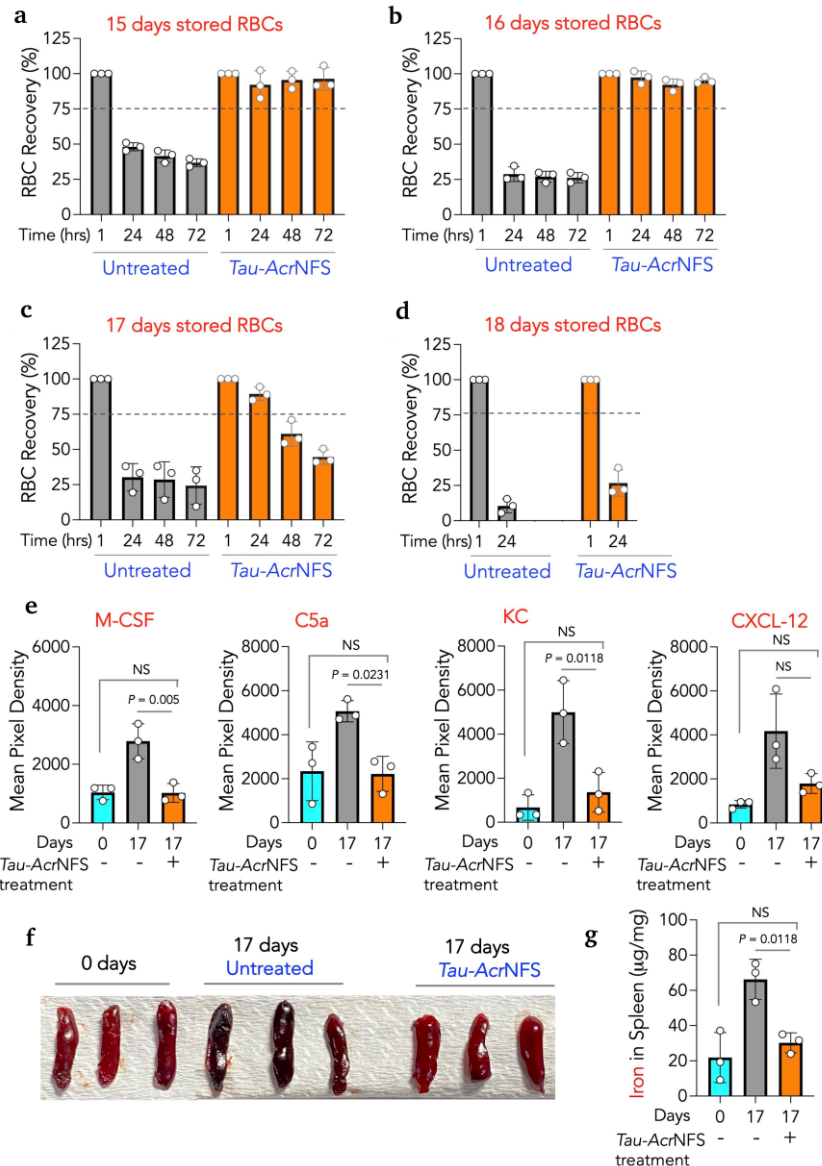


Fig 2.10 a–g.) Recovery of storage-aged RBCs either untreated or intermittently treated with Tau-AcrNFS on the 5th & 10th days. Recovery data suggests that the quality of untreated RBCs that are stored for 15–17 days declines drastically, and RBCs are eliminated from circulation immediately after transfusion. Only 15–20% of transfused RBCs were found in circulation after 24 h (a–d), which is well below the accepted 75%. On the contrary, intermittently treated RBCs, even after storing for 17 days, >95% of transfused RBCs were found in circulation at 24 hrs (a–c), suggesting that intermittent removal of DAMPs has decreased RBC damage, and enhanced the shelf-life of RBCs at least by 22%. Two hours of post-transfusion, serum inflammatory cytokines were quantified (e), spleens were collected and imaged (f), and iron in the spleens was quantified using ICP-AES (g). Data are mean \pm s.d. (n = 3, from independent experiments).

2.4 Discussion

Transfusion of RBCs is a lifesaving practice, which is limited by the progressive deterioration of stored RBCs as a function of time. In situ generated DAMPs such as cell-free DNA, cell-free nucleosomes, free Hb, free iron, and polyunsaturated fatty acids are the primary cause of declining RBCs condition. Large observational studies have reported a mean duration of RBC storage of between 16 and 21 days, with a maximum storage duration before transfusion is limited to 42 days with standard preservative solutions⁴². Until now, existing strategies are limited to developing preservatives and rejuvenating solutions. Additionally, engineered-proteins-containing filters have been developed to absorb pathogen-specific toxins^{43,44}. However, there was no effort made to scavenge DAMPs during RBC storage to prevent RBC damage. Interestingly, these DAMPs carry either cationic or anionic charges⁴⁵. Therefore, we have designed polymers with an appropriate hydrophilicity/hydrophobicity balance, and carrying anionic (due to taurine) or cationic (due to acridine) charges. Robust electrospun nanofibrous sheets (Tau-AcrNFS) were generated using these polymers. The charged Tau-AcrNFS do not cause hemolysis, and upon incubation, RBCs or any other cells do not adhere to these sheets, suggesting that Tau-AcrNFS are robust and consist of key parameters that play a critical role in translating this technology into a medical device.

Interestingly, scavenging DAMPs using Tau-AcrNFS has a dual advantage. When Tau-AcrNFS treatment is used terminally at the end of the storage, they efficiently scavenge in situ generated DAMPs from stored blood. On the other hand, intermittent treatment of stored blood using Tau-AcrNFS can prevent the damage of RBCs, hence, significantly lowering the production of DAMPs until their maximum storage time. We have identified a suitable storage day for intermittent treatment through systematic time-dependent experiments. We found that 28th-day treatment results in a maximum reduction of DAMPs and retains healthier RBCs on the 42nd day. This observation could be because the accelerated deterioration of RBCs majorly occurs between the 28th day and to 42nd day of storage.

The structural integrity of RBCs plays a vital role in increasing their post-transfusion circulation time. The osmotic fragility measurements revealed that compared to untreated RBCs, removal of DAMPs on the 28th day using Tau-AcrNFS enhanced 42

days stored human RBC's membrane integrity and reduced PS-exposure on the outer leaflet of the membrane to reduce 'eat-me' signals. It was envisaged that maintaining membrane integrity should lead to extended survival in circulation. Post-transfusion recovery of murine RBCs affirmed this hypothesis. Murine RBCs can be stored a maximum of 14 days, akin to 42 days for human RBCs. Remarkably, mouse RBCs recovery suggested that intermittent removal of DAMPs enhanced the quality of 14 days-stored old RBCs equivalents to freshly collected RBCs. In addition to improving the quality, intermittent removal of DAMPs has increased the shelf-life by at least 22%. Systemic immunomodulation is one of the hallmarks of the transfusion of stored old RBCs. As we demonstrated here, intermittent removal of DAMPs has lessened the accumulation of DAMPs in stored old RBCs and ultimately reduced immunomodulation. Therefore, it is anticipated that this approach would reduce transfusion-related complications. We are designing a Tau-AcrNFS-based insert in blood bags as a medical device, which will be feasible to use in the blood bank processing setting without the need for multiple interventions during the storage of RBCs.

2.5 Conclusion

In summary, our study implicates in situ generated DAMPs in causing accelerated deterioration of RBCs. We establish a novel approach to slow down the damage of stored RBCs through intermittent scavenging of the storage lesion. In the future, treating with the nanofibrous sheets before the transfusion could significantly reduce DAMPs and prevent transfusion-related complexities. Furthermore, the intermittent treatment adds a new dimension to maintaining RBC quality. Finally, our investigation establishes the concept of scavenging DAMPs during storage, leading to increased quality and shelf-life of RBCs. Furthermore, the use of charged electrospun nanofibrous sheets may lead to the development of novel blood bags or an insert-based medical device. Therefore, it may significantly impact healthcare by improving the RBC transfusion quality.

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Chapter 3

Improving the quality of leukoreduced RBCs by scavenging erythrocytes-associated storage lesions by electrospun nanofibrous sheets

3.1 Introduction

Leuko-reduction (LR) or leuko-depletion is widely employed to reduce the concentration of NETs and histones by filtering out leukocytes from whole blood. The presence of leukocytes in blood components is responsible for several transfusion-related complications¹. It prevents the formation of cytokines/chemokines, avoiding febrile non-hemolytic transfusion reaction (FNHTR) and reducing the transmission of cytomegalovirus²⁻⁵. The LR technique reduces the leukocytes count from 5×10^9 to 5×10^6 per unit. However, residual white blood cells (WBCs) in packed blood units might be linked to an increased risk of infection and organ dysfunction⁶. Past studies suggest that despite the use of additive solutions, changes in RBC morphology and metabolism are expected during the storage of blood bags with and without leuko-reduction⁷⁻¹⁰. It has been extensively studied that the storage of erythrocytes causes some complex structural and biochemical alterations called red cell storage lesions (RCSL)¹¹. Among transfusion of blood or blood components, the non-leukoreduced (NLR) blood transfusion is most common in medical practice in India (or developing countries). Classically, white blood cells (WBCs) and/or platelets (PLTs) are considered as the source of most bioactive lipids, and thus Leukoreduction has been seen as a remedy to this issue; however, this notion has been challenged. The Leukoreduction technology does not remove the RBCs associated lesions, leading to the deterioration of RBCs quality with storage time. In particular, bioactive lipids have been described as accumulating throughout RBC storage and have been implicated as a potential contributor to acute lung injury. Indeed, nonpolar lipids, including arachidonic acid (AA) and its products of oxidation, 5-,12-, and 15- hydroxy eicosatetraenoic acids (HETEs) present in the supernatants of stored RBCs¹² have been identified as a second hit in models of transfusion-related acute lung injury (TRALI) (**Fig 3.1**). In addition, these bioactive lipids can have immunomodulatory effects. They could potentially harm and/or benefit, depending on the clinical situation of the transfusion recipient¹³. Besides the production of bioactive lipids, there is a production of cell-free hemoglobin that leads to impaired vascular function after transfusion¹⁴.

Since the leukoreduction process is expensive; hence, most blood banks in developing countries like India do not use leukoreduction processes routinely¹⁵. The other factor is the cost implicated in universal leukoreduction policy, which is not practically feasible, especially in developing countries and other under-resourced nations¹⁶.

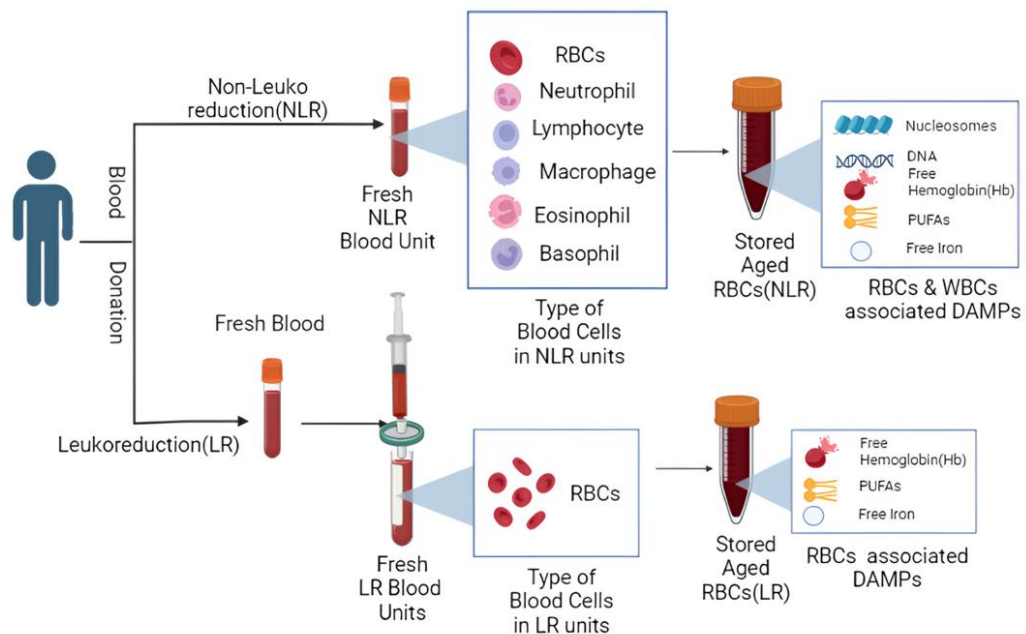


Fig 3.1 Stored NLR RBCs produce storage lesion or damage-associated molecular patterns (DAMPs) such as DNA, nucleosomes, heme (Hb), iron, and polyunsaturated fatty acids (PUFAs). After leukoreduction, LR stored blood leads to the production of RBCs associated DAMPs such as Free Hb & PUFAs. Transfusion of RBCs associated DAMPs could lead to transfusion-related complications, including systemic inflammation and organ injury. The presence of DAMPs progressively reduces the quality of stored LR-RBCs and limits their shelf-life.

Before initiating the project, we received feedback from several blood banks across India regarding using Leukoreduction. Surprisingly, only <10% of blood banks use the leukoreduction process when the recipient is immunosuppressed/ immunocompromised. To maximize the effectiveness of the stored LR blood, elements like blood quality and shelf life need to be considered. Transfusing 42D-old leukoreduced blood with the heavy load of RBCs associated lesions could lead to a greater risk of transfusion-related complications. Here, we have taken the previous approach of intermittent scavenging of DAMPs using electrospun charged nanofibrous sheets that

slows the deterioration of stored LR-RBCs, increasing their quality and shelf-life¹⁷. Critical RBCs associated DAMPs, such as free hemoglobin (Hb), and PUFAs, are charged molecules with anionic or cationic nature. Therefore, we applied the previous “proof of concept” using charged nanofibrous sheets (*Tau-AcrNFS*) made with cationic and anionic polymers to scavenge DAMPs through ionic interactions (**Fig.3.2**). We have demonstrated that a combination of *Tau-NFS* and *Acr-NFS*, hereafter *Tau-AcrNFS*, is efficient in scavenging RBCs associated storage lesion such as free Hb, and PUFAs from stored human LR units, *ex vivo* (**Fig 3.2**). Two significant phenomena were demonstrated using *Tau-AcrNFS*; i) after storage lesions formed in human LR old RBCs (stored for 42 days), *Tau-AcrNFS* could efficiently scavenge and remove accumulated free Hb & PUFAs and ii) the intermittent scavenging of lesions using *Tau-AcrNFS* either at 21st or 28th day resulted in significantly less production of RBCs associated lesions at maximum storage time (on 42nd day), and reduced the loss of LR-RBC membrane integrity. Additionally, the recovery data showed that twice intermittent treatment of 17-day-old LR-RBCs prolonged their circulation in mice for up to 72h. These results are significant, and we aim that newly developed *Tau-AcrNFS* could scavenge DAMPs from leukoreduced blood and improve the quality of 42-day-old LR blood.

3.2 Material

Chamber slides (HI Media), blood collection tubes (BD Vacutainer), citric acid monohydrate, trisodium citrate dihydrate, sodium dihydrogen phosphate, dextrose, sodium chloride, glucose anhydrous, mannitol adenine, APTMS (3-(Aminopropyl)trimethoxy silane), glutaraldehyde, cacodylate buffer, osmium tetroxide, hexamethyldisilane, ethanol, double distilled water and deionized water were used. Unless mentioned otherwise, all chemicals were procured from Sigma-Aldrich.

Method

Packing of human LR RBC units

Blood was collected according to the approved protocols from the Institutional Human Ethical Committee (inStem/IEC-10/003) of the Institute for Stem Cell Science and Regenerative Medicine (inStem). Informed consent has been obtained from the participants. RBC units were prepared from whole human blood (both male and female, n = 3) of different blood groups collected in 20 ml vacutainers containing citrate-

phosphate-dextrose with adenine (CPD-A) that contains citric acid monohydrate (3.577 mg/mL), trisodium citrate dihydrate (29.972 mg/mL), sodium dihydrogen phosphate (2.496 mg/mL) and dextrose (25.5 mg/mL). The blood was passed through leukofilters (Aerodisc WBC 25mm PSF, product id: AP-4952)

The leukofiltered blood was centrifuged for 20 min at 500g, and the supernatant was removed. The removal of supernatant was followed by the addition of Optisol (AS-5, preservative), which contains sodium chloride (8.77 mg/mL), glucose anhydrous (8.18 mg/mL), mannitol (0.01% w/v) and adenine (0.03 mg/mL) to obtain a hematocrit of ~65%, which was transferred into standard PVC bags, and stored at 4 °C in the dark.

3.2.1 Scavenging of DAMPs from stored human RBCs using *Tau*-NFS and *Acr*-NFS, *in vitro*

On different days of stored RBCs (0, 14, 21, 28, and 42) were incubated with *Tau*-NFS and *Acr*-NFS individually and in combination for 5 min in total to scavenge DAMPs. In both treatment conditions, the nanofibrous sheets were prewetted with distilled water for 30 min before adding RBCs. After removing the water, the scavenging experiments were performed on ice under the aseptic condition. *Tau-Acr*NFS was mounted on the chamber slides, and the stored RBCs samples were added. The RBCs samples were ensured to cover the entire surface area and be in contact with nanofibers. Scaffold treatment of RBCs was done at 4 °C for 5 min to keep the cold chain storage. The total surface area of the scaffold was kept at 24 cm² for each experiment.

