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Transfusion of Older Stored Blood Worsens Outcomes in Canines Depending on the Presence and Severity of Pneumonia

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Abstract

BACKGROUND—In experimental pneumonia we found transfused older blood increased mortality and lung injury that was associated with increased *in vivo* hemolysis and elevated plasma cell-free Hb (CFH), non-transferrin bound (NTBI), and plasma labile (PLI) iron levels. In the present study, we additionally analyze identically treated animals that received lower or higher bacterial doses.

STUDY DESIGN AND METHODS—Two-year-old purpose-bred beagles (n=48) challenged intrabronchially with *Staphylococcus aureus* [0 (n=8), 1.0 (n=8), 1.25 (n=24) and 1.5 (n=8) × 10⁹ CFU/kg) were exchange transfused with either 7- or 42-day-old canine universal donor blood (80 ml/kg in 4 divided doses).

RESULTS—The greater increases in cell-free Hb (CFH) with older blood over days following exchange proved relatively independent of bacterial dose. The lesser increases in CFH observed with fresher blood were bacterial dose-dependent potentially related to bacterial hemolysins. Without bacterial challenge, levels of CFH, NTBI, and PLI were significantly higher with older vs. fresher blood transfusion but there was no significant measurable injury. With higher dose bacterial challenge, the elevated NTBI and PLI levels declined more rapidly and to a greater

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extent after transfusion with older vs. fresher blood, and older blood was associated with significantly worse shock, lung injury, and mortality.

CONCLUSION—The augmented *in vivo* hemolysis of transfused older red cells appears to result in excess plasma cell-free Hb and iron release, which requires the presence of established infection to worsen outcome. These data suggest that transfused older red cells increase the risks from infection in septic subjects.

Keywords

Red cells; hemoglobin; iron; absorption and metabolism; Transfusion medicine; Vascular biology; animal models of vascular pathology; Blood storage lesions

INTRODUCTION

In the United States, red blood cells (RBCs) for transfusion are stored for up to 42 days and standard inventory practice is to issue the oldest blood first.^{1,2} Although six weeks stored blood meets current Food and Drug Administration standards, clinical and laboratory studies have raised concerns that the changes that develop as RBCs age in storage (“storage lesions”) render “older” blood less safe than blood stored for shorter durations.^{3–6} Hemolysis with release of plasma cell-free Hb (CFH) and the resultant nitric oxide (NO) scavenging, vasoconstriction, and vascular injury,^{7–9} as well as release of RBC iron promoting bacterial growth, have been implicated among the possible mechanisms for the reported toxicity of older stored blood.^{10–13} A recent meta-analysis has further raised concern that patients receiving older blood have increased mortality rates.¹⁴ Four randomized controlled trials addressing this issue are being conducted in Canada,^{15,16} Australia,¹⁷ and the United States,¹⁸ but the results may not be available for several years. Even these studies may not provide definitive results. Because the optimal study design may not be possible in clinical trials, we developed an animal model of blood transfusion injury to investigate the risks, determine the mechanisms of injury, and facilitate development of new therapeutic approaches.⁷

In our initial study,⁷ purpose-bred beagles with *S. aureus* pneumonia were randomized for exchange transfusion with blood that was relatively fresh (7 days old) or at the end of storage (42 days old). The older blood increased mortality and lung injury that was associated with increased *in vivo* hemolysis and elevated plasma CFH levels over days. After transfusion of older blood, pulmonary pressures were markedly elevated and necrosis was increased at the site of infection. The increased plasma CFH and NO consumption capability were strongly correlated with increased mortality. We concluded that NO depletion by CFH and vasoconstriction played a critical role in the increased lung injury and mortality associated with older blood.⁷ The levels of non-transferrin-bound iron (NTBI) and plasma labile iron (PLI) were only transiently elevated during transfusion and not significantly correlated with outcome.⁷

In the present study, we reanalyzed the 24 animals previously published⁷ that received 1.25×10^9 CFU/kg *S. aureus*, and analyzed for the first time an additional 24 identically treated animals that received lower [0 (n=8) or $1.0 (n=8) \times 10^9$ CFU/kg] or higher bacterial doses

