Mechanisms of Red Blood Cell Transfusion-Related Immunomodulation (TRIM)

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Abstract

Red blood cell (RBC) transfusion is common in critically ill, post-surgical, and post-trauma patients in whom both systemic inflammation and immune suppression are associated with adverse outcomes. RBC products contain a multitude of immunomodulatory mediators that interact with and alter immune cell function. These interactions can lead to both pro-inflammatory and immunosuppressive effects. Defining clinical outcomes related to immunomodulatory effects of RBCs in transfused patients remains a challenge, likely due to complex interactions between individual blood product characteristics and patient-specific risk factors. Unpacking these complexities requires an in depth understanding of the mechanisms of immunomodulatory effects of RBC products. In this review, we outline and classify potential mediators of RBC transfusion-related immunomodulation and provide suggestions for future research directions.

INTRODUCTION

In the United States, 11 to 16 million red blood cell (RBC) units were administered annually during the last decade, equating to a RBC transfusion every 2 seconds.[1-5] RBC transfusion is particularly commonplace in emergency departments, intensive care units (ICUs) and operating suites, with 37-60% of ICU patients receiving a transfusion during hospitalization.[6-12] Nonetheless, RBC transfusion may have deleterious immunologic effects, particularly for critically ill patients.[13,14] Mounting evidence from predominantly observational studies demonstrate independent associations between RBC transfusion, dysregulated immunity and increased mortality and morbidity; mechanisms of which are only partly understood.[15-26] The following review will summarize current literature on mechanisms of RBC transfusion-related immunomodulation, classify potential mediators, and propose a research agenda to fill critical knowledge.

Red Blood Cell Transfusion-Related Immunomodulation

Beginning in 1973, Opelz and colleagues provided initial evidence for RBC transfusion-related immunomodulation (TRIM) with the observation that the survival rate of transplanted kidneys was significantly higher in cadaveric renal transplant patients who received RBC transfusion.[13,27] These findings strongly suggested immunosuppressive effects of non-leukoreduced allogeneic RBC transfusion. More recent findings suggest both pro-inflammatory and immunosuppressive effects of RBC product exposure, including pre-storage leukoreduced blood products. Clinically, RBC transfusion is associated with new or worsening organ dysfunction, the development of nosocomial infection, and cancer recurrence, suggesting dysregulated recipient immune responses.[13,14,21,28-32] The extent to which RBC transfusion

directly contributes to immunologic dysregulation in transfused patients remains unclear, though a wealth of pre-clinical evidence demonstrates that RBC products can directly modulate immune cell function. In a variety of pre-clinical models, RBC product exposure results in inflammatory effects including: leukocyte priming, enhanced neutrophil chemotaxis, monocyte/macrophage activation, and inflammatory cytokine release.[13,17,21,31,33-35] Immunosuppressive effects include impaired natural killer cell function, alterations in T lymphocyte ratios, defective antigen presentation, suppression of lymphocyte proliferation, and decreased macrophage phagocytic function.[14,36-40] While evidence supporting both pro-inflammatory and immunosuppressive effects of RBC transfusion may seem contradictory, given the complex nature of transfused blood products and the multitude of potentially immunomodulatory mediators contained therein, mixed effects are not surprising. Indeed, mixed immunomodulatory potential of RBC transfusion may be particularly relevant for critically ill patients in whom both excess inflammation and immune suppression are significantly associated with adverse outcomes.[14] Overall, defining the sum total immunomodulatory effects of particular RBC products in individual patients remains challenging. Future research to determine the effects of individual blood products on individual patients and to mitigate potential risks depends on understanding mechanisms of RBC transfusion-related immunomodulation.

While mechanisms for RBC transfusion-related immunomodulation are not yet fully characterized, many potential mediators have been identified. These include leukocyte-derived mediators, component hemolytic contents (heme, iron release), platelet-derived factors, and extracellular vesicles (Figure 1).