3.2.2 Quantification of free Hb using Cyanomethaemoglobin (Drabkin's) method

Drabkin's reagent lyses RBCs and oxidizes all forms of Hb, except for the minimally present sulphaemoglobin, to the stable HiCN 22. The supernatant of the stored RBCs unit (scaffold-treated or untreated) was diluted (1:10) with Drabkin's reagent (Sigma, St. Louis, MO, USA). Human Hb diluted with Drabkin's reagent (1:10) (Sigma Aldrich, USA) was used to prepare the standards (0–40 mg/dl) and the calibration curve. The samples and standards were incubated in the dark at RT for 15 min. Absorbance was recorded at 550 nm using a Varioskan LUX Multimode Microplate Reader (ThermoFisher, Massachusetts) USA). The Hb concentration of each sample was calculated from the human Hb calibration curve.

3.2.3 Quantification of free DNA by PicoGreen assay

RBC supernatants from scaffold-treated and untreated groups were thawed on ice. Supernatants were diluted (1:50) with PicoGreen® reagent and Tris-EDTA (TE) buffer (Quant-iT™ PicoGreen™ dsDNA Assay Kit, Invitrogen, USA), followed by 5 min incubation in the dark. Free-DNA estimation was performed using a fluoro spectrophotometer (Horiba Fluro log QM, France)¹⁸.

3.2.4 Quantification of nucleosomes

Stored RBC supernatant was diluted in PBS (1:5). The Anti-Histone-Biotin-Monoclonal antibody (cloneH11-4) and Anti-DNAPOD-Monoclonal antibody from mouse (clone MCA-33) were used for quantifying nucleosomes by ELISA (Catalog No.11920685001, Cell Death Detection kit, Roche, Indianapolis, IN), according to manufacturer's instructions.

3.2.5 Osmotic fragility test

The stored RBC units (treated and untreated group) were added to 9 tubes containing saline concentration series ranging from 0% (distilled water) (Tube 1) to 0.9% (Tube 9). The tubes were gently mixed and incubated at room temperature for 30 min. The incubation was followed by centrifugation at 500g for 20 min, and supernatants were collected. The optical density of the supernatant was measured by Varioskan LUX Multimode Microplate Reader (Thermofisher, Massachusetts, USA) at 540 nm. Hemolysis in each tube was expressed in percentage. The maximum absorbance value of haemolyzed RBCs was taken at 100% in the distilled water. The erythrocytes treated with normal saline were used as a negative control (0% hemolysis).

3.2.6 Bioactive lipid estimation by LCMS

Chemicals and standards

Eicosanoids; 5(S)-HETE, 12(S)-HETE, 15(S)-HETE, and AA were purchased from Cayman Chemical Co. (Ann Arbor, Michigan, USA). 5(S)-HETE-d8 (Cayman Chemical Co) was used as an internal standard (IS) for this study. Acetonitrile (Baker analyzed® LC-MS reagent), water (Baker analyzed® LC-MS reagent), formic acid, ethanol, ethyl acetate (EtOAc), and glacial acetic acid were used in sample preparations for LC-MS/MS measurements.

Preparation of stock solutions

5-(S)-HETE, 12(S)-HETE, 15(S)-HETE, AA, and IS 5(S)-HETE-d8 were constituted in ethanol by the provider. 5, 12 & 15 (S)-HETE was further processed and serially diluted to obtain solutions ranging from 0.05 to 100 ng/ml. AA solutions ranged from 0.5 to 1000 ng/ml. The working concentration of the pure IS was 1000 ng/ml.

Sample preparation

Lipid extraction from stored RBCs supernatant was performed by gently mixing the samples with 0.5 mL of EtOAc containing 0.13% acetic acid (v/v). All samples were spiked with 5 μ l of the IS. After 10 min of vigorous shaking (1500 rpm), samples were centrifuged (10,000 rpm, 10 min, 4 °C), and 450 μ l of the organic layer was transferred to fresh tubes and evaporated in speed vac (Scan Vac, Labogene, Demark) at 4 °C. The dry residue was reconstituted in 25 μ L of nitrogen-purged EtOH and then directly injected (2 μ l) into their LC–MS/MS system.

LC-MS/MS conditions

HPLC analysis was done using a Shimadzu Prominence HPLC system. The instrument was equipped with a communication module (CBM-20A), degassing unit (DGu-20A5R), solvent delivery units (LC-30AD), column oven (CTO-20AC), and an autosampler (SIL-30AC). The column oven temperature was maintained at 40 °C while the autosampler was set at 4 °C. The lipids were separated using an Acquity UPLC® BEH C18 1.7 μ m column (2.1 \times 50 mm). The mobile phase comprised solvent A, i.e., water with 0.1% formic acid (FA) and acetonitrile with 0.1% FA as solvent B. The flow rate was maintained at 0.2 ml/min, and the injection volume was 2 μ l. The gradient for lipid elution (20.10 min) was set as 50% A and 50% B for one minute. This was followed by a linear gradient to 20% A and 80% B for 8 min to elute 5, 12, and 15 HETE. This was followed by a linear gradient to 100% B for 2 min and kept for 5 min to elute AA. Finally, the column was equilibrated at 50% A and 50% B for 4 min before the next injection. LC-MS analysis was performed by QTRAP 5500 (Sciex, Framingham, Massachusetts, USA), a triple quadrupole mass spectrometer. LC–MS analysis was performed by MultiQuant™ 3.0.3 Software (SCIEX). The operating parameters for the mass spectrometer were as follows: declustering potential (DP):

−209.8 V, entrance potential (EP): −5.7 V, collision cell exit potential (CXP): −16.9 V, ion spray voltage: −4500 V, source temperature: 500 °C, curtain gas: 35 psi, ion source gas 1: 40 psi and ion source gas 2: 50 psi. All the spectra of lipids were recorded and quantified in multiple reaction monitoring (MRM) modes. Negative electrospray ionization was employed for all the lipids and the deuterated IS. The ion Q1/Q3 transitions and other parameters can be seen in Table 2.1.

3.2.7 *In vivo* experimental design

Mice

Both male and female C57BL/6J mice aged 8–12-weeks-old were used for the experiments. The animals were housed in the animal facility at the National Center for Biological Sciences, Bengaluru. Animals were caged (maximum, four per cage) during the experiment, and food and water were provided ad libitum. Mice were housed in a facility subject to a 12/12 h light/dark cycle at a temperature of 20–25 °C and humidity of 35–65%. All mouse studies strictly adhered to institutional and national guidelines for humane animal use. The experimental protocols were approved by the Institutional Animal Ethics Committee (IAEC) at the Institute for Stem Cell Science and Regenerative Medicine (INS-IAE-2020/16(R1)).

3.2.8 Packaging and storage of mice LR RBCs and their treatment with *Tau-AcrNFS* to scavenge DAMPs

Blood collected in CPD was immediately transferred and centrifuged at 500g for 20 min at 4°C. Plasma was carefully aspirated, and the RBCs were pooled together. The RBCs were passed through Leukofilters, and then Optisol (AS-5) was added to the LR pRBCs at 65% hematocrit. Totally 550 µL and 250 µL blood units were aliquoted from these pRBCs and stored in sterile Eppendorf tubes (0.6 and 0.3 ml). Mouse pRBCs were treated with *Tau-AcrNFS* (24 cm²) for 5 min on the 5th and 10th day of storage at 40 °C. After the scavenging procedure, the RBCs were again transferred into autoclaved 2 ml Eppendorf tubes, leaving a small residual space, and stored till the 17th day. Its supernatant was taken for extracellular Hb and nucleosome estimation.

3.2.9 Transfusion of RBCs

Biotinylation of RBCs, *in vivo*

Briefly, Sulfo–NHS Biotin (ThermoFisher Scientific, US) powder was dissolved in sterile PBS, vortexed, and passed through a 0.22 µm filter. It was diluted to a final concentration of 1 mg biotin–sulfo–NHS per 300 µL 1× PBS. Mice were restrained, and the biotin reagent solution was administered intravenously. After 48 h of the biotin administration, under mild anesthesia, around 1 ml of biotinylated blood was collected through a cardiac puncture in CPD-containing vials and immediately centrifuged at 500g for 20 min at 4 °C. Plasma was carefully aspirated, and pRBCs were pooled together. The biotin-labeled blood was processed and stored using Optisol as a preservative in sterile Eppendorf tubes (0.5 and 1 ml).

Treatment of Biotin-labeled RBCs with *Tau-AcrNFS*

Functionality tests Biotin-labeled RBCs were divided into treated and untreated groups. The treatment group (n = 3) pRBC units were treated with *Tau-AcrNFS* on the 5th and 10th days of storage and then packed until 18 days. The untreated group pRBCs units (n = 3) were not treated with sheets during the storage timeline. Both groups were analyzed for phosphatidylserine (PS) exposure during evaluating the enhanced shelf-life of biotin-labeled RBCs by recovery experiment on different days of storage (0, 7, 14, 15, 16, 17, and 18 days), biotinylated pRBC units with and without treatment were diluted (1:1) with PBS. 200 µl of diluted biotinylated pRBC was administered to the restrained mice via intravenous injection 24. At specific time points, 6 µl of blood was collected via tail snip and stored in microfuge tubes containing 250 µL of sterile PBS. To detect biotinylated RBCs through flow cytometry, approximately 10 –10 RBCs (100 µL of the diluted sample) were added to 0.125 µg of streptavidin– APC-eFluor® 780 Conjugate (1:20 of 0.2 mg/mL; eBioscience™). Samples were incubated in the dark for 5 min, washed with 400 µL PBS, and centrifuged at 1000 g for 4 min. The RBC pellet was resuspended in 200 µL PBS. Samples were analyzed using BD LSR Fortessa cytometer (BD biosciences, USA). The red laser excites the dye (λemission = 780 nm) with a bandpass filter of 780/60.

$$\frac{\text{Post transfusion RBC recovery (\%)} \text{ at } t(t)}{\text{Post transfusion Rbc Recovery at } t(1h)} \times 100$$

where (t) stands for post-transfusion time point, i.e., 1, 24, 48, and 72 h. This formula is used for all pRBCs units stored for different days and then transfused in a different set of mice. The data was collected with FACSDiva v8.02 and data analysis was performed FlowJo v10.0.8.

3.2.10 Statistical analysis

Repeated measures of one-way ANOVA were used in experiments with multiple groups, which are time-course studies. Ordinary one-way ANOVA with Tukey's post hoc test was used in multiple-group experiments. The two-tailed Student's t-test with Welch's corrections was used to compare two experimental groups. The probability value (P) < 0.05 was considered a statistically significant difference. Statistical analysis and graphing were performed with GraphPad PRISM 9.

3.3 Results

3.3.1 Significant production of RBCs associated lesions in LR units during storage and the role of charged *Tau-Acr*NFS in scavenging them.

Before evaluating the DAMPs-scavenging efficacy of NFS, we quantified the production of DAMPs (extracellular DNA, Hb, and PUFAs) in stored human leukoreduced RBCs in a time-dependent manner for up to 42 days. Various analytical techniques such as PicoGreen assay, ELISA, Drabkin's assay, and LC-MS were used to quantify extracellular DNA, nucleosomes, Hb, and PUFAs, respectively (Methods). The extracellular DNA level did not increase significantly with time in LR units, suggesting that removing WBCs prevents the production of its associated DAMPs such as DNA. (**Fig 3.2a**).

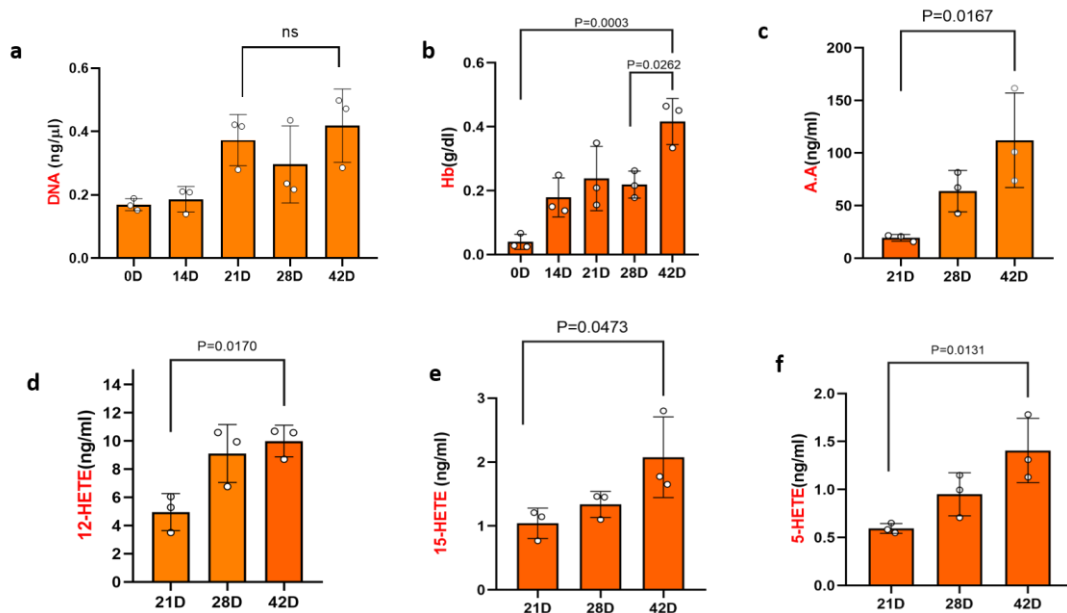


Fig 3.2: Storage-induced production of DAMPs as a function of time. The removal of WBCs has significantly reduced the production of free DNA(a). RBCs stored at 4 °C progressively produce DAMPs such as free hemoglobin (Hb, b), and polyunsaturated fatty acids (PUFAs) such as AA (c), 5-HETE (d), 12-HETE (e), and 15-HETE (f). Data are mean \pm s.d. (n = 3, from independent experiments).

However, the quantitative value of the RBCs associated storage lesion indicated that extracellular Hb (**Fig. 3.2b**) and PUFAs such as arachidonic acid (AA, **Fig. 3.2c**) and eicosatetraenoic hydroxy acids (5-HETE, 12-HETE, and 15-HETE, **Fig. 3.2d-f**)

increase significantly over time. The quantification of free Hb concentration on the 42nd day showed an increase of ~20-fold compared to 0 day.

In the case of bioactive lipids, the increase in production on 42D (~111ng/ml) was approximately two folds higher than 28D (~63ng/ml), affirming the formation of lesions associated with RBCs (**Fig 3.2b**). The oxidation products of AA are known as eicosanoids, a class of bioactive lipids mainly produced through enzymatic activity¹⁸⁻²⁰. Cyclooxygenases convert AA into prostaglandins, whereas lipoxygenase (LOX) activity leads to the production of HETEs and leukotrienes. Cytochrome P450s (CYPs) are also reported to aid the production of HETEs²¹⁻²³. Our study in Chapter-2 found that HETEs significantly increased with the duration of human RBC storage and correlated with the AA generation. 12-HETE (NLR units) recorded an exponential increase in its concentration from the 28th to the 42nd Day of storage. However, a similar trend was not observed in LR units from 28D to 42D (**Fig 3.2 d-f**). The probable reason for this observation can be attributed to the 12-LOX activity of WBCs in NLR units. The kinetics data gave a comprehensive insight into the RBCs associated DAMPs production in stored RBCs units w.r.t time. These observations are in agreement with previous studies²⁵⁻²⁸.

In Chapter-2, we systematically optimized the required surface area of nanofibrous sheets and incubation time to scavenge DAMPs. The same parameters were followed to scavenge all DAMPs (extracellular DNA, Hb, and PUFAs) from old LR RBCs. The stored LR RBCs (1ml) were incubated with cationic/anionic nanofibrous sheets (*Tau-AcrNFS*) for 5 min at 4 °C mentioned in **Fig 3.3a**. Data in **Fig. 3.3c–g** suggests that *Tau-AcrNFS* efficiently scavenges RBCs associated with DAMPs. Since there was less production of free DNA in LR units by the 42nd Day, the data showed no significant change in free DNA concentration after treatment (**Fig 3.3b**). On the other hand, the data showed that *Tau-AcrNFS* could effectively scavenge 50% of Hb (**Fig. 3.3c**), 70% of AA (**Fig. 3.3d**), 30% of 12-HETE (**Fig 3.3e**), 70% of 5-HETE

(**Fig 3.3f**) and 50% of 15-HETE (**Fig.3.3g**). These results suggest that *Tau-AcrNFS* can efficiently scavenge and significantly reduce the concentrations of RBCs associated with DAMPs in 42 days of stored old RBCs.