[1.5×10^9 CFU/kg (n=8)]. We demonstrate that without bacterial infection there are no measurable increased risks from older blood despite CFH, NTBI and PLI levels being more elevated compared to fresher blood. However, with increasing doses of bacterial challenge, both iron values decline more rapidly and completely over time with older blood, and their disappearance from the plasma is associated with greater clinical de-compensation and increased mortality. The bacterial dose effect suggests that plasma iron released from RBCs is potentially available to be utilized by the bacteria to promote growth and worsen infection^{12,13,19} and, along with NO depletion,⁷⁻⁹ contributes to clinical effects of the storage lesion.^{5,10,11}

MATERIALS AND METHODS (Please see online supplement for more complete Materials and Methods)

Study design

Forty-eight purpose-bred beagles (12- to 28-mo-old, 10–15kg) were employed to investigate if the presence and severity of infection alters effects of transfused older stored blood. Animals were challenged with none or one of four increasing intrapulmonary doses of *Staphylococcus aureus* at 0h [0 (n=8), 1 (n=8), 1.25 (n=24), 1.5 (n=4), and 2.0 (n=4) $\times 10^9$ CFU/kg]. The results of the 24 animals that received 1.25×10^9 CFU/kg *S. aureus* were previously published,⁷ but otherwise treated identically. Investigators and all other staff were blinded to animal treatment groups throughout the study. This study was approved by the Animal Care and Use Committee of the Clinical Center at the National Institutes of Health.

In this study, animals with a tracheostomy were sedated and mechanically ventilated. At time 0 (T0), *S. aureus*, oxacillin sensitive, isolates were administered into the right lower lobe, sub-segmental airspace via a bronchoscope as previously described.²⁰ The doses of *S. aureus* (1, 1.25, 1.5, and 2 $\times 10^9$ CFU/kg in 1 mL PBS) were determined spectrophotometrically and confirmed turbidometrically. The 0 bacterial dose group (n=8) received a volume of saline via bronchoscope. Oxacillin (30 mg/kg IV) was started 4h after bacterial inoculation and administered every 4h until 96h or until death in all animals.

Starting at 4h after bacterial challenge and immediately after starting antibiotics, animals were randomized to receive an exchange transfusion of commercially available canine universal donor, leukoreduced blood (DEA 1.1 ABRINT, Dixon, CA) stored for 7- or 42-day as previously described.⁷ Each animal had 25% of total blood volume (20 mL/kg) removed over 30 minutes and replaced with an equivalent volume of stored RBCs (10 mL/kg) at room temperature and thawed fresh frozen canine universal donor plasma (10 mL/kg) over 30 minutes. The exchange transfusion was repeated at 7, 10, and 13h. From time 0 to 96h or until death, conventional intensive care unit support was employed, including mechanical ventilation, fluid support, and vasopressors titrated to physiological endpoints based on previously established algorithms.²⁰ Animals alive at 96h were considered survivors and euthanized while still sedated (Beuthanol; 75 mg/kg IV).

RESULTS

Serial mean shock scores

The shock score is used to reflect mean arterial pressure (MAP), and accounts for the amount of vasopressor needed (norepinephrine) to maintain MAP at a preset normal level for canines (mean 80 mmHg).^{21,22} At 4h, immediately before exchange transfusion, there was no significant difference in the two groups in the mean shock score over all bacterial doses (0, 1, 1.25, or 1.5×10^9 CFU/kg, respectively) (all, $p=ns$) (Figure 1A.). However, by 13h, systemic hypertension developed in all animals, resulting in an “improved” overall (higher) mean shock score with old compared with new blood transfusion at all bacterial doses (combined all doses, $p=0.01$) (Figure 1B).

At 16h, the effect of older blood on the shock score was bacterial dose-dependent; the shock score with older blood declined from a value more elevated at the 0 than the 1.0×10^9 CFU/kg bacterial doses to values sufficiently decreased that animals required vasopressor support to manage hypotension at the 1.25 and additional vasopressors at the 1.5×10^9 CFU/kg doses (bacterial dose response, $p=0.03$) (Figure 1C). In contrast, at 16h, there was no significant change or worsening of the shock score in animals transfused with new blood even as bacterial dose increased ($p=ns$) (Figure 1C). By 24h, animals in both old ($p<0.0001$) and new ($p=0.02$) blood groups showed a dose-related progressive abatement of the hypertensive effect seen with the zero or no bacterial challenge dose. As the bacterial dose further increased, progressive hypotension and a worsening or more negative shock score developed. As bacterial dose increased, shock score decreased more markedly with old than with new blood ($p=0.03$ interaction) (Figure 1D). For completeness, the serial mean values at different bacterial doses of the shock score components (MAP and norepinephrine requirement) are shown in the online supplemental Figure 1A-H.