PROPOSED MECHANISMS

1. Leukocytes and Leukocyte-derived Mediators

The observation that pre-storage leukoreduction may mitigate TRIM suggests that either intact leukocytes and/or soluble leukocyte-derived mediators play a role in its development.[41-44] Leukoreduction removes most residual white blood cells from stored blood components and appears to improve clinical outcomes. Randomized trials in surgical patients receiving either leukoreduced versus non-leukoreduced RBCs, autologous versus allogeneic RBC transfusions, or restrictive versus liberal RBC transfusion thresholds demonstrate that in each case, subjects in the leukoreduced, autologous or restricted transfusion arms developed fewer nosocomial infections.[15,45-47] Likewise, meta-analyses demonstrate that leukoreduction, autologous RBC transfusions (which prevent exposure to allogeneic WBCs) and restrictive transfusion thresholds (which decrease exposure to residual allogeneic WBCs) are each associated with decreased risk of post-operative infection.[15,45,47] RBC unit leukoreduction may also attenuate the systemic inflammatory response following cardiac surgery, with a dose-dependent increase in survival when leukoreduced RBCs are utilized.[48] Lastly, animal models demonstrate that leukoreduction may reduce transfusion-associated cancer metastasis and T cell apoptosis.[29,49] Taken together, these data suggest that residual leukocytes or leukocyte-derived mediators in RBC products may be harmful via immunomodulatory mechanisms. Although in the US, 75-80% of RBC units transfused are pre-storage leukoreduced to mitigate these risks, it is worth noting that a substantial number of residual leukocytes (~5000 to ~ 5 x 10^6 leukocytes/unit) remain despite current leukoreduction technologies.[50-52]

Residual leukocytes

Antigen presenting cells (*i.e.* monocytes and dendritic cells) carry major-histocompatibility complex (MHC) II molecules (*i.e.* HLA-DR) on their cell surfaces. MHC II molecules function to present processed antigens and activate lymphocytes. Following transfusion, interactions between donor MHC II molecules on residual leukocytes and recipient lymphocytes may result in either alloimmunization or immune suppression.[53-56] Features such as the degree of HLA compatibility, the functionality of donor antigen presenting cells (APCs), and the inflammatory state of the recipient likely determine whether residual allogeneic leukocytes induce immune tolerance or alloimmunization.[21] In the case of immune suppression, residual allogeneic APCs which engage recipient T cells without necessary secondary or co-stimulatory signals would be expected to produce antigen-specific T cell anergy.[21] The resulting immune tolerance is a proposed mechanism for allogenic RBC transfusion-related adaptive immune cell (T cell) suppression.[21] T cell immune tolerance may also be responsible for development of microchimerism in allogenic blood transfusion recipients, whereby donor leukocytes fail to elicit an immune response and become "accepted" by the recipient.[57] Microchimerism may be common in trauma patients and may persist for up to two years following transfusion.[57,58] Moreover, immune tolerance and associated microchimerism may explain the observed shift to immunosuppressive T_H2 responses following blood transfusion.[38,59-62] However, clear demonstration of direct causal links between HLA molecules on residual allogeneic APCs and post-transfusion immune suppression is currently lacking.

In addition to residual functional allogeneic leukocytes, it is possible that apoptotic leukocytes in RBC products may also induce immune suppression.[63] During collection and storage, leukocytes undergo apoptosis.[64] One of the early steps in apoptosis involves exposure of

phosphatidyl serine on the outer leaflet of the cell membrane. Interaction between immune cells and phosphatidyl serine has been shown to induce immunosuppressive signals, including release of anti-inflammatory cytokines IL-10 and TGF-β, inhibition of pro-inflammatory cytokine release, inhibition of APC activation, and predominance of immunosuppressive regulatory T cells.[63,65] The degree to which apoptotic residual leukocytes in RBC units contribute to recipient immune suppression in the clinical setting remains unknown. However, it is worth noting that similar responses may also be seen in response to phosphatidyl serine-containing membrane fragments or microparticles.

Soluble leukocyte-derived mediators

Removal of supernatant from stored RBC units by washing reduces the inflammatory response in pediatric cardiac surgery patients and pre-clinical studies suggest that RBC-induced immunomodulation can be recapitulated using RBC unit supernatants.[24,25,66,67] Thus, it seems likely that soluble mediators also play a role in TRIM pathogenesis.

There are multiple soluble leukocyte-derived factors, including cytokines, white blood cell degranulation products, soluble FAS-L, and soluble HLA molecules, which directly inhibit the immune response.[68,69] Of these, sFAS-L and the anti-inflammatory cytokine, TGFβ have the strongest evidence suggesting that they may promote TRIM, particularly in non-leukoreduced blood products.[36,68] *In vitro* studies indicate that sFAS-L and TGFβ found in blood components may directly induce innate immune cell apoptosis, impair neutrophil chemotaxis, and decrease natural killer cell activity.[36,69,70] Immunosuppressive effects may not be limited to these, as TGFβ is a known anti-inflammatory cytokine with broad immunosuppressive effects.