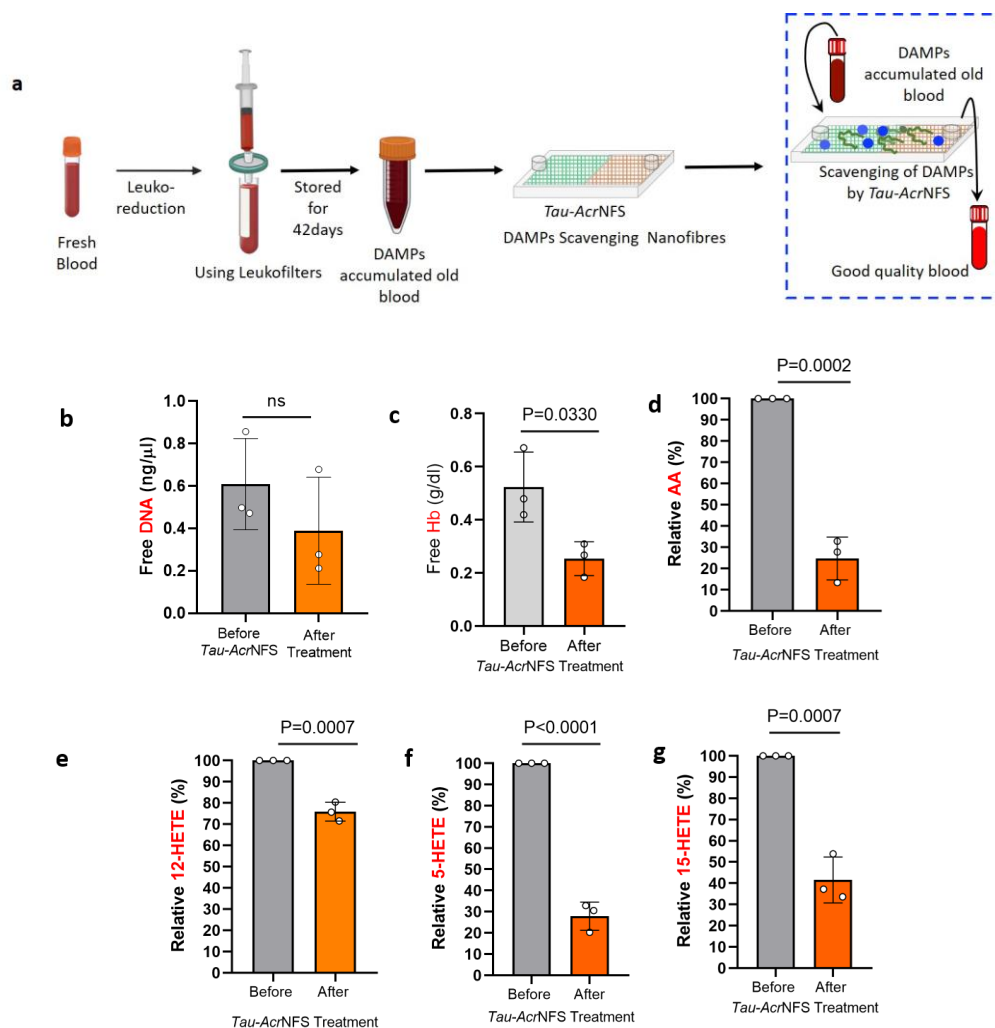


Fig 3.3: a) A schematic representation of our approach where collected RBCs units were passed through leukofilters and stored for 42days. On 42nd day, charge-bearing electrospun nanofibrous sheets (*Tau-NFS* and *Acr-NFS*) were prepared to scavenge charged RBCs associated DAMPs from stored LR-RBCs. Incubation of DAMPs accumulated 42 days-stored RBCs with anionic and cationic NFS, *Tau-AcrNFS*, for 5 min at 4 °C efficiently scavenged and reduced the concentration of DAMPs (b-g). The relative concentration of DAMPs before and after *Tau-AcrNFS* treatment has significantly reduced accumulated Hb (c), and PUFAs (e-g). Data are mean ± s.d. (n = 3, from independent experiments).

3.3.2 Intermittent scavenging of RBCs associated DAMPs in stored LR units by *Tau-AcrNFS* significantly enhances their quality

The storage lesion is a denomination that generally includes all changes that occur as RBCs age while in storage solution. They can be classified as "young" (<14–21days) and "old" (>21 D) RBC units, based on the concentration of generated DAMPs. The kinetics data in **Fig. 3.2a–f** showed a significant increase of DAMPs during 21st to 42nd Day of storage. There is a possibility that accumulated DAMPs until 21 days might have a synergistic effect on accelerating the deterioration of the quality of LR-stored RBCs. Hence, large quantities of DAMPs are produced within 42 days. This could be a reason for limiting the storage shelf life of LR-RBCs units. It was hypothesized that reducing DAMPs from the blood unit at a particular point in the storage timeline (0–42D) may enhance healthier RBCs on the 42nd Day of storage. To identify that distinct point in the storage timeline, the kinetics data of DAMPs production in stored RBCs was revisited (**Fig. 3.2a–f**). Therefore, we predicted that intermittent removal of DAMPs on the 21st or 28th Day using *Tau-AcrNFS* might prevent further damage to RBCs and enhance the quality of 42 days-stored LR RBCs. To test this hypothesis, either on the 21st or 28th Day, stored RBCs were incubated with *Tau-AcrNFS* for 5 min (**Fig. 3.4a**), stored back at 4 °C, and measured the DAMPs on the 42nd Day. The data in **Fig.3.4(b–g)** suggest that intermittent removal of DAMPs on the 28th Day significantly reduces the concentration of DAMPs present on the 42nd Day. However, the 21st D treatment did not impact the lysis rate of RBCs in LR units, whereas the 28th D treatment limited the free Hb production by 50%, compared to the untreated group (~0.4g/dl to ~0.2g/dl) (**Fig.3.4b**). Still, it was further strengthened with the evaluation of bioactive lipids. The abundant AA in LR units was reduced to ~33 ng/ml and ~38ng/ml when treated on the 21st and 28th D compared to the untreated group(~112ng/ml) (**Fig.3.4c**). This study suggests that intermediate treatment on the 21st and 28th Day significantly reduces the production of RBCs associated with DAMPs at 42D. This strategy is expected to enhance the overall quality of the stored LR blood. Intermittent treatment could provide a significant breakthrough in preventing transfusion-related complications. Stored blood with 28th-day intermediate treatment is

expected to enhance the quality of stored blood and drastically bring down the concentration of RBCs associated lesions during storage.

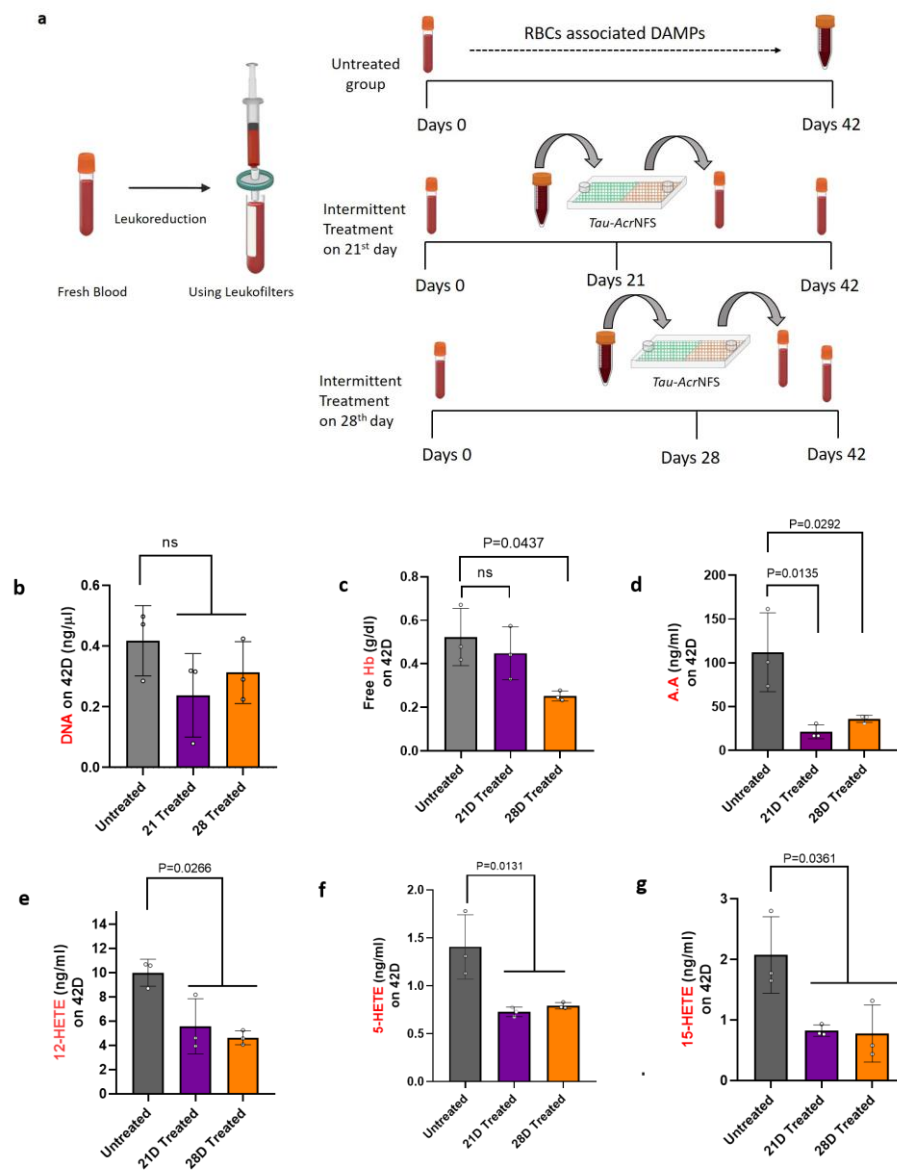


Fig 3.4: a.) A schematic of DAMPs production in untreated and Tau-AcrNFS treated stored LR RBCs. In the untreated group, RBCs were stored at 4 °C for 42 days, in treated groups, RBCs were incubated with *Tau-AcrNFS* for 5 min on either the 21st or 28th day, and stored at 4 °C for 42 days, and DAMPs were quantified on 42nd day for all groups (b–h). Intermittent treatment of stored RBCs with *Tau-AcrNFS* on either the 21st or 28th day significantly reduced the accumulation of DNA (b), Hb (c), and PUFAs (d–g) on the 42nd day. Data are mean \pm s.d. (n = 3, from independent experiments).

The morphology of human red blood cells (RBCs) deteriorates progressively throughout hypothermic storage. Echinocytosis is the prevalent pathway that leads to these morphological deformities. As a result, each unit of stored blood contains a heterogeneous mixture of cells (discocytes, spherocytes, and spherocytes) at various stages of echinocytosis. Discocytes are regarded as healthy RBCs²⁹⁻³⁰. Investigating more into stored RBCs quality, the Osmotic fragility test (OFT) is also a crucial parameter in characterizing erythrocyte membrane health. It depends on the degree of resistance of red blood cells (RBC) to lysis when introduced in a hypotonic environment. OFT is also determined by 50% of the hemolysis at a specific NaCl concentration (%) known as MCF (Mean cell fragility).

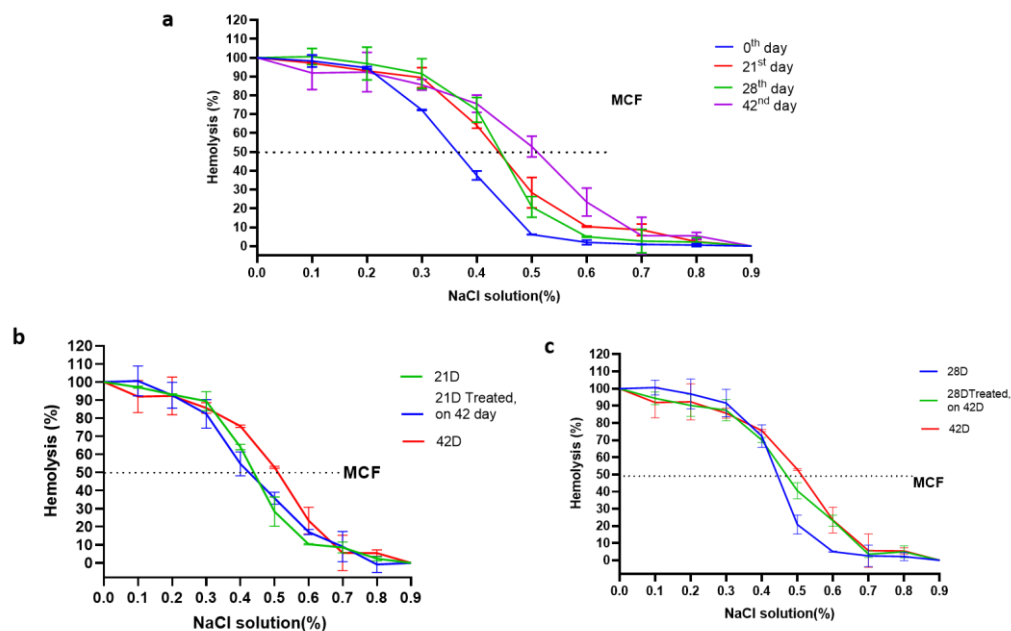


Fig 3.5. Osmotic fragility test revealed that intermittent treatment had enhanced membrane integrity of stored LR RBCs. a.) The lower MCF values are equivalent to higher membrane integrity. MCF values of stored RBCs progressively increased as a function of storage time, suggesting that loss of membrane integrity of RBCs during the storage. MCF values of 42 days of stored RBCs that were intermittently treated were lower than untreated stored old RBCs (b, c), suggesting that intermittent removal of DAMPs reduced the damage of RBCs and enhanced the membrane integrity of stored old RBCs (n = 3, from independent experiments).

The value of MCF rises with the increase in the storage period of the pRBCs unit. Surprisingly, the LR units also showed an increase in MCF values from approx. 0.35 to 0.5 stating the extent of RBC's health damage (**Fig3.5a**). The data suggest that LR RBCs also get fragile during storage. In order to evaluate the impact of intermittent treatment given to LR units on 21st /28th D of storage with *Tau-Acr*NFS, LR units were treated with sheets at either 21st or 28th day and stored for 42 days. MCF were measured on 42nd day. The data suggest that the 21st D intermittent treatment has significantly reduced the MCF value to approximately 0.52 (**Fig 3.5b**), whereas the 28th D intermediate treatment led the MCF of 42D RBCs to coincide with the 28th D stored unit (**Fig.3.5c**). The 21st and 28th D intermittent treatment in LR units reduced the MCF values from the untreated group, shifting the hemolysis curve to the left side, i.e., the healthy side. The reduction in MCF values suggests that RBCs were healthier after intermediate treatment and are more resistant to lysis (**Fig 3.5b&c**). The data also suggest that intermittent removal of DAMPs slows down the deterioration of RBC membrane quality and lead to healthier RBCs. The extensive study on the RBCs membrane integrity (human) laid the foundation to understand more about the functionality of the treated RBC

3.3.3 Intermittent scavenging of storage lesions with *Tau-Acr*NFS increases the circulation time of transfused LR RBCs in mice

Designing a functionality experiment for human LR-RBCs after transfusion was not ethically feasible, leading to its scrutinizing via a murine model. The shelf life of C57 BL/6 mice RBCs is reported to be 14 days which is equivalent to human RBCs (42D)³¹. The mice's blood was drawn via retro-orbital puncture and processed as described (**Methods**) for the functionality test (**Fig 3.6 a**). The stored red cell functionality was investigated through the survival of biotin-labeled RBCs in circulation in male/ female mice (8-12 weeks) after transfusion. The sulfo-NHS-biotin was administered intravenously to bind and label RBC membrane proteins covalently³². After 48 h of post-administration, blood was collected and quantified for labeling efficiency via flow cytometry. (**Fig 3.6**). Monitoring the rate of successful LR pRBCs transfusion in studies, biotin-labeled RBCs were stored up to 18D (with and without treatment) were transfused in a new set of mice.

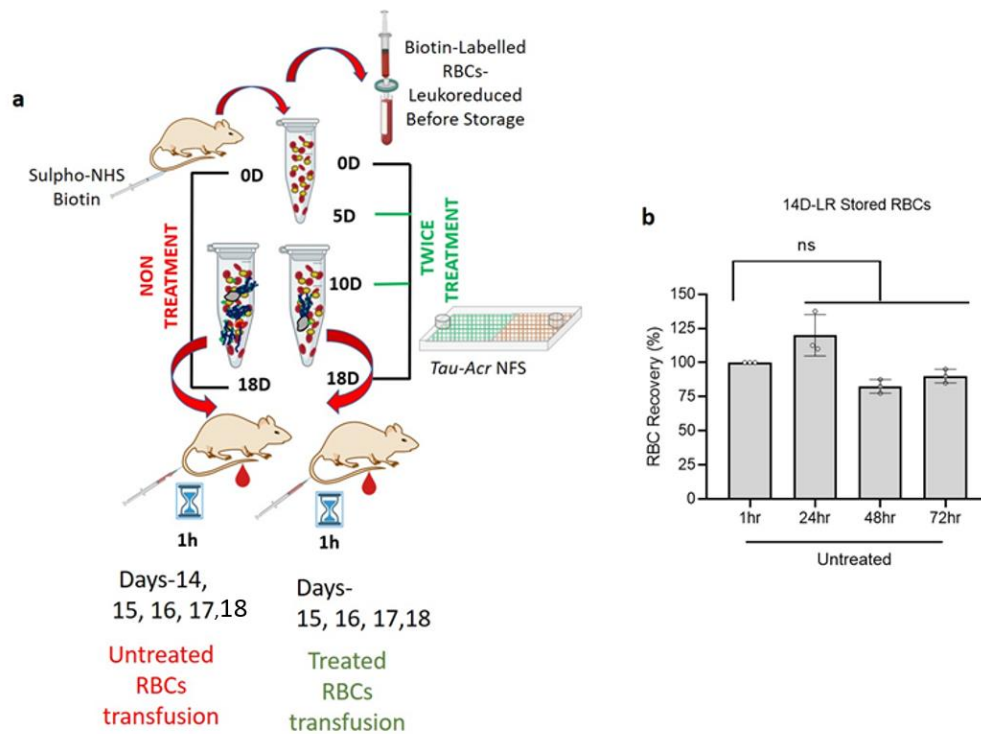


Fig 3.6 a.) Schematic of RBC recovery experiments, *in vivo* The number of labeled RBCs present in circulation has been quantified (b) Untreated 14 days-stored biotinylated LR RBCs, were transfused into mice, collected blood at post-transfusion 1, 24, 48, and 72 h, and labeled with Streptavidin APC-eFluor™, and quantified via flow cytometry (n = 3, from independent experiments). In untreated stored LR RBCs, ~90% of RBCs were in circulation up to 72h suggesting increased quality of stored old RBCs after leukoreduction. Data are mean ± s.d. (n = 3, from independent experiments).