Serial mean pulmonary artery pressures (MPAP)

At 4h after bacterial challenge, there were no significant differences with old or new blood in MPAP at any bacterial dose or overall bacterial doses combined (all, $p=ns$) (Figure 2A). However, by 13h (during transfusion), as seen in the systemic circuit, MPAP increased with old compared to new blood at all bacterial doses (all doses combined, $p=0.01$) (Figure 2B). In contrast, by 16h, the effect of older blood disappeared (all, $p=ns$). Further, by 24h, MPAP with old and new blood rose in parallel with increasing bacterial dose; the increase in MPAP was significantly greater with old compared to new blood transfusion at the 1.25×10^9 CFU/kg bacterial dose versus the other bacterial doses ($p=0.03$, interaction). With old compared to new blood, the rise in MPAP was greater at the 1.25×10^9 CFU/kg *S. aureus* dose than at the 0 and 1×10^9 CFU/kg doses ($p=0.04$), and the MPAP of old blood at the 1.25×10^9 CFU/kg bacterial dose was greater than that at the 1.5×10^9 CFU/kg bacterial dose ($p=0.01$) (Figure 2D).

Serial mean lung injury score (LIS)

To examine lung injury, we used a previously developed scoring system.²¹ At 4h after bacterial challenge, no significant changes in mean LIS with old or new blood could be detected as bacterial dose increased (all, $p=ns$) (Figure 2E). Furthermore, without bacterial

challenge there was no increase or difference in mean LIS score between old and new blood transfused animals throughout (all, $p=ns$). However, at 13 and 16h, as bacterial dose increased, there was a similar worsening of the LIS with old ($p=0.055$ and $p=0.02$, respectively) and new blood transfusion ($p=0.0001$ and $p=0.02$, respectively) (Figure 2F and G). In contrast to 13 and 16h, at 24h there were significant differences with increasing bacterial doses between old and new blood in the rise in mean LIS ($p=0.01$ interaction) (Figure 2H), similar to those found in MPAP at 24h (Figure 2D). At the 1.25×10^9 CFU/kg bacterial dose, the increase in LIS was more profound when comparing old *vs.* new blood transfusion than at the 0 and 1.0×10^9 CFU/kg bacterial dose ($p=0.01$) and the LIS of old blood at the 1.25×10^9 CFU/kg bacterial dose was greater than that at the 1.5×10^9 CFU/kg bacterial dose ($p=0.004$) (Figure 2H).

For completeness, the serial values for old and new blood for the components of the LIS are shown in online supplemental Figures 2A-H and 3A-H. When the MPAP values are excluded from calculating the LIS, the results are unchanged. Upon examination of tissue specimens taken immediately post mortem from the right infected lung and compared to the left uninfected lung in each animal, patterns similar to the LIS were seen. Only the 1.25×10^9 CFU/kg bacterial dose was significantly associated with greater hemorrhage, necrosis, and thrombosis with old compared to new blood transfusion ($p=0.03$, previously reported). There were no significant differences in these pathologic findings between old and new blood at the other bacterial doses (0, 1, or 1.5×10^9 CFU/kg) (all, $p=ns$) (see online supplemental Figure 4).

Serial mean levels of plasma cell free hemoglobin (CFH) and plasma NO consumption capability

At 4h after bacterial challenge, there were no significant elevations (or differences) in mean CFH plasma levels comparing old and new blood transfusions (all, $p=ns$) (Figure 3A). In contrast, at 13h, the CFH levels (Figure 3B) were elevated with old compared to new blood at all bacterial challenge doses studied (0, 1, 1.25 and 1.5×10^9 CFU/kg; all doses combined, $p<0.0001$).

At 24h, the difference in CFH levels between old and new blood changes significantly as bacterial dose increases ($p=0.04$ interaction) (Figure 3C). The difference between the elevated CFH levels comparing old *vs.* new blood transfusion declines as bacterial dose increases. This narrowing reflects the more marked increases in plasma CFH levels with new blood as bacterial dose increases ($p=0.03$ dose effect).