In addition to anti-inflammatory cytokines, pro-inflammatory cytokines may also accumulate in blood products during storage.[71-74] However, in some reports pre-storage leukoreduction appears to substantially decrease the accumulation of pro-inflammatory cytokines in RBC products such that levels are undetectable.[72,74] When cytokines are detected, it is unclear whether their concentrations are high enough to strongly influence recipient immune function.[73,74] In addition to cytokines, white blood cell degranulation products such as histamine and eosinophil cationic protein have been detected in red blood cell components.[75] Each of these mediators has immunomodulatory potential. For example, histamine has been shown to inhibit neutrophil chemotaxis and decrease T cell proliferation, while eosinophilic cationic protein may also reduce T cell proliferation.[76,77]

While leukocytes and leukocyte-derived soluble mediators appear to promote TRIM, such effects are likely reduced by pre-storage leukoreduction. Because evidence for TRIM remains in the post-leukoreduction era, it is likely that non-WBC derived factors are also involved.[14]

2. Red Blood Cell Storage Lesion and Decompartmentalized RBC Contents

Another potential mechanism for TRIM arises from the RBC, itself. As RBC units age under refrigerated conditions, a well described "storage lesion(s)" develops. The RBC storage lesions are characterized by altered RBC morphology, rheological changes, metabolic derangements, changes in oxygen affinity, changes in osmotic regulation, and changes in the ability to vasoregulate.[78-85] In addition, RBC hemolysis (both during storage and post-transfusion) can lead to reduced pH, increased lactate and other metabolic wastes, release of microparticles, as well as accumulation of cell-free hemoglobin (CFH), heme, and iron.[26,78,86-90]

Iron content can be in the form of transferrin bound iron (TBI), non- transferrin bound iron (NTBI), or plasma labile iron (PLI). Given the well-described bioactivities of these species, RBC hemolysis can disturb plasma redox balance and broadly disrupt normal signaling in coagulation, vascular, and immune systems.[4,22,23,78,86,91,92]

In normal physiology, plasma haptoglobin sequesters CFH, forming a complex for removal by macrophages *via* CD163.[18,22,23,93] However in critical illness, even moderate intravascular hemolysis may overwhelm plasma-binding capacity resulting in unbound extracellular hemoglobin. When extracellular hemoglobin is unbound, it becomes oxidized to methemoglobin, releasing free heme. Free heme can then undergo the Fenton Reaction to cause further release of iron..[67,93-97] Accumulation of un-complexed heme and iron in plasma is associated with significant tissue damage, presumably by iron-catalyzed generation of reactive oxygen species (ROS), promotion of other radical chains, increases in leukocyte activation and migration, upregulation of adhesion molecules, and subsequent deleterious effects to tissue barriers and to immunity.[22,93,98-104] In murine models, transfusion of long-stored RBCs led to increased iron in the form of NTBI and augmented circulating pro-inflammatory cytokine release.[22,23,105,106] However, in human healthy volunteers, while transfusion with older versus fresher RBCs significantly increased circulating NTBI levels, a pro-inflammatory cytokine response was not observed. [91,105,107] The lack of observed inflammatory response in the human studies may relate to differences between mice and humans, relative transfusion dose; or the inflammatory response to RBC transfusion may not be apparent in healthy subjects (without underlying inflammation). That said, in a study of 33 premature neonates, while levels of NTBI were increased post transfusion, NTBI levels were not associated with increases in plasma

inflammatory cytokines.[108] These data suggest that pro-inflammatory effects of NTBI may be minimal.