Flow cytometry tracked the labeled RBCs for their survival post-transfusion after 1, 24, 48, and 72 h. Since transfused labeled RBCs get mixed with the non-labeled RBC of recipient mice, % of labelled population obtained post 1h transfusion is considered as 100% that is circulating in recipient mice. In the previous chapter, 14D-UT stored NLR RBCs in recipient mice showed more than 75% recovery after 24h. When the time points were extended up to 72 hrs, a noticeable drop of labeled RBCs was observed after 48h, and the survival population was reduced to 50%¹⁷. As per the FDA guidelines, the survival studies have been performed within 24h, which is the "gold standard"

criteria. Thus, our study tweaks the current experimental settings and shows the labeled RBCs survival up to 72h. The post-24 h recovery data gives a novel insight into the consequence of RBCs quality that plays a pivotal role in its function and survival in circulation.

The recovery experiment of untreated 14D- LR RBCs showed more than 75% recovery up to 72hrs. Here, the 48h-72h recovery (%) sets to prove that the

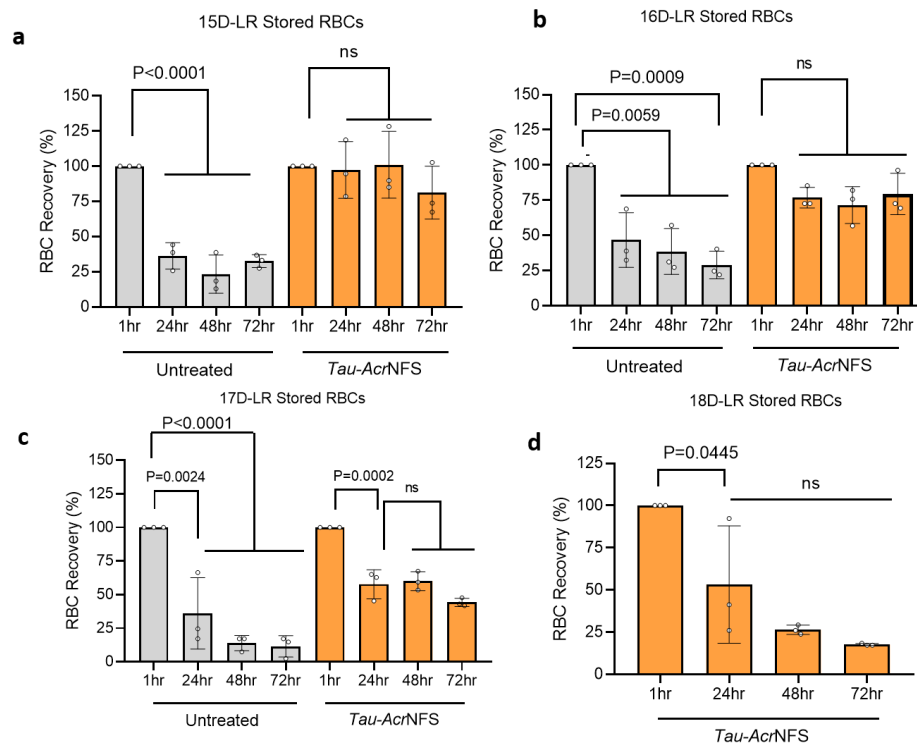


Fig 3.7 Recovery of storage-aged RBCs either untreated or intermittently treated with *Tau-AcrNFS* on the 5th & 10th days. Recovery data suggests that the quality of untreated LR RBCs that are stored for 15–17 days declines drastically, and RBCs are eliminated from circulation immediately after transfusion. Only 20–25% of transfused RBCs were found in circulation after 24 h (a–c), which is well below the accepted 75%. On the contrary, intermittently treated RBCs, even after storing for 17 days, transfused RBCs were in circulation up to 72h (a–c), suggesting that intermittent removal of DAMPs has decreased RBC damage, and enhanced quality of RBCs. However, irrespective of whether intermittent treatment or not, 18 days-stored RBCs were eliminated from circulation, with only 50% of RBCs found after 24 h and the population was declining afterwards. Data are mean \pm s.d. ($n = 3$, from independent experiments).

transfused RBCs are in good health. The survival of LR RBCs in circulation up to 72hrs suggests that removing WBC-associated lesions improves the quality of stored blood units and thereby increases circulation time. This also effectively asserts that the quality of RBCs plays a crucial role in circumventing their clearance from mice circulation by the spleen. However, transfusing 15D untreated LR RBCs in mice led to 40% labeled RBCs survival after 24h, and it kept declining with time (**Fig.3.7**). A similar trend was observed for 16D and 17D stored LR RBCs without *Tau-AcrNFS* treatment as they were removed from circulation within 24h of transfusion. The data suggest that the generation of DAMPs due to the biochemical changes in RBC accelerates its deterioration and thus limits the shelf life of leukoreduced RBCs. On the contrary, 15-17 days-stored RBCs when they were intermittently treated with *Tau-AcrNFS*, post-transfusion, the labeled population was in circulation up to 72 h. The data shows that intermittent treatment with *Tau-AcrNFS* improves the quality of stored leukoreduced blood, allowing them to stay in circulation for longer. Post-blood transfusions, RBCs associated DAMPs are known to cause systemic immunomodulation. So, the detailed systemic inflammation studies need to be done to test the efficacy of *Tau-AcrNFS* treatment to prevent transfusion-induced immunomodulation.

3.4 Discussion

Despite the progress in red blood cell (RBC) storage followed by Leukoreduction, storage lesions still occur, leading to complement activation and removal of RBCs from circulation. Our study was not restricted to the NLR RBCs unit. the kinetic study of DAMPs production in LR units was investigated in detail. Though their WBCs associated lesions production rate was insignificant, like NLR units, the release of free hemoglobin and bioactive lipids raised interest. Extensive experimental studies have reported that there is a significant production of RBCs associated with DAMPs after 42 days of storage that has the potential to induce Lung injury³²⁻³⁵. Until now, existing strategies are limited to developing preservatives and rejuvenating solutions. However, no effort was made to scavenge the entire spectrum of DAMPs during RBC storage to prevent RBC damage. Using the previously generated proof of concept¹⁷, robust electrospun nanofibrous sheets (*Tau-AcrNFS*) were generated using polymers. The data suggested that cleaning LR units before transfusion could significantly reduce the concentration of DAMPs

associated with RBCs. In this case, the concept of intermittent treatment worked efficiently where the free Hb and bioactive lipids concentration was significantly reduced on the 42nd day when treated on the 21st or 28th D of storage.

Here, the concept of structural integrity of RBCs was explored concerning their residual circulation time. The osmotic fragility data supports that the scaffold-treated RBCs (28th-day intermediate treatment) were healthier on the 42nd Day of storage. Their membrane integrity was highly intact compared to the untreated LR RBCs. It was envisaged that maintaining membrane integrity should lead to extended survival in circulation (enhanced functionality). In order to prove this hypothesis, the murine model was chosen for the functionality experiment. Since Murine RBCs have a shelf life of 14D, the stored RBCs were intermittently treated twice on the 5th and 10th D of storage with *Tau-Acr*NFS. The recovery experiment presents a new standard to assess transfused LR RBCs' quality and functionality. The 14D stored RBCs without treatment showed a recovery of >75% at 24h post-transfusion, and sustained till 72h. This was as per FDA standards. This data was interesting because NLR labeled RBCs population declined post 24 h in Chapter-2. This was attributed to their deteriorated condition, contributed by DAMPs formation by WBCs and RBCs¹⁷. This phenomenon was not witnessed in the 14D-untreated LR group, as labeled RBCs were in circulation for 72 h. However, post 14 days, the untreated labeled LR RBCs were not in circulation, and a similar trend was seen up to 17 days. In the case of intermittently treated LR RBCs, 17-day-old RBCs were sustained in circulation for up to 72h. The data suggest that intermittent removal of DAMPs has improved the quality of stored LR RBCs, more importantly their shelf-life. Systemic immunomodulation is one of the hallmarks of the transfusion of stored old RBCs. Therefore, extensive study has to be performed to evaluate that intermittent removal of DAMPs has reduced immunomodulation.

3.5 Conclusion

In this Chapter, intermittent cleaning of RBCs associated lesion in LR units improve the quality of RBCs and increases the circulation time. Gazing at the bright front, scaffold treatment before the transfusion could significantly reduce the storage lesion and prevent transfusion-related complexities. Further, the intermittent treatment adds a new dimension to maintaining leukoreduced RBC's

quality. The detailed investigation here explains the concept of scavenging LR-DAMPs during storage, which ultimately leads to the increased circulation of RBCs. This research has a massive potential to address the transfusion-related complexities associated with old stored blood.

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Chapter 4

Removal of DAMPs from stored RBCs units using nanofibre scaffolds to prevent transfusion related acute lung injury (TRALI)

4.1 Introduction

During storage of whole blood, leukocytes (mostly neutrophils) undergo NETosis lead to release of extracellular DNA as NETs (neutrophil extracellular traps) and histones up to ~100 folds higher than fresh blood unit. The production of extracellular DNA, histones, bioactive lipids, and free hemoglobin known as Damage Associated Molecular Patterns (DAMPs), deteriorate blood quality, and limits packed RBCs' shelf life up to 35–42 day¹⁻³. Transfusion of old stored NLR(Non-Leukoreduced) blood consisting of the whole spectrum of DAMPs is a significant mediator in the pathophysiology of fatal diseases like Transfusion-Related Acute Lung Injury (TRALI). It is a respiratory distress syndrome with a high rate of morbidity and mortality caused due to transfusion of blood products⁴⁻⁵. The incidence rate of TRALI is 0.08 to 15.1% per patient transfused and 0.01 to 1.12% per product transfused⁶. According to US Food and Drug Administration (FDA), 41% of fatalities occurred due to TRALI alone, including other transfusion-related diseases⁷. The conventional occurrence of TRALI, according to the development of acute lung injury, prevails within 6 h after blood transfusion. A high incidence of TRALI has been seen in critically ill patients (50-100 times) than general hospital population.

To explain the pathogenesis in critically ill patients "two-hit model" has been proposed⁸. The first hit refers to the patient's clinical condition, such as sepsis, haematological malignancy, heart surgery, massive blood transfusion, liver surgery, acute renal failure, and patient age. These conditions have been discovered as risk factors that lead to pro-inflammatory pulmonary conditions by activating lung endothelium with subsequent sequestration and priming of polymorphonuclear neutrophils (PMN)⁹⁻¹⁰. The second hit is mediated by the cellular blood products transfused to the patient, leading to activation of pulmonary neutrophils and resulting in capillary leakage and subsequently pulmonary edema (respiratory distress). The second hit can be induced through antibodies present in blood products against human leukocyte antigens (HLA) or human neutrophil antigens (HNA)¹¹⁻¹³. Though various mitigation strategies such as screening donor blood for HNA/HLA antigens can reduce

the antibody-mediated TRALI reports. Still, non-antibody mediated TRALI is still a major concern of transfusion-related morbidity and mortality.

The incidence rate of non- antibody-mediated TRALI has directly associated with transfusion of longer stored blood products. Researchers have proposed several mechanisms, including accumulated cell-derived substances in donor blood (bioactive lipids, free heme, NETs and microparticles), changes during RBC storage (erythrocyte deformation) that cause TRALI¹⁴. It is evident from the literature that DAMPs released in stored blood products are a potential candidate for causing TRALI through activating primed neutrophils, increased superoxide production and enhanced phagocytic ability of neutrophils¹⁵. Hence, eliminating the DAMPs before transfusion to patients is imperative to prevent the incidence of TRALI events and reduce mortality.

In this Chapter, we have developed a novel approach to induce Two Hit non-antibody mediated TRALI in C57BL/6J male mice aged 8–12 weeks (**Fig.4.1**). We establish that the concentration of LPS and the age of stored mice blood play a pivotal role in precipitating the symptoms of TRALI. The “first hit” of LPS activates the pulmonary endothelium and promotes the recruitment and adhesion of neutrophils to the capillary endothelium, and later causes a disarray of the lung alveolar-capillary permeability barrier. We have demonstrated that the transfusion of 17day old mouse blood aggravated the extent of lung injury in LPS-administered mice compared to 14-day-old blood. We demonstrated that the stored mouse blood units showed an elevated concentration of critical DAMPs, such as extracellular DNA, nucleosomes, free hemoglobin (Hb), and PUFAs, significantly contributing to the second hit. The two-hit model was efficiently optimized based on vasculature permeability, NETs biomarkers, histopathological parameters, and inflammatory cytokines in plasma and BALF (Bronchoalveolar Lavage). In light of preventing the occurrence of TRALI, we have used the concept of using *Tau-AcrNFS* from our previous study to scavenge storage lesions. We have shown that intermittent scavenging of DAMPs on the 5th and 10th day of storage by *Tau-AcrNFS* resulted in significantly less production of DAMPs at maximum storage time (on 14th day). This also increased the shelf life of mouse RBCs by 22%. We have demonstrated that two phenomena to prevent TRALI events using *Tau-AcrNFS*; i) The intermittent scavenging of 17-day-old mouse blood on the 5th and 10th day of storage, significantly prevented mice from developing TRALI symptoms, such as vasculature leakage, infiltration of neutrophils in alveolar space and elevated cytokines levels. ii) cleaning with *Tau-AcrNFS* on maximum storage day (14th & 17th

day) removes the accumulated DAMPs, hence not stimulating the second event of TRALI. These results are interesting and we aim to generate either novel blood bags or an insert-based device using Tau-AcrNFS. Additionally, the concept of cleaning RBC before transfusion also opens up a new avenue where stored blood might pass through medical device i.e., bedside purification to reduce TRALI incidence.

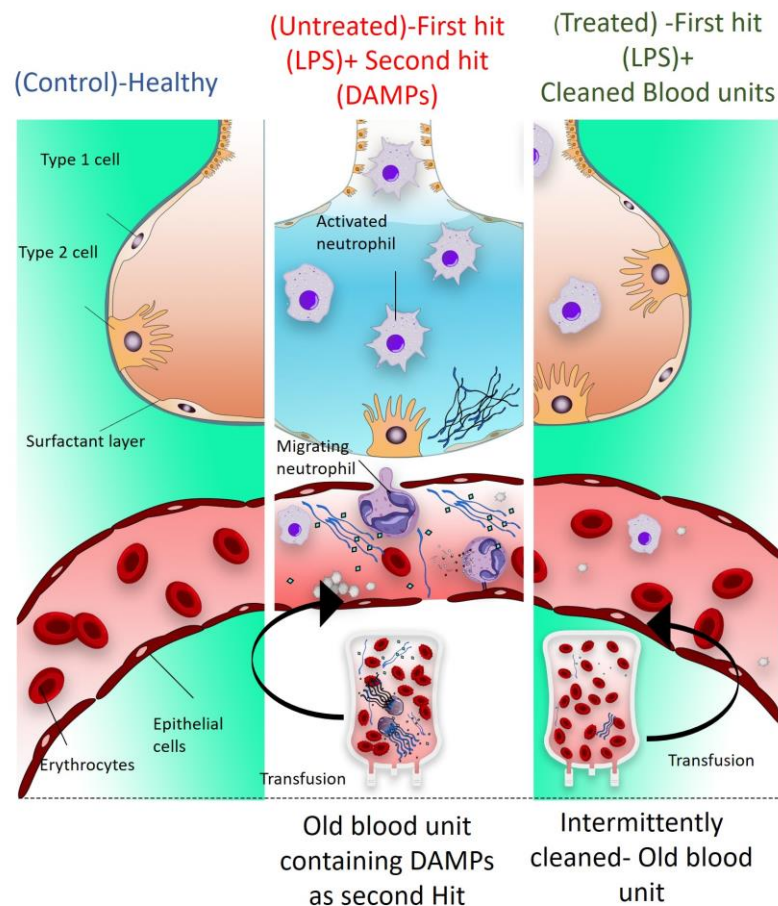


Fig 4.1. The schematic representation of Two hit non-antibody mediated TRALI model: The “Control” section shows the normal condition of lung and blood vessels, “Untreated” shows the two-hit non- antibody mediated model of TRALI with hypothesized pathophysiology. A ‘first hit’, an underlying clinical condition of the patient. The ‘second hit’, the transfusion of using 17D stored mice blood, causes activation of the neutrophils and coagulation pathways resulting in TRALI. and Section 3 describes the concept of treatment with *Tau-AcrNFS* improves the quality of 17D old mice blood.