The assay of NO consumption capability of plasma^{23,24} results were similar to the above CFH data (Figure 3D-F). The only exception was that at 13h the increases in NO consumption capability of plasma with new blood became statistically significantly bacterial dose-dependent. The CFH level elevation with new blood became significantly bacterial dose-dependent later at 24h.

Serial mean non-transferrin-bound iron (NTBI), labile plasma iron (PLI) and transferrin-bound iron (TBI) levels

At 4h after bacterial challenge and before exchange transfusion, there were no significant differences between old or new blood in mean NTBI (Figure 4A) and TBI (Figure 4G) (both $p=ns$). At 4h, however, PLI increased with new blood compared to old blood transfusion across all doses of bacteria combined (Figure 4D) ($p=0.004$), although still below the level considered abnormal ($0.20 \mu M$).

In contrast, at 13h and 24h alike with zero or no bacterial challenges, there were marked elevations in mean NTBI (Figure 4B and 4C), PLI (Figure 4E and 4F) and TBI levels (Figure 4H and 4I) in both groups, greater with old blood for NTBI and PLI but similar with old and fresher blood for TBI. At 13 and 24h, NTBI, PLI and TBI levels declined as bacterial dose increased ($1.5 > 1.25 > 1.0 > 0 \times 10^9$ CFU/kg bacterial dose). Notably, this fall in NTBI and PLI associated with increasing bacterial dose ($1.5 > 1.25 > 1.0 > 0 \times 10^9$ CFU/kg bacterial dose) was significantly greater with old compared to new blood at 13h ($p=0.04$ interaction and $p=0.06$ interaction, respectively) and at 24h ($p=0.01$ interaction and $p=0.03$ interaction, respectively).

In contrast, at 13h and 24h, TBI levels declined similarly ($p=0.23$ and $p=0.73$ for interaction) with older and fresher blood as bacterial dose increased ($p=0.0009$ and $p=0.0005$ for old blood, $p<0.0001$ and $p=0.001$ for fresher blood at 13h and 24h, respectively) (Figure 4H and 4I). Furthermore, for the control animals (0×10^9 CFU/kg bacterial dose), TBI levels after the first transfusion (7h) were high and remained similarly high after the second and third transfusion of older blood (see Supplemental Figure 5). However, not until the third transfusion of new blood (13h) do TBI levels reach similar high levels seen with older blood transfusion.

Using a plasma iron depletion score to measure the relative amount of NTBI and TBI disappearing from the plasma at 24h

We normalized the NTBI and TBI levels to those expected, based on levels recorded with the 0 or no bacterial challenges and old blood exchange. Using this normalized scoring-system, the old blood animals “consumed” more iron (measured by the sum of NTBI and TBI levels) than did the new blood animals ($p=0.005$) at the 1.25×10^9 CFU/kg bacterial dose, but not at the 1×10^9 CFU/kg bacterial dose ($p=0.93$) or the 1.5×10^9 CFU/kg dose ($p=0.57$) (Figure 5). The results are similar if the levels of PLI are added to the iron calculation.

Survival

There were dose-dependent increases in mortality with increasing doses of bacteria with older ($p=0.0002$) and new blood transfusion ($p=0.006$) (see online supplemental Figure 6A-B). There were no deaths with older or newer blood transfusion at the zero or no bacterial dose challenge, but at all other bacterial doses, older blood compared to fresher produced either nominally more deaths or more rapid deaths (Figure 6). However, similar to the LIS and MPAP data, significant increases in mortality occurred with older vs. new blood only at

the 1.25×10^9 CFU/kg dose ($p=0.0005$, previously reported) and not at the 0, 1, or 1.5×10^9 CFU/kg bacterial doses (all, $p=ns$) (Figure 6).

Other laboratory values

For completeness, commonly measured laboratory parameters are shown in online supplemental Tables 1 through 3. None of these data show marked abnormalities with old vs. new blood that can easily explain our results.