4.2 Materials:

Blood collection tubes (BD Vacutainer), citric acid monohydrate, trisodium citrate dihydrate, sodium dihydrogen phosphate, dextrose, sodium chloride, glucose anhydrous, mannitol, adenine, evans blue dye, lipo-polysaccharide, hematoxylin, eosin, formamide, methylene blue, toluidine blue, ethanol, phosphate buffer saline, double distilled water and deionized water were used. Unless mentioned otherwise, all chemicals were procured from Sigma-Aldrich.

4.2 Methods:

4.2.1 Experimental Mice:

Both male and female mice (C57B6/J strain) of age 8- 12-weeks-old were used for the experiments. The animals were housed in the animal facility at the National Centre for Biological Sciences, Bengaluru. Animals were caged (maximum, four per cage) during the experiment, food and water were offered ad-libitum. All mice study strictly adhered to institutional and national guidelines for humane animal use. The experimental protocols were approved by the Institutional Animal Ethics Committee (IAEC) at the Institute for Stem Cell Science and Regenerative Medicine (INS-IAE-2017/06(ME))

4.2.2 Packaging and Storage of Mice RBCs and their treatment with *Tau-Acr*NFS scavenge DAMPs

Mice blood collected in CPD was immediately transferred and centrifuged at 500g for 20 min at 4 °C. Plasma was carefully aspirated, and the pRBCs (packed RBCs) were pooled together. Optisol, also known as AS-5, was added to the pRBCs at 65% hematocrit. 550 μ l and 250 μ l blood units were finally aliquoted from these pRBCs and stored in sterile Eppendorf tubes (0.6 & 0.3 ml). Mice pRBCs were treated twice in combination with *Tau-Acr*NFS scaffolds (24cm²) for 5 min on the 5th and 10th Day of storage at 4°C. After the scavenging procedure, the RBCs were again transferred in autoclaved 2ml Eppendorf tubes, leaving a small residual space, and stored till the 17th Day. The groups considered for the scaffold treatment or non-treatment process are provided in the table.

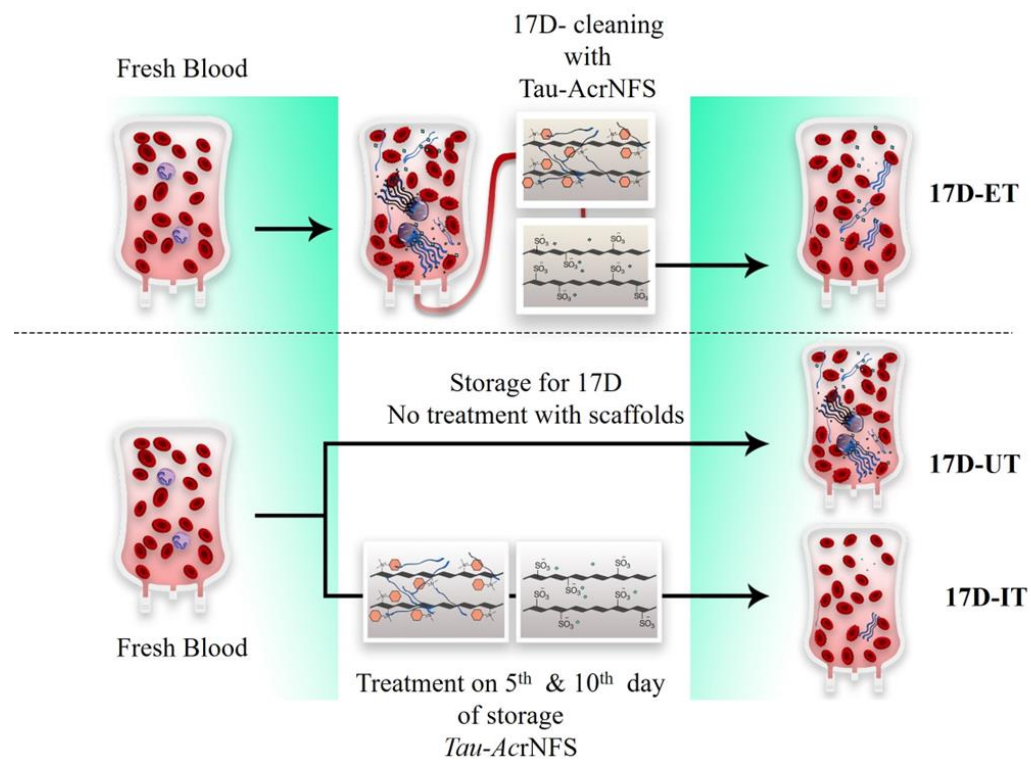


Fig 4.2: Schematics of mouse blood treatment with *Tau-AcrNFS*: Mouse blood stored for 17days without treatment termed as 17D-UT and transfused into the mice as a 2nd hit in TRALI model. In treatment group, End-day treatment with *Tau-AcrNFS* on 17th Day of storage labeled as 17D-ET and Intermittent treatment on 5th and 10th day during storage and transfused into new set of mice on 17th day grouped as 17D-IT.

4.2.3 The non-antibody mediated two-event TRALI *in vivo* model

An anemia led two hit, TRALI model was developed as following.

Inducing Anemia: Blood collection- Mice were made anemic by drawing 20 μ l/g of blood via retro-orbital puncture with the help of sterile capillary tubes and collected into a sterile Eppendorf tube containing 50 μ l of anticoagulant solution, citrate phosphate dextrose (CPD).

First Hit: Post-20 h of blood withdrawal from mice, 100 μ l of LPS was injected via intraperitoneal injection. In all the experiments, 4mg/kg dose of LPS was given.

Second hit: Post-4h of LPS injection, 200µl of stored blood (OD and 17D) with and without treatment (17D-UT, 17D-ET, 17D-IT) and 200µl of 14D-supt (S) with and without intermittent treatment were transfused into the mice intravenously divided into groups as described (**Fig4.2**)

4.2.4 Vasculature permeability through EBD (Evans Blue Dye) assay: Vascular endothelium leakage results in the albumin accumulation the tissue. EBD binds to this albumin, which is later quantified. The amount of EBD in the tissue (associated with albumin) depicts the extent of vascular endothelium rupture and acute lung injury (ALI). EBD was administered intravenously (100µl of 1% (w/v)) in saline 10 minutes post EBD injection, the vasculature was intracardiacaly perfused with 30 mL phosphate-buffered saline (PBS) using a peristaltic pump system of flowrate (200ml/hr). Left lungs harvested and lyophilized. The weighed lyophilized lungs were incubated in 500µl of formamide (55°C overnight) to extract EBD. This was followed by centrifugation (13000 rpm, 10 min, 25 °C. 50µL of supernatant(S) of different group mentioned in table1 was quantified in triplicates in a 96 well plate along with the EBD standards of (0-100µg/ml) was measured at 620nm using Multi-Mode Microplate Reader (Thermo fisher, Massachusetts, USA). EBD concentration was calculated from a standard curve and was expressed as mg of EBD per lung tissue.

4.2.5 Lung tissue histology and Scoring

Lung tissue stained with hematoxylin and eosin (H&E) was analyzed by a histopathologist, blinded to the experimental groups. Twenty random microscopy fields (left and right) were scored for each mouse in a group; three mice were taken in each group. Parameters like neutrophils in the alveolar and interstitial spaces, formation of hyaline membranes, proteinaceous debris filling in airspace and alveolar septal thickening are considered markers of lung injury¹⁶. These parameters were scored on a scale of 0–2: 0 = normal lungs denoted (-), 1 = moderate lung involvement (+), 2 = serious lung involvement (++)

4.2.6 MPO (Myeloperoxidase) assay

The collected blood from each animal in a group was centrifuged at 500g for 20 minutes. The plasma was collected, aliquoted, and stored at –80°C for subsequent batch analysis. The thawed plasma was analyzed for MPO activity using solid-phase

sandwich enzyme-linked immunosorbent assay (ELISA) kits (DY3667; R&D Systems, Minneapolis, MN).

4.2.7 Bronchoalveolar lavage fluid (BALF) collection

BALF was collected from the lungs by pumping in 1 mL of sterile PBS and carefully aspirating the same. This was repeated with another 1 mL of sterile PBS. About 800-900µl of the pumped PBS was recovered successfully at each repeat. The collected fluid was centrifuged at 500 g at 4 °C for 10 minutes. The supernatant was collected and centrifuged again at the same conditions to ensure the complete recovery of cells in BALF pellet. The supernatant was further collected and stored at -80 °C for protein, cytokine and NET-DNA analysis in BALF.

4.2.8 Differential counting of Immune cells in BALF

Cells were stained with eosin (0.075%) followed by Methylene Blue (0.06%) and Toluidine (0.04%). Each staining step was followed by washing. In each group, 3 mice were taken, and counting a minimum of 500 cells per slide from their BALF were done. Total cell number was counted and expressed as cells/ml. The differential cell counts were further evaluated. Macrophages, lymphocytes and neutrophils were determined using standard morphology criteria.

4.2.9 Protein estimation in lung BALF

25 µl of each standard and sample solution was pipetted into 96 -plate wells and 200µl of the BCA working Reagent was added to each well and mixed thoroughly on a plate shaker for 30 seconds. The plate was covered and incubated at 37°C for 30 minutes and the plate was cooled RT. The absorbance was measured at 562 nm on a plate reader and Compared to a standard curve of concentration (0-1500µg/ml).

4.2.10 BALF DNA measurements

Free NET-DNA was measured in mice supernatant via PicoGreen® assay ¹⁷. BALF was diluted (1:50) with PicoGreen® reagent and Tris-EDTA (TE) buffer (Quant-iT™ PicoGreen™ dsDNA Assay Kit, Invitrogen, USA), followed by 5 min incubation in the dark. Free DNA estimation was done using a fluoro spectrophotometer (Horiba Fluoro log QM, France). PicoGreen® binds to dsDNA and exhibits emission maximum at 530

nm (Free PicoGreen® reagent exhibits minimal fluorescence in solution). DNA was quantified via a standard curve ranging from concentration (0 – 10µg/ml).

4.2.11 Cytokine Measurements in Plasma and BALF: Mice

The collected plasma and BALF from each experimental groups were quantified ELISA kits that includes VEGF, Surfactant Proteins, TNF-alpha (DY410), CXCL1/KC (DY453), IL-6 (DY406) and IL-1BETA/1L-1F2(DY401) (R&D Systems, Minneapolis, MN). The manufacturer's protocol was followed during the measurements.

4.2.12 Statistical analysis

Repeated measures of one-way ANOVA were used in experiments with multiple groups, which are time-course studies. Ordinary one-way ANOVA with Tukey's post hoc test was used in multiple-group experiments. The two-tailed Student's t-test with Welch's corrections was used to compare two experimental groups. The probability value (P) < 0.05 was considered a statistically significant difference. Statistical analysis and graphing were performed with GraphPad PRISM 9.

4.3 Results:

4.3.1 Intermittent treatment with *Tau-Acr*NFS on 5th and 10th day enhances quality and shelf life of stored old mouse RBCs.

The data in **Fig 4.3(a-f)** suggest that DAMPs are minimal until 10 days of storage, subsequently, an exponential production of DAMPs occurs. The reason could be the higher concentration of DAMPs, might have cumulative effect on accelerating the deterioration of stored mouse RBCs, hence large quantities of DAMPs are produced within 17days.

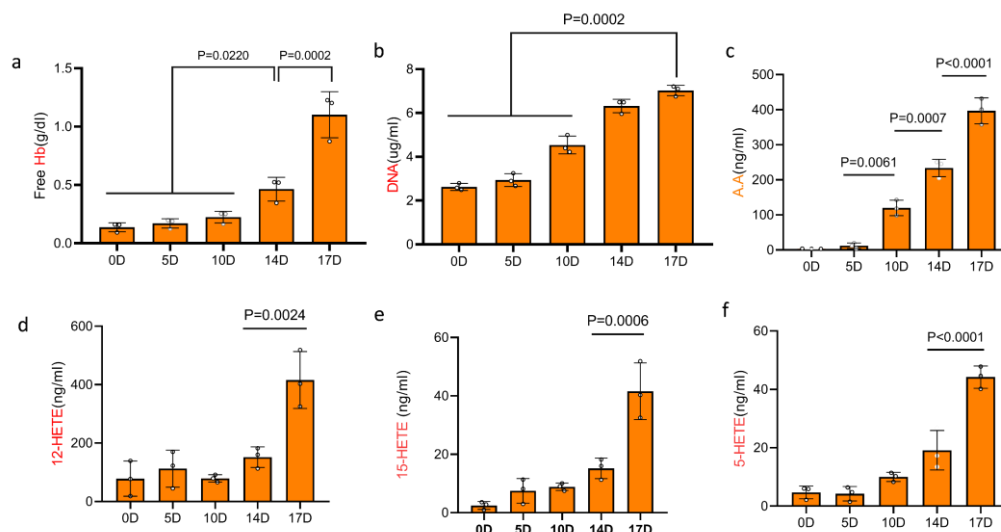


Fig 4.3: Hypothermal storage of mouse RBCs induced DAMPs production: a-f, Storage-induced production of DAMPs as a function of time 0(0D),5(5D),10(10D),14(14D) and 17days(17D) of storage, respectively. RBCs stored at 4°C progressively produce, free hemoglobin (Hb, a), produce DNA (b) and polyunsaturated fatty acids (PUFAs) such as AA (c), 12-HETE (d), and 15-HETE(e),5-HETE(f). Data are mean \pm s.d. (n = 3, from independent experiments).

Previously in our studies, we have shown that intermittent removal of DAMPs on the 5th and 10th day using *Tau-Acr*NFS prevented further damage to RBCs and enhanced the quality of 14 days-stored RBCs. To test same hypothesis with 17 days stored old

blood, they were incubated with *Tau-AcrNFS* on 5th and 10th for 5 mins, and stored back at 4°C, and measured the DAMPs on the 17th day. The data in **Fig. 4.4 (a-g)** suggest that intermittent removal of DAMPs either on 5th and 10th day significantly reduces the quantity of DAMPs present on the 17th day. It was observed that untreated stored old blood condition gets worst when mouse RBCs crosses the shelf life of 14 days and reaches to 17th day. In the span of 3 days, the rate of DAMPs production was increased approximately by 2 folds. In order to confirm the enhanced shelf life, stored

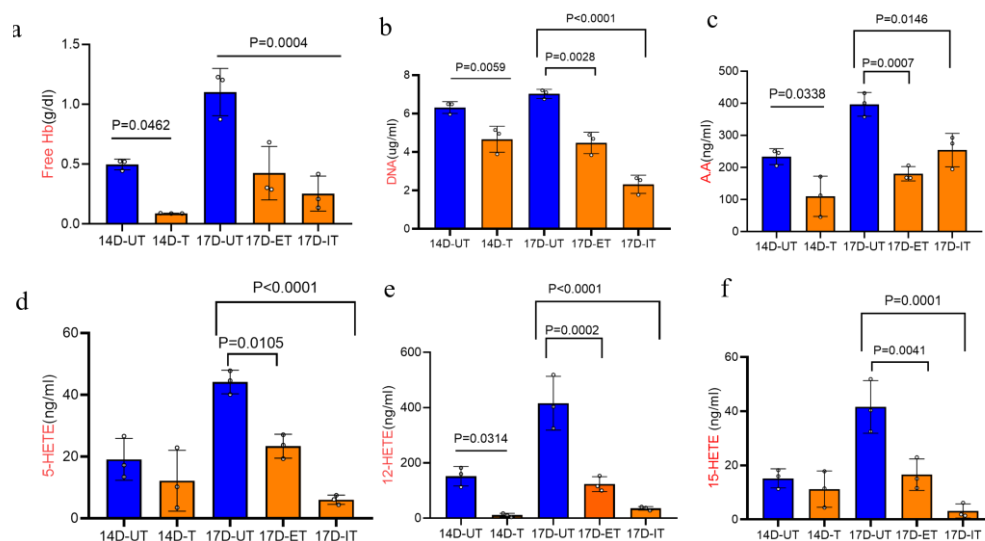


Fig 4.4: Incubation of DAMPs accumulated in 14 & 17 days-stored mouse RBCs with anionic & cationic NFS, *Tau-AcrNFS*, for 5 mins at 4°C efficiently scavenged and reduced the concentration of DAMPs significantly: The Intermittent scavenging of DAMPs with *Tau-AcrNFS* treatment in 17-IT group has shown significant reduced accumulated (a) Free Hemoglobin (Hb), (b) DNA, (c) nucleosomes, and PUFAs (d-g) compared to 17D-UT. The Data are mean \pm s.d. (n=3, performed at least twice).

old mouse RBCs in 17D-IT group were treated with *Tau-AcrNFS* on 5th and 10th day of storage and measured DAMPs level on 17th day. The intermittent treatment has remarkably reduced the formation of cell-free DNA, nucleosomes, cell-free Hb and PUFAs by 60%, 40%, 50%, and 50-75%, respectively, compared to 17days stored untreated RBCs (Fig. 4.4a-g). Similarly, the end day scavenging of DAMPs on 14th and 17th day (14D-T&17D-ET) suggests that *Tau-Acr NFS* are efficient in

scavenging high concentration of DAMPs. Upon incubation, nanofibrous sheet scavenged 50-70% of DAMPs and significantly reduced their concentration in 17 days stored old blood (**Fig. 4.4a-g**

4.3.2 Intermittent cleaning of stored mouse blood on 5th and 10th day with *Tau-AcrNFS* maintains vasculature integrity and prevent TRALI condition

The optimization of LPS dose was essential to establish the non-antibody mediated TRALI model where it was administered intraperitoneally as the "First hit". The first hit is necessary in Two-Hit Model for lung injury amplification that comprises modulation of neutrophil effector functions, increased expression of cognate antigens, or recruitment of additional cellular elements¹⁸⁻¹⁹. Based on the hypothesis that changing the concentration of LPS may affect the magnitude of the neutrophil priming and activation. We administered two different concentrations of LPS (2 mg/kg & 4 mg/kg) in different group of mice. The magnitude of vasculature permeability in lung injury was assessed via EBD (Evan's Blue Dye) assay. The amount of EBD deposited in the lungs after *ex vivo* perfusion shows the extent of vascular leakage in TRALI model. The data in **Fig. 4.5b** suggests that LPS concentration of 2 mg/kg is not effective to prime significant number of immune cells and their activation might requires higher LPS doses. The EBD deposition in LPS administered mice after the transfusion of 0D and 14D supernatant(S) in two different groups, didn't show any significant vasculature leakage in **Fig 4.5b**. To further increase the circulating neutrophil counts and increased sequestration of neutrophils in lung microcirculation in mice, LPS of concentration (4 mg/kg) was administered as first insult. For a second insult, mice were transfused with 0D and 14D old blood (S), respectively in two different group. The EBD data in **Fig. 4.5b** showed a 1.5-fold increase in the extent of vasculature permeability than 2 mg/kg LPS group. Also, compared with 0D(S) transfusion, the 14D(S) showed an increase in of EBD by 2-fold. This trend complies with the threshold model and correlates with the literature that patients' predisposition determines the extent of lung injury²⁰⁻²².

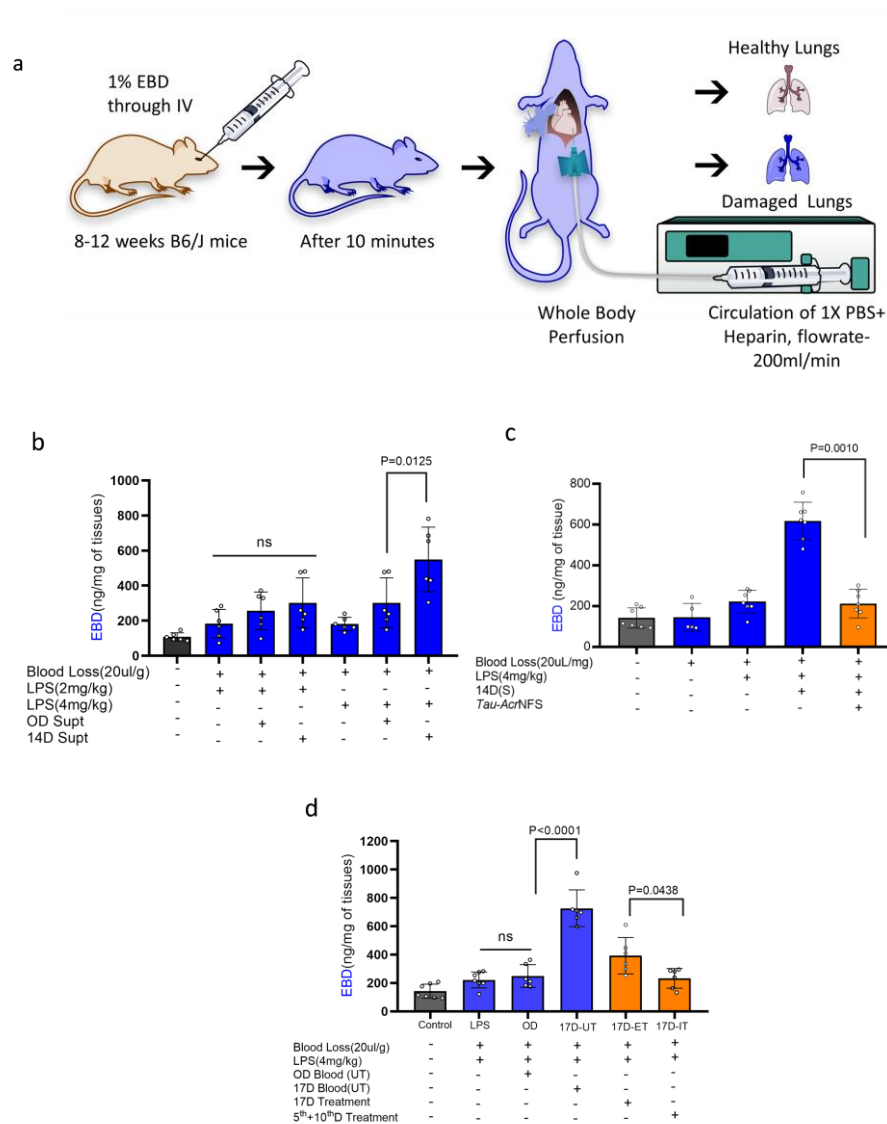


Fig 4.5 a.) Representation of whole-body perfusion performed in after administering EBD to evaluate vasculature permeability. b) The two different concentrations of LPS (2 mg/kg & 4 mg/kg) were administered. The data showed LPS (4 mg/kg) as the first hit showed a significant amount of lung injury after 14day old blood (14D(S)) transfusion compared to LPS (2 mg/kg). c.) A significant increase in the dye leakage was observed in lungs transfused with 14D-supt(14D(S)) compared to only LPS administered animals. The low concentration of EBD in 17D (ET-End day Treatment)&17D (IT-Intermittent Treatment) group confirms lower vasculature permeability than the untreated group. The Data are mean \pm s.d. (n=6, performed at least twice).

According to the threshold model, either "first" or "second" hit or both the hits should reach a certain threshold for TRALI to precipitate. In such conditions, a patient can develop mild or severe TRALI depending on the dose of the first hit, which is the patient's clinical predisposition. A patient with much severe infection is expected to develop severe TRALI compared to an individual with a mild infection²³. Similarly, mice with a mild dose of 2 mg/kg LPS developed mild lung injury than 4 mg/kg LPS administered group. This difference explains that LPS 2 mg/kg does not prime the neutrophils to a required extent such that the second hit of stored blood(S) does not activate the neutrophils way beyond the threshold. Thus, LPS 4 mg/kg was optimized as the first hit.

To further optimize the "second hit" in two hit animal model, the age of stored blood played a critical role in precipitating severe TRALI symptoms. In this scenario, second hit activates neutrophils way above the threshold required to precipitate TRALI. Past studies have shown that the administration of LPS and aged pRBC in rats leads to pulmonary inflammation and coagulopathy in TRALI model²⁴⁻²⁷. Also, previous experiments in this model showed that the injuring second insult is contained in the supernatant of aged human blood products such as bioactive lipids, exosomes and microparticles. Confirming the similar phenomena, the EBD dye deposition in lungs after 14D (S) transfusion was ~3-fold higher than only LPS-administered animals. The increase in dye accumulation after 1st hit (~200ng/mg of tissues) to 2nd hit (~550ng/mg of tissues) shows the role of DAMPs from 14D (S) in disrupting the alveolar-capillary barrier leading to TRALI in **Fig. 4.5c**. To prevent the incidence of TRALI in LPS administered mice, the 14D(S) was passed through *Tau-AcrNFS*. The reduction in DAMPs after *Tau-AcrNFS* treatment has significantly reduced the EBD leakage compared to the untreated group. The data in **Fig. 4.5c** advocates that cleaning DAMPs from 14D (S) with a scaffold alleviates the DAMPs-induced augmentation of vasculature permeability.

However, stored 14 days old blood supernatant as the 2nd hit didn't fit the existing clinical scenario. It was further, substituted with 17day old mouse blood and vasculature permeability was evaluated. Our previous study recommends intermittent cleaning of mice stored blood with *Tau-AcrNFS* on the 5th and 10th day

of storage. This will enhance their quality and shelf life by up to 17 days. In light of preventing the severe TRALI symptoms, the intermittent (IT) and end-day treated blood (ET) with *Tau-Acr*NFS were administered in different groups of LPS-administered mice (4 mg/kg). In the presence of the first hit, the transfusion of 0D, 17D(IT), 17D(ET), and 17D(UT) was considered as the "second hit". The EBD data in **Fig. 4.5c** suggests that transfusion of 17D UT blood significantly increased the EBD accumulation (~709 ng/mg of tissue), confirming the extreme vasculature permeability. In the treatment groups, the 17D-IT showed the lesser dye deposition in lungs (~233ng/mg of tissues). Interestingly, the EBD leakage in the group of LPS, 0D, and 17D treated (IT) seen quite similar. This clearly state that the intermittent removal of DAMPs from 17D-old stored blood produces less DAMPS, hence does not lead to endothelial injury. In the other group, 17D ET showed (~400ng/mg of tissues) remarkably low vascular leakage compared to the untreated group. The end day treatment also opens the new direction of bedside purification of stored blood to reduce the risk of TRALI (**Fig. 4.5c**).

4.3.3. Age of stored blood for transfusion determines the severity of TRALI and removing their DAMPs limits neutrophil activation in LPS primed mice

The severity of non-antibody mediated TRALI was assessed by histological examination. Following LPS priming and challenge with old stored blood transfusion, lung damage was evaluated based on five histological findings: (1) neutrophils in alveolar spaces, (2) neutrophils in interstitial space (3) hyaline membrane formation, (4) alveolar septal thickening and (5) pink proteinaceous debris filling the airspace 16. All the slides show lung tissue consisting of numerous alveoli.

In between the alveoli, there are bronchioles lined by ciliated columnar epithelium. Grading of the parameters was done in 20 high power fields, and the sum of each of the five independent variables was normalized to the number of fields16. After grading and normalization (n=3) from each group, the values were represented by symbols i.e., <0.5(-), <1.5 (+) and <2.5(++) mentioned in Table1

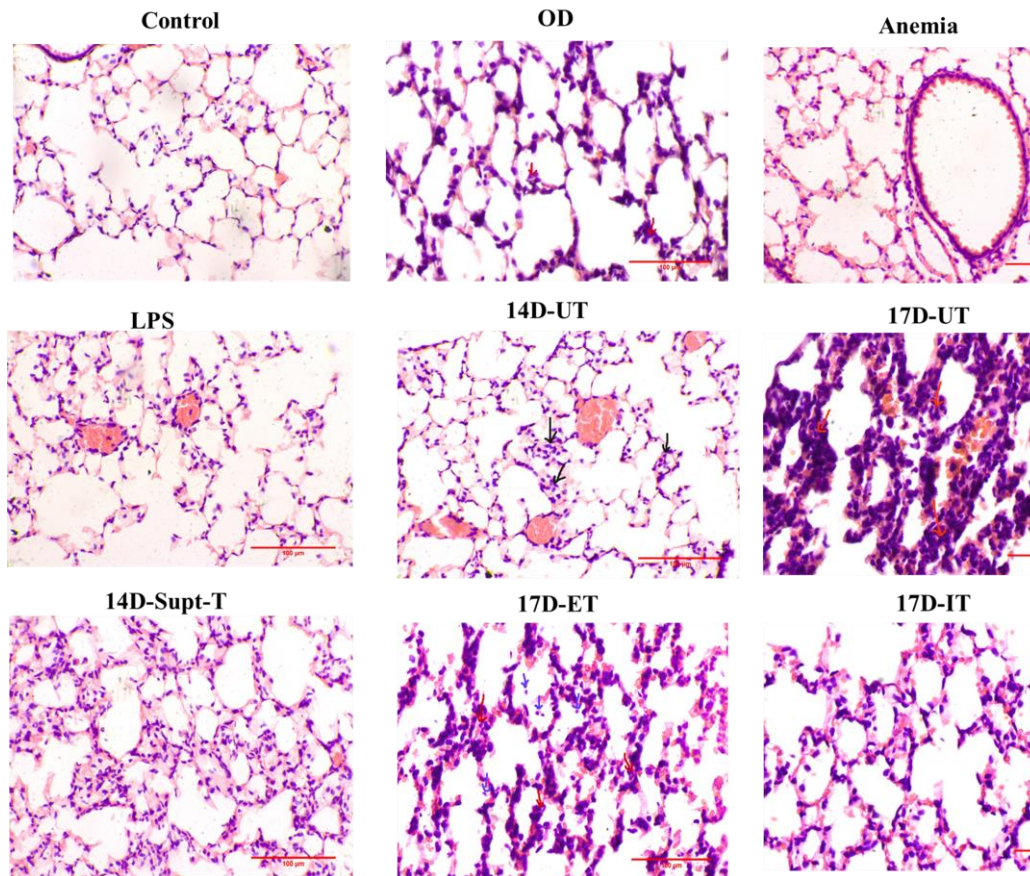


Fig 4.6: Histological evidence of lung morphology with and without scaffold treatment in TRALI mouse model: The severity of non-antibody mediated TRALI was assessed by histological examination. Following LPS priming and challenge with old stored blood transfusion, lung damage was evaluated based on five histological findings. The 17D-UT groups showed the severe lung injury compared to 14D-UT group supporting the concept that stored blood's age plays a critical role to elicit second insult. In case of treatment groups revealed mild inflammatory cell infiltration in interstitial spaces. However, the extent of damage was far reduced on the 17D IT group.

In the 14D(S) transfused TRALI model, the tissue sections show diffuse inflammatory cell infiltration (acute and chronic) in the interstitial and alveolar spaces. In 14D-T, the tissue section revealed mild inflammatory cell infiltration (acute and chronic), and no thickening of the alveolar septum or edematous areas (Fig. 4.6 & Table 1). Though the treatment group showed a lesser extent of acute lung injury than the untreated group, the difference in histological features in both groups was insignificant, not leading to any conclusive argument

. This observation was due to the mild dose of the second hit, which could reach a certain threshold to induce multiple histological features depicting acute lung injury in the 14D-UT group. In order to intensify the extent of lung injury, the whole experiment was replicated in 17D-UT group where 17-day old blood was transfused. The histological evaluations revealed diffuse inflammatory cell infiltration (acute and chronic) in interstitial spaces. The inflammatory cells like neutrophils and lymphocytes were seen in the alveolar space, widening the alveolar septum compared with 14D(S). Other histological injury features like edematous areas and congested blood vessels were aggravated in the 17D-UT group (**Fig. 4.6 & Table3.1**). The observations in the 17D ET and IT groups were encouraging and indicated the advantage of DAMPs removal via scaffold treatment. The treatment groups revealed mild inflammatory cell infiltration in interstitial spaces. However, the extent of damage was far reduced on the 17D IT group. Interestingly, the observations in 17D IT group were similar to the OD treated animals suggesting 17D(IT) blood quality is as good as fresh blood (**Fig 4.6**).

Table4.1: Histological analysis of stored blood transfusion with (17D-ET (End Treatment),17D-IT (Intermittent Treatment)/without treatment (17D-UT(Untreated) induced ALI in LPS mice displayed different parameters:

All the slides show lung tissue consisting of numerous alveoli.

Sample	1. Neutrophils interstitial space	2. Neutrophils- alveolar space	3. Hyaline membranes	4. Proteinaceous Debris	5. Alveolar septal thickening
Control	-	-	-	-	-
LPS only	-	+	-	-	-
0D-Transfusion	-	+	-	-	-
14D-Supt- Transfusion	-	+	-	-	-
17D-UT-Blood- Transfusion	+	++	-	-	+
17D-ET-Blood Transfusion	-	++	-	-	-
17D-IT-Blood	-	+	-	-	-

4.3.4 Intermittent treatments with scaffolds reduce circulating NET biomarkers and immune cells in BALF (bronchoalveolar lavage fluid) and plasma of TRALI mice model

Understanding the inflammatory responses, MPO (Myeloperoxidase) activity serves as a surrogate for activated neutrophils accumulation that indicates their migration in the airspaces of the lungs³⁰. MPO was measured by commercial ELISA kits. In **Fig. 4.7a**, transfusion of 17D-UT blood as 2nd hit increased the MPO level in plasma up to 2 folds (~8 ng/ml) compared to 0D transfused animals. In the 17D-ET group, there was decrease in 1.3-fold (~6 ng/ml) compared to 17D-UT. After the intermittent treatment (17D-IT), the level was dropped down to ~4 ng/ml suggesting the significantly less migration of neutrophils in the lung airspaces. The level of MPO in BALF suggested the similar trend as compared to MPO level in plasma in **Fig. 4.7b**. The data shows the reduced activation and migration of neutrophil in lung fluid after the transfusion of cleaned stored blood.

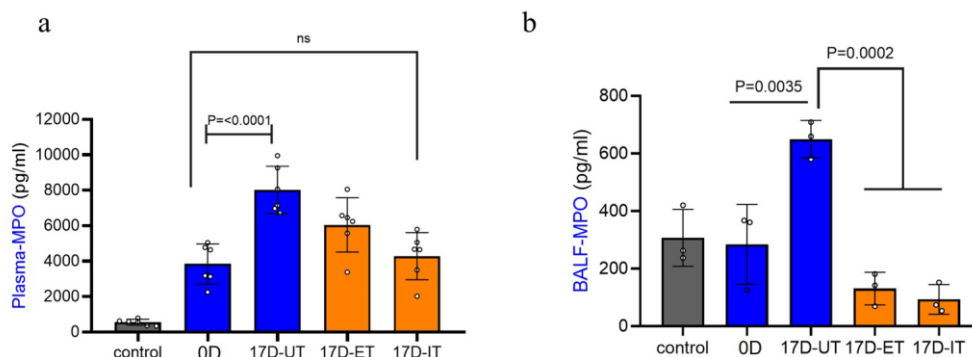


Fig 4.7: MPO activity in the plasma and BALF of mice: It showed that 17D-IT group didn't show the inflammatory response compared to 17D-UT group. This suggests the significantly less migration of neutrophils in the lung airspaces. The Data are mean \pm s.d. (n=3, performed at least twice).