DISCUSSION

In this study, we found that during transfusion at all bacterial challenge doses, older blood produced elevations in CFH and NO consumption capability of plasma (Figure 3) that was associated with hypertension (Figure 1B). This effect persisted for hours after transfusion only with the zero or the very lowest bacterial doses (Figure 1C and D). In contrast, by 10 hours after transfusion the shock score with older blood went from being “improved” (hypertension) at low bacterial doses compared to new blood transfusion to significantly worse at higher bacterial doses (Figure 1C and D). This effect was not evident during the transfusion. During this time, and in parallel with the bacterial dose-dependent worsening of shock, a greater bacterial dose-dependent disappearance in transfusion-related elevations of NTBI and PLI levels occurred with older compared to newer blood (Figure 4B, C, E, and F).

These data suggest that early into exchange transfusion with older blood, the vasoconstrictive effects of elevated CFH predominate and can persist for hours even with no or low level bacterial infection. However, the larger the bacterial challenge, the more marked the disappearance of iron from the plasma after transfusion, resulting over time in worse shock. These data are consistent with two temporally distinct mechanisms of injury related to infusion of older blood: an acute mechanism apparently independent of bacteria and associated with the ability of CFH released from older RBCs to cause NO scavenging and vasoconstriction;^{8,9,25–30} the second effect, related to the ability of *in vivo* hemolysis to potentially supply iron to the infused bacteria, which could increase bacterial growth, and over time worsen the infection and result in more profound shock.^{5,12,19,31–33} The iron-donating mechanism requires sufficient time for bacterial growth to develop and for increasing infection to counteract the hypertensive manifestations of CFH and result in a picture of worsening shock. Notably the increased CFH and plasma iron causes no injury in the absence of infection.

Although the shock score reflects a systemic response to infection, the animals in this study succumb to deteriorating gas exchange from pneumonia rather than to shock. As expected, mortality and lung injury were worse with higher bacterial dose challenges with both old and fresh blood. However, the only marked difference between older and fresher blood in injury directly related to the dose of bacteria challenge [i.e., lung injury (Figure 2H) and mortality (Figure 6C)] was at the dose that produced an overall 50% mortality (1.25×10^9 CFU/kg). We believe that at the very low level infection found with zero or the 1.0×10^9 CFU/kg bacterial dose (12.5% mortality, 1) (Figure 6A and B), the NO scavenging effect in the absence of severe infection does not produce injury sufficient to increase lethality, and 2) the increased iron has no effect on outcome in the absence of bacteria and utilization at the

lower bacterial doses does not augment bacterial growth enough to significantly increase mortality. Moreover, at the highest bacterial dose (100% mortality), no significant differences except for the level of shock between the transfusions of older or fresher blood are observed (Figure 6D) because bacterial load is already so great that the increased iron utilization and NO scavenging add little to the already marked lethality of the bacterial challenges.

If this interpretation is correct, bacteria only at the middle bacterial dose should consume more iron with old *vs.* fresher blood. To examine this possibility, we calculated the amount of iron available in the plasma for bacterial consumption based on proportionality between CFH, an indicator of the level of hemolysis present and total potential plasma available iron stores, and the available iron levels actually measured (NTBI and TBI levels). The difference with increasing bacterial dose between the expected iron levels and the measured should indicate the amount that disappears or is possibly consumed by bacteria. Consistent with this hypothesis, the levels of iron consumed with the older blood *vs.* new blood indicated by the bacterial consumption score is significantly greater only at the 1.25×10^9 CFU/kg middle bacterial challenge dose (50% mortality), and not at low minimally lethal (12.5% mortality) or very high (100% mortality) lethal bacterial challenge doses.

We also found during transfusion unexpectedly bacterial challenge dose-dependent increases in plasma CFH and NO consumption capability after fresh blood transfusion. After 24h the levels with the highest bacterial doses were close to those found with older blood transfusion (Figure 3C). The comparable levels at 24h of CFH might account for the absence of any difference in mortality between the two groups at this time point. One possible explanation is the recognized ability of bacteria, and particularly *S. aureus*, to produce hemolysins and lyse RBCs.^{32,34} Normal host defense mechanisms limit iron availability to bacteria by avoiding protein binding (transferrin and lactoferrin) and by intracellular sequestration.^{35–38} Bacteria have evolved to acquire iron by releasing hemolysins,^{35,39,40} and by developing high affinity iron-binding siderophores that compete with plasma proteins to obtain extracellular iron.^{37,41,42} Some bacteria such as *S. aureus* have evolved cell surface receptors which recognize heme and transport it across the cell wall into the cytoplasm.^{35,37,43,44} These opposing mechanisms may account for the increases in CFH several hours after the infusion of the highest dose bacterial challenge and partial obliteration of the differences in new and older blood seen at lower bacterial doses.