To confirm the formation of NETs in the lungs, the NET-DNA levels, an important marker of NETs formation in BALF were measured²⁸⁻³⁰. In the BALF of 17D UT group showed ~8ug/ml concentration of NET-DNA, i.e., 4 folds higher compared to

0D group (~2ug/ml) (**Fig 4.8a**). Intermittent treatment with Tau-Acr NFS decreased the NET-DNA concentration in the BALF to ~3ug/ml (**Fig 4b**). The final day removal of DAMPs in the ET group showed a lower concentration of neutrophil granules, i.e., ~5ug/ml, which was 1.5-fold lower than the untreated group (**Fig4.8a**). To critically assess the vasculature leak in the TRALI model, the total protein levels in BALF were also quantified. In the 17D IT group, the total proteins concentration (~50ug/ml) was lower compared to the 17DUT group (~120ug/ml), which reflects its reduced accumulation due to reduced er alveolocapillary membrane permeability (**Fig4.8b**). The total protein concentration in both groups, i.e., 0D & 17D IT, was similar and statistically insignificant. However, periodic cleaning of stored blood leads to lower accumulation of DAMPs by the end of 17D. The cleaned blood when transfused, does not lead to protein permeability.

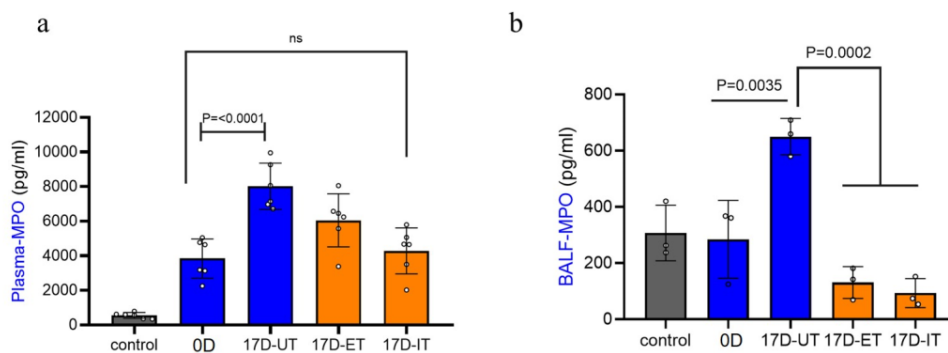


Fig 4.8: Intermittent treatments with scaffolds decreases a.) circulating NET and b.) protein permeability in BALF (bronchoalveolar lavage fluid) and of TRALI mouse model. The Data are mean \pm s.d. (n=3, performed at least twice).

To further analyse the number of immune cells infiltration in the alveolar space, the cytological analysis of the BALF was performed (**Fig4.9 a-b**). On average, 500 cells were counted based on their morphology from all the groups. We could find low counts of neutrophils in the BALF of the 17D-UT group of mice. However, total immune cells comprising lymphocytes and macrophages in BALF of 17D-UT group significantly increased by 2.5 folds compared with 0D-group (**Fig 4.9c-d**). A nearly equal population of lymphocytes and macrophages was observed in the untreated group of mice, and a similar trend was seen in other groups. The data was compared with the treatment group where 17D-ET and 17D-IT showed 1.5- & 2.5-folds lesser presence lymphocytes and macrophages, respectively. The differential count data suggests that the intermittent removal of DAMPs from the 17D-IT group has significantly reduced the cellular infiltration by maintaining the alveolar-capillary barrier.

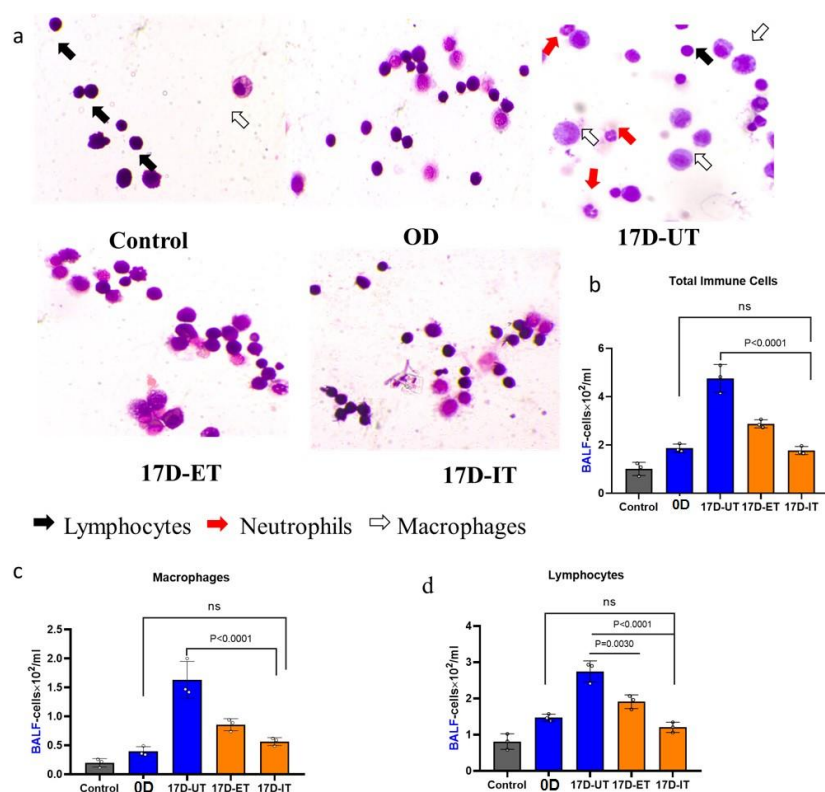


Fig 4.9: Intermittent treatments with scaffolds reduce immune cells in BALF (bronchoalveolar lavage fluid) TRALI mouse model. The Data are mean \pm s.d. (n=3, performed at least twice).

4.3.5. Removal of DAMPs from stored blood units significantly reduces the expression of inflammatory cytokines in BALF and plasma of TRALI mice model

To test augmented neutrophil and endothelial expression of adhesion molecule, the cytokines levels (VEGF and Surfactant protein D) in the plasma of LPS challenged mice transfused with 14D(S) UT were measured.

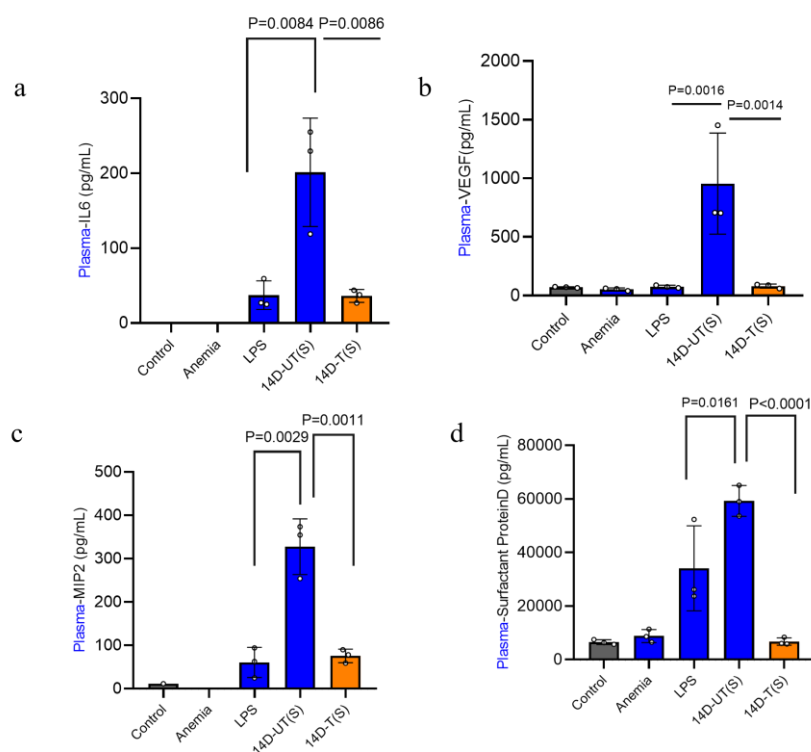


Fig 4.10: Reduction in acute phase inflammatory response in mice plasma when pre-treated with scaffold: The DAMPs in 14D(S) stimulated VEGF expression and surfactant protein D relative to the LPS administered mice. The Data are mean \pm s.d. (n=3, performed at least twice).

The DAMPs in 14D(S) stimulated VEGF expression and surfactant protein D relative to the LPS administered mice. There was an increase in 2.5 & 4 folds of VEGF (~354 pg/ml) and Surfactant protein(~60ng/ml) level, respectively, in the 14D(S) compared to only LPS administered animals (**Fig. 4.10a-d**). The removal of DAMPs from 14D(S) with scaffold decreased VEGF (~81 pg/ml) and Surfactant protein D (~6.7 ng/ml) concentration and showed no sign of lung injury and permeability (**Fig. 4.10a-d**).

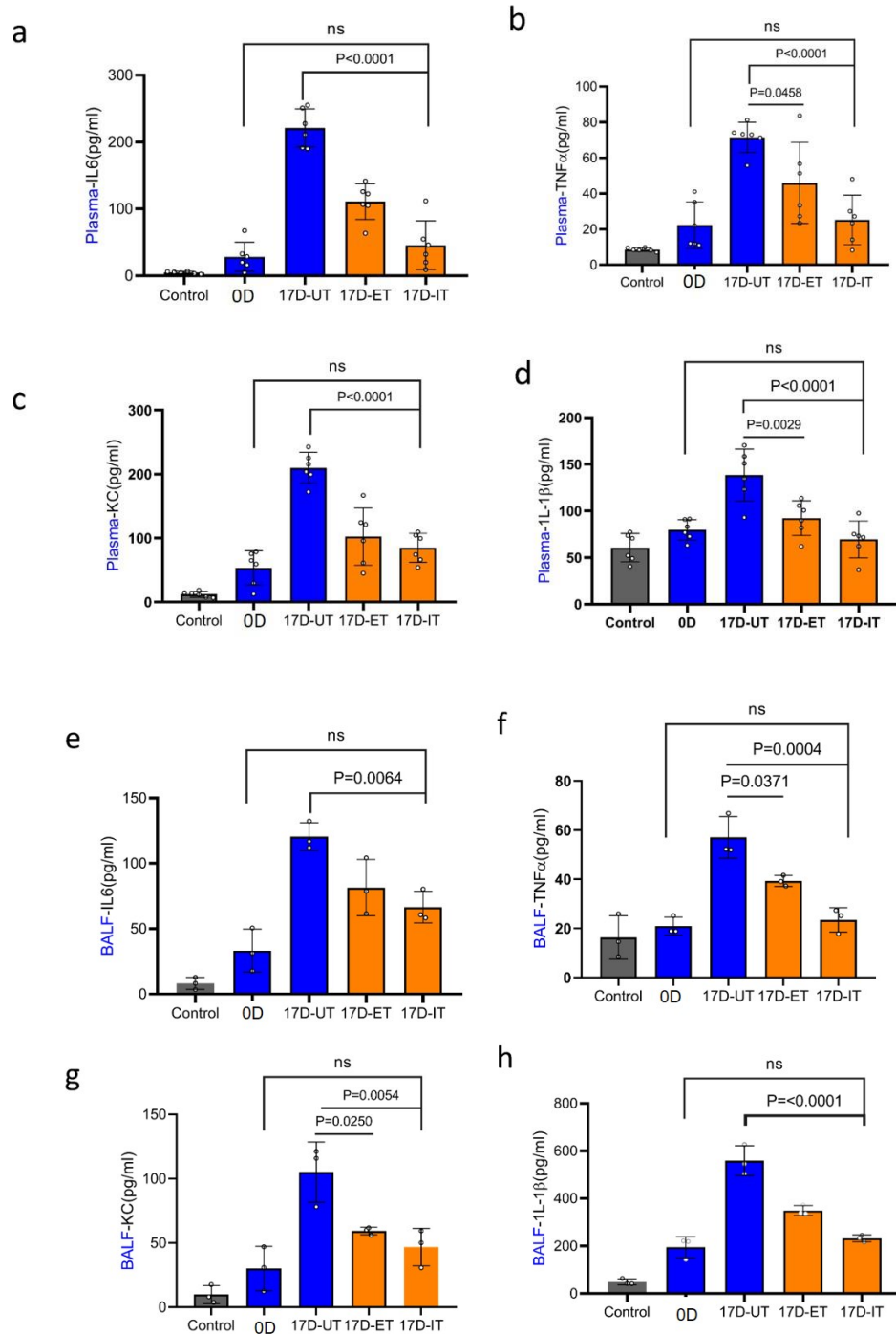


Fig 4.11: Removal of DAMPs from stored blood units significantly reduces the expression of Inflammatory cytokines in BALF and plasma of TRALI mice model. These events lead to the upregulation of pro-inflammatory cytokines in plasma and BALF of mice. An inflammatory response most frequently accompanies ALI, and it was profound in the 17D UT group of mice as reported in this study, periodic cleaning of DAMPs keeps the blood unit healthy, and their transfusion as 2nd hit leads to reduced inflammatory cytokines. The Data are mean \pm s.d. (n=3, performed at least twice).

A similar experiment was also performed with 17D-UT group where innate and adaptive immunity got activated in mice. These events lead to the upregulation of pro-inflammatory cytokines in plasma and BALF of mice. In BALF, the levels of IL-6 and TNF-alpha in 17D UT were 1.5 and 3 folds, respectively higher than in the fresh blood (OD) transfusion group (Fig. 4.11a-d). The increase in these cytokines expression clearly states the role of DAMPs as "second hit" to rupture the endothelial barrier and the increase in vasculature permeability. (**Fig. 4.11a-h**) In the case of the intermittent treated group, there was a reduction in systemic pro-inflammatory cytokines level to ~70 pg/ml and ~30 pg/ml. compared to the untreated group. The cytokine data supports that removing DAMPs intermittently can also significantly reduces the expression of other cytokine profiles (IL-1beta, KC) in plasma. A similar trend of down-regulation of pro-inflammatory cytokines was observed in the BALF of 17D-IT & ET group suggesting reduced inflammation in lungs. (**Fig. 4.11 e-h**).

4.4 Discussion

Transfusion-related acute lung injury (TRALI) is a respiratory distress syndrome and sometimes caused by transfusion of aged blood products. It is a significant cause of transfusion-related mortality³¹⁻³⁵. Previous studies have established that the transfusion of aged RBCs cause increased neutrophil counts, cytokine and chemokine concentrations in the lung followed by increased pulmonary microvascular permeability³⁶⁻³⁸. Progressing in a similar direction, we developed a non-antibody mediated TRALI model in C57B6/J mice after optimizing the dose of LPS as "first hit" and old stored RBCs transfusion as the "second hit". Using the concept of our previous study where intermittent treatment with combination of cationic and anionic scaffolds (*Tau-AcrNFS*) improves the quality and shelf life of stored mouse blood. Our initial study in this work determines the extent of lung injury by increased vascular permeability, responsible for the increased EBD in lung tissue. LPS (4 mg/kg) as the first hit showed a significant amount of lung injury after 14day old blood (S) transfusion. Thus, the dose of 4 mg/kg was sufficient to primes neutrophils and led to the event of TRALI. Also, the accumulation of dye and temporal variation of protein levels in BALF was seen 24h after the transfusion of 14D (S) comprising DAMPs. It was observed that 24 hours is essential to activate the primed neutrophils that have undergone NETosis to cause endothelial barricade rupture. This allows the flow of plasma proteins from blood into the alveolar spaces. To further explore the non-

antibody mediated two-hit TRALI model, we transfused 17D mice blood as a 2nd hit. The critical point here is using a 17D old blood unit that has already crossed the mice's blood shelf life of 14D and contains a heavy load of DAMPs to develop serious lung injury. In order to make sure that the severity of the injury is exaggerated after the transfusion of 17D old blood in LPS challenged mice, we compared the untreated data (17D-UT) with only LPS administered animals. The 17D-UT group demonstrated a significant increase in vasculature permeability by impairing alveolar-capillary barrier function. Interestingly, in the 17D-ET group, end day removal of DAMPs didn't lead to severe vasculature permeability compared to 17D-UT group. The observations were supported by the Evans Blue dye experiment and further confirmed by histological studies. The data suggests the scavenging of DAMPs via scaffolds from stored blood does not lead to severe TRALI symptoms in mice. This concept of end day treatment (ET) was followed by intermittent treatment during storage (IT) to prevent blood stored units from undergoing biochemical and structural changes. This technique involves periodical removal of DAMPs using scaffolds from stored blood units. In the meantime, our previous study shows that the intermittent removal of DAMPs using *Tau-AcrNFS* on the 5th and 10th day of storage time significantly improved the quality of the blood units. Also, the shelf life of mice RBCs was pushed to 17 days by slowing the rate of deterioration during storage³⁹. The same concept explored in the TRALI model, where the stored blood was treated on the 5th and 10th day of storage (IT) and transfused to the mice as 2nd hit. The low concentration of EBD in 17D old (IT) group confirms lower vasculature permeability than the untreated group. The fascinating point is that the extent of vasculature permeability in 17D(IT) mice were similar to freshly transfused blood (0D). This indeed confirmed that the quality of 17D blood (IT) was maintained by periodic cleaning of DAMPs. To further confirm the development of TRALI symptoms in LPS administered mice by stored blood transfusion, the histology of lungs was performed and studied.