The iron hypothesis as it relates to transfusion of older RBCs worsening outcome has been investigated extensively in a mouse model and in normal volunteers.^{5,10,11} In the mouse model, explosive proliferation of the ordinarily self-limited pathogen *S. typhimurium* was associated with older stored blood transfusion.¹⁰ In normal volunteers, transfusion with older blood increased extravascular hemolysis, circulating levels of iron, NTBI, and ferritin.¹¹ Moreover, in both models, the serum samples from transfusions with older stored blood produced markedly increased proliferation of bacteria *in vitro*.¹¹ Other data indicate that sera from patients administered oral iron and undergoing renal dialysis maintain increased NTBI levels for hours and similarly enhance *S. aureus* growth *in vitro*.⁴⁵ Furthermore, transfusion-related iron overload is associated with increased risk of bacteremia.⁴⁶ Our data suggest bacterial presence is critical in otherwise healthy subjects for

older blood to increase risks. Further, after transfusion, elevations of iron in the form of NTBI and TBI are available and potentially utilized by bacteria to enhance growth.^{13,37,41,42,45}

In patients with sepsis, elevated CFH levels are independently associated with an increased risk of death,⁴⁷ and critically ill patients, including those with sepsis or at risk for sepsis, have increased levels of CFH.⁴⁸ This CFH elevation during sepsis could be related to *in vivo* hemolysis caused by the infecting bacteria producing hemolysis and accumulating extracellular iron. The use of blood as a resuscitation fluid has been advocated for the early resuscitation of septic shock.⁴⁹ Our data suggest if older blood is used for resuscitation this may increase risks during septic shock because of increased *in vivo* hemolysis being a source of iron, promoting bacterial growth and worsening outcome.^{15–18}

We recognize that a limitation of our finding is absence of direct evidence that bacterial growth is related to increased iron availability and of direct evidence of bacterial hemolysin production. We also cannot rule out other storage lesions may contribute to the increased lethality of older stored blood transfusion in this model.^{6,50} The generalization of our current findings to human sepsis may be limited by differences in the storage of canine and human blood. It is also possible in subjects that are not otherwise healthy, such as those with coronary artery disease, the increased risk of vasoconstriction related to augmented CFH release with older blood transfusion may also worsen outcome independent of infection. We also cannot exclude the possibility that iron disappears because bacteria activate host factors that remove the NTBI, TBI and PLI from the plasma. However, this cannot easily explain the progressive lowering of TBI plasma levels associated with progressive increasing bacterial dose challenge, which would require bacterial siderophores which tightly bind transferrin for the iron to be removed.^{37,41,42} It is also possible that increased release of hepcidin which commonly occurs as a result of inflammation, contributes to the disappearance of iron in the circulation.⁵¹

In conclusion, we demonstrate *in vivo* evidence of two mechanisms by which CFH levels may increase during infection and worsen outcome: release of CFH from older transfused RBC due to hemolysis independent of bacterial dose effects, and with fresher transfused RBCs bacterial dose-dependent hemolysis presumably by bacterial hemolysins causing increased levels of CFH.^{35,39,40} We also demonstrate two mechanisms by which *in vivo* hemolysis of older blood may worsen outcome: acute vasoconstriction due to NO scavenging related to high CFH levels,^{9,25,28,29} and, increased iron released from older transfused blood, potentially augmenting bacterial growth, and worsening outcome.^{5,12,13,52} The latter mechanism, based on the greater disappearance of iron associated with increasing bacterial challenge and worse outcomes, could be an important mechanism of injury during septic shock.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Mean (± SE) shock scores at serial time points (panels A-D)

The shock score accounts for the level of vasopressor support (norepinephrine) needed to maintain the mean arterial pressure at a preset normal level for canines (mean 80 mmHg).^{21,22} In each panel the shock score is plotted at increasing doses of bacterial challenge for animals receiving older blood (solid bars) and fresher blood (open bars). Elevations in shock score represent elevations in mean arterial pressure and/or decreasing vasopressors requirement and *vice versa*. The p-value in each panel reports differences between old and new blood over all bacterial doses. The explanation for this difference is denoted by asterisks and shown below the figures.