Since lungs may be the most susceptible organ for protein debris and NETs deposition after transfusion, the activated neutrophils would probably be trapped in lung microcirculation and transmigrate locally while forming NETs. However, these histological observations were slightly seen in the 14D(S) transfusion model, but the degree of inflammation was not aggravated, leading to inconclusive results between the treated and non-treated groups. While in the 17D-UT group, the severe inflammation was primarily associated with neutrophil infiltration and deposition of proteinaceous

debris or fibrin strands in the airspaces with thickened alveolar walls. Clumping of RBCs was also observed. It was either due to the increased expression of advanced glycation end products (AGE) on stored RBCs or increased inflammatory cytokines in stored blood. Therefore, observing clumps of RBCs within the lung microvasculature may be considered a sign of inflammation⁴⁰. In the treatment group of 17D (ET), the tissue section showed lesser inflammatory cell infiltration (acute and chronic) in interstitial and alveolar spaces compared to the untreated group. There was a minor widening of the alveolar septum and expansion of interstitial space due to inflammatory cells and congested blood vessels. The congested blood vessel could be due to the presence of lysed/ aged RBCs in 17D old blood units and the removal of DAMPs led to reduced inflammation. There was no widening of the alveolar septum or edematous areas in the 17D (IT) group. Moreover, 17D(IT) group's histological findings appears to be similar to the fresh blood transfusion group.

The findings discussed above prove that the quality of the blood plays a pivotal role to prevent mice from developing TRALI symptoms. Similarly, there was an increase in NETs formation, during 17D pRBCs transfused LPS challenged mice, demonstrated by the widespread colocalization of extracellular DNA, and myeloperoxidase enzyme in BALF and plasma. The total protein concentration and total cell count in the BALF reflect extravascular protein accumulation primarily from increased permeability. However, removing the DAMPs using scaffolds on the last day of storage (17D-ET) leads to lesser infiltration of immune cells, decrease in NETs biomarkers and extracellular proteins. In the case of the 17D-IT group, intermittent removal of DAMPs significantly ameliorated the condition of lung injury in LPS administered mice compared to the untreated group. Excessive NETs formation and poor clearance may induce severe inflammatory processes involving leukocyte accumulation, diffuse alveolar damage and epithelial injury. These events result in the massive release of inflammatory cytokines, locally and in the circulation via increased alveolar permeability. Concentrations of pro-inflammatory mediators in TRALI are invariably associated with elevated pro-inflammatory mediators, including tumour necrosis factor (TNF), IL-1 beta, IL-6, Keratinocytes derived chemokine (KC), Macrophage inflammatory protein (MIP-2), Surfactant protein D and Vascular endothelial growth factor (VEGF)⁴¹⁻⁴³. These mediators are readily measured by commercial ELISAs or multiplex assays using either Balf fluid or mice plasma. An inflammatory response most frequently accompanies ALI, and it was profound in the

17D UT group of mice as reported in this study, periodic cleaning of DAMPs keeps the blood unit healthy, and their transfusion as 2nd hit leads to reduced inflammatory cytokines like IL-6, TNF- α , KC and Plasma-1L-1 β levels in the Balf and plasma of mice.

4.5 Conclusion

In this work, we prove that our non-antibody mediated two-hit TRALI model can develop TRALI symptoms mimicking the same trend observed in humans. The concept of scavenging DAMPs before transfusion by incubating old stored blood with *Tau-AcrNFS* scaffolds efficiently reduces the transfusion-related complications. The periodic cleaning of DAMPs by scaffolds improves the quality of stored blood by the end of their shelf life and does not lead to TRALI events after transfusion.

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Conclusions & Future Directions

Transfusion of healthy red blood cells (RBCs) is a lifesaving process, and this process is severely impacted by the production of DAMPs in RBCs during storage. The quality of stored RBCs declines during storage due to the production of a wide range of damage-associate molecular patterns (DAMPs). Since these DAMPs consist of either positive or negatively charged species, the developed polymers were smartly designed to have an appropriate hydrophilicity/hydrophobicity balance, and carry anionic (due to taurine) or cationic (due to acridine) charges.

The current work has two novelty factors. 1). Demonstrating the concept of intermittent scavenging of DAMPs during storage could slow the aging of RBCs to enhance the quality and shelf-life of stored oldRBCs. 2) Material design to efficiently scavenge DAMPs through ionic interactions and intercalation process. A suitable storage day for intermittent treatment is identified through systematic time- dependent experiments. It is reported that 28th-day treatment results in a maximum reduction of DAMPs and retains healthier RBCs on the 42nd day. This observation could be because the accelerated deterioration of RBCs majorly occurs between the 28th day and to 42nd day of storage. The developed technology has the potential to remove these DAMPs intermittently and enhance the quality of stored-old RBCs equivalent to freshly collected RBCs and increases their shelf-life by ~22%.

Currently, to combat post transfusion related complexities, leukoreduced blood are widely used. Given, the leukoreduction process is expensive; hence, most of the blood banks in India and developing countries do not use leukoreduction processes. Thus, the cost implicated in universal leukoreduction policy is not practically feasible, especially in developing countries and other under-resourced nations. It is observed that leukoreduced blood units have lesser WBCs associated lesions like extracellular DNA and free histone by the end of the storage period. However, several poly-unsaturated fatty acids (PUFAs) and oxidized products are still accumulated in leukoreduced blood units akin to non-Leukoreduced blood units. Indeed, PUFAs and their oxidized products are known to cause transfusion-related acute lung injury (TRALI)).

Therefore, we have also quantified the DAMPs and performed the recovery experiment with leukoreduced stored RBCs. The data suggest that DAMPs such as free Hb and PUFAs are significantly produced at 42 days. Newly developed Tau-AcrNFS could

scavenge DAMPs from leukoreduced blood and reduce transfusion-related systemic inflammation. Similarly scavenging DAMPS from end day of RBCs storage can prevent the incidence of TRALI in mice. Furthermore, intermittent treatment adds a new dimension to maintaining RBC health by preventing the inflammatory response in TRALI mice model. This suggest that quality of old storage blood is as good as freshly stored blood after intermittent treatment. This technology may help in preventing transfusion related complexities and solving a problem of huge blood wastage.

We are considering making this technology as a medical device instead of a storage bag. We are brainstorming to have a final 'product design' to translate the technology. In this work, as a proof-of-concept, we establish that intermittently scavenging DAMPs using Tau-AcrNFS can enhance the quality and shelf-life. Now, we are focusing on designing a clinical product. The electrospun nanofibrous sheets may lead to the development of novel blood bags or an insert-based medical device, which will be feasible to use in the blood bank processing setting without the need for multiple interventions during the storage of blood. Therefore, we are designing an insert in a PVC bag that could scavenge DAMPs and could be removed. In the next product development stage, it will be finalized.






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Appendix B: Patent grant

 <p>INTELLECTUAL PROPERTY INDIA PATENTS DESIGNS TRADE MARKS GEOGRAPHICAL INDICATIONS</p>	 <p>सत्यमेव जयते</p>	<p>क्रमांक : 044136098 SL No :</p>	
<p>भारत सरकार GOVERNMENT OF INDIA पेटेंट कार्यालय THE PATENT OFFICE पेटेंट प्रमाणपत्र PATENT CERTIFICATE (Rule 74 Of The Patents Rules)</p>			
पेटेंट सं. / Patent No.	:	382827	
आवेदन सं. / Application No.	:	201841006678	
फाइल करने की तारीख / Date of Filing	:	21/03/2018	
पेटेंटी / Patentee	:	1.INSTITUTE FOR STEM CELL BIOLOGY AND REGENERATIVE MEDICINE (INSTEM) 2.PRAVEEN KUMAR VEMULA	
<p>प्रमाणित किया जाता है कि पेटेंटी को उपरोक्त आवेदन में यथाप्रकटित A CONJUGATE, A COMPOSITION, AN ARTICLE, PROCESSES OF PREPARATION AND APPLICATION THEREOF नामक आविष्कार के लिए, पेटेंट अधिनियम, १९७० के उपबंधों के अनुसार आज तारीख 21st day of March 2018 से बीस वर्ष की अवधि के लिए पेटेंट अनुदान किया गया है।</p>			
<p>It is hereby certified that a patent has been granted to the patentee for an invention entitled A CONJUGATE, A COMPOSITION, AN ARTICLE, PROCESSES OF PREPARATION AND APPLICATION THEREOF as disclosed in the above mentioned application for the term of 20 years from the 21st day of March 2018 in accordance with the provisions of the Patents Act,1970.</p>			
<p>INTELLECTUAL PROPERTY INDIA PATENTS DESIGNS TRADE MARKS GEOGRAPHICAL INDICATIONS</p>			
			
अनुदान की तारीख : 26/11/2021 Date of Grant :	पेटेंट नियंत्रक Controller of Patent		
<p>टिप्पणी - इस पेटेंट के नवीकरण के लिए फीस, यदि इसे बनाए रखा जाना है, 21st day of March 2020 को और उसके पश्चात प्रत्येक वर्ष से उसी दिन देना होगा। Note - The fees for renewal of this patent, if it is to be maintained will fall / has fallen due on 21st day of March 2020 and on the same day in every year thereafter.</p>			

Appendix C: First Paper

nature communications



Article

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Intermittent scavenging of storage lesion from stored red blood cells by electrospun nanofibrous sheets enhances their quality and shelf-life

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Check for updates

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Transfusion of healthy red blood cells (RBCs) is a lifesaving process. However, upon storing RBCs, a wide range of damage-associated molecular patterns (DAMPs), such as cell-free DNA, nucleosomes, free-hemoglobin, and poly-unsaturated-fatty-acids are generated. DAMPs can further damage RBCs; thus, the quality of stored RBCs declines during the storage and limits their shelf-life. Since these DAMPs consist of either positive or negative charged species, we developed taurine and acridine containing electrospun-nanofibrous-sheets (*Tau-AcrNFS*), featuring anionic, cationic charges and an DNA intercalating group on their surfaces. We show that *Tau-AcrNFS* are efficient in scavenging DAMPs from stored human and mice RBCs ex vivo. We find that intermittent scavenging of DAMPs by *Tau-AcrNFS* during the storage reduces the loss of RBC membrane integrity and reduces discocytes-to-spherocytocytes transformation in stored-old-RBCs. We perform RBC-transfusion studies in mice to reveal that intermittent removal of DAMPs enhances the quality of stored-old-RBCs equivalent to freshly collected RBCs, and increases their shelf-life by ~22%. Such prophylactic technology may lead to the development of novel blood bags or medical device, and may therefore impact healthcare by reducing transfusion-related adverse effects.

Blood transfusion is often a lifesaving practice for patients in the intensive care unit (ICU), where ~50–70% of ICU patients are transfused with blood units during their stay¹. Typical indications for blood transfusion include sickle cell crisis, anemia, and severe blood loss². The highest transfused blood components are red blood cells (RBCs), with >85 million RBC units are transfused annually worldwide^{3,4}. While RBC transfusion has therapeutic benefits, transfusion of storage-aged RBCs may cause deleterious effects on the recipients. These

deleterious effects have been attributed to the storage lesion consisting of damage-associated molecular patterns (DAMPs) generated due to biochemical, morphological, and structural changes in RBCs during the storage-aging process⁵. The most studied DAMPs in this context are as follows: (i) extracellular free-iron and free-hemoglobin (Hb) generated due to lysis of RBCs, (ii) bioactive lipids such as poly-unsaturated fatty acids (PUFAs), (iii) extracellular DNA, and (iv) nucleosomes generated by neutrophils. These DAMPs are a potential

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Appendix C: Second Paper

SCIENCE ADVANCES | RESEARCH ARTICLE

NEUROPHYSIOLOGY

Prevention of pesticide-induced neuronal dysfunction and mortality with nucleophilic poly-Oxime topical gel

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Organophosphate-based pesticides inhibit acetylcholinesterase (AChE), which plays a pivotal role in neuromuscular function. While spraying in the field, farmworkers get exposed to pesticides through the dermal route. Internalized pesticide inhibits AChE, which leads to neurotoxicity, cardiotoxicity, cognitive dysfunction, loss of endurance, and death in severe cases. Here, we present a nucleophilic pyridine-2-aldoxime-functionalized chitosan-based topical gel (*poly-Oxime gel*) that rapidly deactivates organophosphates, methyl parathion (MPT), on the skin of rats, which leads to reduced AChE inhibition in the blood and tissues. Testing the robustness of *poly-Oxime gel*, we report reduction in AChE inhibition following repeated dermal administration of MPT in the presence of *poly-Oxime gel*. Furthermore, *poly-Oxime gel* prevented MPT-induced neuromuscular dysfunction, loss of endurance, and locomotor coordination. We observe a 100% survival in rats following topical MPT administration in the presence of *poly-Oxime gel*. This prophylactic gel may therefore help farmworkers by limiting pesticide-induced toxicity and mortality.

INTRODUCTION

Acetylcholinesterase (AChE) is abundant in both central and peripheral nervous system (1, 2). In addition to chemical warfare agents (CWAs), organophosphate pesticides [such as methyl parathion (MPT), malathion, paraoxon, and chlorpyrifos] are AChE inhibitors. Inhibition of AChE leads to the accumulation of the neurotransmitter acetylcholine, resulting in neurological disorders, suffocation, paralysis, and death in severe cases (3, 4). India is one of the prime countries in pesticide usage in the world, with a vast majority of agricultural workers being repeatedly exposed to pesticides in the field (5, 6). In addition to neurotoxic effects, AChE inhibition leads to cardiotoxicity, reduced immunity, infertility, and delayed sexual maturation (7). Cohort studies reveal that pre- and postnatal exposures to pesticides impede neurodevelopment in children living in agricultural communities and increase susceptibility to neuropsychiatric disorders (8, 9).

According to the World Health Organization (WHO) Recommended Classification of Pesticides by Hazard, organophosphate pesticides oxydemeton-methyl and methamedophos belong to class I (extremely/highly hazardous); dimethoate, fenthion, quinalphos, chlorpyrifos, prothiofos, and diazinon belong to class II (moderately hazardous); and malathion and pirimifos-methyl belong to class III (slightly hazardous) (10, 11). It is clear that systemic exposure to pesticides through the dermal route is a health hazard. Preventive strategies to eliminate or minimize dermal exposure to reduce pesticide-induced toxicity are a less attended clinical need. Although the personal protective equipment (PPE) such as suits, gloves, face masks, headgear, and boots are available, they are scarcely used, mainly because of high cost and discomfort under tropical conditions (12–14). The most effective way to prevent pesticide exposure is chemical deactivation of pesticides, and existing PPE lack this ability. There have been attempts to formulate physical barrier creams in the past to attenuate exposure to pesticides or CWAs (15–17). This design comes with an inherent limitation of expo-

sure, as the pesticide that is arrested on the surface can still enter the system through the oral or ocular route via hand contact. In addition, each pesticide brand has different additive formulations, and these formulations have variable penetrance through physical barrier creams. The noncatalytic nature of this approach also limits the amount of pesticide the barrier creams can arrest. Therefore, physical barrier creams are not effective in preventing exposure. The metal nanoparticles, like CeO₂, are studied as potential chemical deactivators of pesticides/CWAs (18, 19). However, CeO₂ nanoparticles are known to cause DNA damage in dermal fibroblasts, an effect that necessarily limits their use in dermal protectants (20, 21).

To understand the awareness of agricultural workers about the pesticide-induced toxicity, we interacted with several farmers and their families in the field. All of them were aware of pesticide-induced toxicity, as they experience pain right after they spray pesticides in the field. However, because of the lack of any protective technologies, they suffer, and they expressed their willingness to adopt any topical technologies that can prevent pesticide exposure. Because of the low purchasing power of this sector, the affordable cost would be a key differentiating factor to have compliance to adopt these technologies by the end user—the farmer. This unmet need inspired us to develop prophylactic technologies to prevent pesticide exposure during spraying in the field. An ideal user-compliant solution would be a low-cost, easy-to-use, nonobstructive biocompatible material, which could inactivate pesticides on the skin and prevent their penetration. A topical gel with catalytic activity to cleave/hydrolyze pesticides before entering into the skin may serve as a prophylactic strategy to reduce chronic exposure (Fig. 1A). The aim/objective of this study was to determine whether a nucleophilic pyridine-2-aldoxime-functionalized chitosan-based topical gel (*poly-Oxime gel*) could be used as a prophylactic gel to deactivate pesticides on the skin before they enter the body. However, multiple technical hurdles exist in designing this catalytically robust prophylactic topical gel. The first is the identification of a reactive nucleophile that can act by rapidly hydrolyzing organophosphate ester on the skin before it enters. Also, it should catalytically, not stoichiometrically, cleave organophosphates. Second, the topical gel should be chemically active in different climate conditions to be effective.

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