* Old vs. New blood, with old blood, there is greater hypertension (higher SS) even as bacterial dose increases. (*Main Effect*)

** Old vs. New blood, as bacterial dose increases, with old blood, hypertension abates and shock increases progressively (more negative SS) with increasing bacterial dose than with new blood. (*Interaction*)

Figure 2. Mean ($\pm$ SE) pulmonary artery pressures and Lung Injury Score (LIS) at serial time points

This figure uses the same format as Figure 1, except now mean pulmonary artery pressure (panels A-D) or lung injury score (panels E-H) are plotted on the y-axis. The LIS in a previously published scoring system increases our ability to find the abnormalities in the lungs which includes mean pulmonary artery pressure, alveolar-arterial oxygen gradient, plateau pressure, O_2 saturation and respiratory rate.²¹ Similarly, p values are denoted by asterisks in each panel and explained below the figures.

* Old and New blood in tandem show a bacterial dose independent rise in mPAP, but it's more pronounced with old blood (*Main Effect*)

** Old and New blood, as bacterial dose increases, mPAP and LIS rise in tandem, but this rise is significantly greater at the 1.25×10^9 CFU/kg bacterial dose with older than new blood (*Interaction*). This interaction is as follows; the old *vs* the new blood rise in mPAP and LIS with 1.25×10^9 CFU/kg dose is greater than with the 0 and 1×10^9 CFU/kg bacterial doses ($p = 0.04$ and $p = 0.01$ respectively) and greater than with the 1.5×10^9 CFU bacterial dose ($p = 0.01$ and $p = 0.004$ respectively).

Figure 3. Mean ($\pm$ SE) levels of cell-free Hb and NO consumption capability of plasma at serial time points

The format is similar to Fig 1, except now plasma levels of cell-free hemoglobin on top (panels A-C), and NO consumption capability of plasma on the bottom (panels D-F), are plotted on the y-axis. P-values are denoted by asterisks and explained below the figures.

* Old vs. New blood overall bacterial doses alike a greater increase in CFH level or NO consumption capability of plasma even as bacterial dose increases (*Main Effect*)

** The difference at each bacterial dose in the level of CFH between Old and New blood progressively decreases as bacterial dose increases (*Interaction*), mostly because the level of CFH rises significantly more with New than Old blood as bacterial dose increases.

Figure 4. Mean ($\pm$ SE) levels of non-transferrin-bound iron (NTBI), plasma labile iron (PLI) and transferrin-bound iron (TBI) at serial time points

The format is similar to Fig 1, except now plasma levels of NTBI (panels A-C), PLI (panels D-F) and TBI (panels G-I) are plotted on the y-axis. P-values are denoted by asterisks and explained below the figures.

* Old vs. New blood, as bacterial dose increases, there is a fall in NTBI and PLI levels, with a significantly steeper fall in these levels with old blood (*Interaction*).

Figure 5. Plasma iron depletion score at 24 h after bacterial challenge

Iron consumption score represents the difference between cell-free hemoglobin (treated as the source of iron available) and iron levels (conceived as the residual iron after bacterial consumption) after both being standardized by the average levels of non-septic animals receiving old blood (dose 0×10^9 CFU/kg). The difference between old and new blood groups in this score is represented for each bacterial dose (1, 1.25 and 1.5×10^9 CFU/kg). Zero on the y-axis represents no difference between iron depletion of older versus fresher blood. Elevations in score represent higher consumption in old blood group. The p-value above each bar represents the significance of that difference. Iron consumption scores (ICS) for each animal in each blood group (old or new) at a particular dose were calculated as cell-free Hb for that animal divided by the average CFH (averaged over all non-septic animals receiving old blood) minus total iron (sum of NTBI and TBI) divided by the average total iron (averaged over all non-septic animals receiving old blood). See Supplementary Methods for additional description.

Figure 6. Survival curves for each *S. aureus* challenge dose

Kaplan-Meier plots over the 96 h study comparing old (solid lines) and new blood (dashed lines) groups at the different bacterial doses studied are represented (A to D).