RBC transfusion may also burden the mononuclear phagocyte system (MPS), delivering large amounts of hemoglobin and RBC contents to monocytes and macrophages.[93] Phagocytosis of RBCs by macrophages (*i.*e. extravascular hemolysis) increases macrophage intracellular heme and iron to a degree that can trigger inflammasome activation and pro-inflammatory cytokine release via NLRP3 and NF-κB signaling; this process is further exacerbated by generation of iron-related reactive oxygen species.[93] Conversely, macrophage exposure to high concentrations of heme may also bias macrophage phenotype from the activated/inflammatory (M1) phenotype toward an immunosuppressive (M2) profile via upregulation of heme-oxygenase 1 and release of the anti-inflammatory cytokine, IL-10.[109] Similarly, macrophage iron loading may promote immune suppression by inhibiting IFN-γ-mediated secretion of pro-inflammatory cytokines, reducing expression of MHC II and impairing nitric oxide synthesis. Cumulatively, these effects compromise phagocytic and microbicidal macrophage activity.[110] Iron overload may also further promote immune suppression by impairing proliferation and activation of T, B, and natural killer cells.[111] Additionally, independent of direct effects on immune cells, un-complexed heme and iron may directly promote bacterial growth.[78,93,105]

Finally, an additional compound of interest is ubiquitin, an intracellular regulatory protein present in a variety of cell types. RBCs carry large amounts of ubiquitin relative to other cell types, and extracellular ubiquitin has been found to accumulate in RBC unit supernatants during storage.[112] Extracellular ubiquitin has varied effects on immune cell function, including blunting LPS-induced TNFα production while augmenting LPS-induced IL-8 production.[112-114] Additionally, extracellular ubiquitin found in RBC units may skew helper T cell function toward

an immunosuppressive Th2 phenotype, as evidenced by increased IL-4 production and decreased IFNγ production by LPS-stimulated PBMCs exposed to 35-day-old stored RBC supernatant or ubiquitin.[112,114] The mix of pro-inflammatory and immunosuppressive effects of extracellular ubiquitin mirrors immunomodulatory effects observed in response to RBC supernatants *in vitro* and may explain mixed responses reported *in vivo*.

In summary, soluble mediators resulting from RBC ageing and breakdown are varied, and individual mediators likely have pleiotropic effects on recipient immune response. Although animal studies show worsened survival and increased inflammation from transfusion with longer stored RBCs, these findings have not been demonstrated in recently published human RCTs[4,16,78,87,115]. This may be because animals studies can carefully delineate "fresh vs. old" RBC cutoffs (i.e. >21 days) whichhas proven difficult in human RCTs, where a mean duration of RBC storage in the US of 17.9 days results in comparisons between "fresh" vs. "middle-age"[87,116]. Additionally, storage duration effects may be more robust if transfusion occurs in the setting of more significant baseline inflammation, though to date this question has not been adequately evaluated. The relative impact of inflammatory and immunosuppressive effects of RBC-derived mediators for individual patients, particularly in the setting of baseline inflammation or immune suppression, remains largely unknown. It is likely that a complex interplay between de-compartmentalized RBC contents and underlying host immune response contributes to patient-specific immune modulation, a topic of active ongoing research.

3. Residual Platelets and Platelet-derived Factors

While less is known about platelet-derived factors as TRIM mediators, emerging data strongly suggests that platelets and platelet-derived factors have important immunomodulatory

potential.[117-119] For instance, platelet-derived microparticles are capable of inducing both immune cell suppression and activation.[120,121] Platelets themselves may play important roles in modulating immune cell response in both health and disease, suggesting that residual platelets found in RBC products likely contribute to immunomodulation. Non-leukocyte reduced RBC units have been shown to accumulate platelet-leukocyte aggregates over time, which correlate with immune cell apoptosis and monocyte tissue factor expression.[122] These changes are expected to be immunomodulatory, however effects of platelet-leukocyte aggregates on recipient immune cells was not evaluated. Likewise, the immunodulatory potential of residual platelets within leukoreduced red blood cell products is unknown.

4. Bioactive Lipids and Extracellular Vesicles

Bioactive Lipids

Bioactive lipids with pro-inflammatory and pro-coagulant activity accumulate during storage in RBC units and may contribute to inflammatory complications of RBC transfusion, including transfusion-related acute lung injury (TRALI).[83,123] Accumulation of some bioactive lipids, such as lysophosphatidylcholines, appears to be reduced by leukoreduction.[124] However, a variety of polyunsaturated fatty acids, including arachidonic acid, linoleic acid, docosahexaenoic acid, and their metabolites accumulate in RBC units despite leukoreduction.[123,125] Arachidonic acid and its oxidized metabolites, when isolated from older stored RBC supernatants, are capable of priming neutrophils *in vitro*. Further, infusion of these bioactive lipids in rats that are primed by LPS, induce acute lung injury - providing evidence that bioactive lipids may provide the second-hit in the two-hit model of non-antibody mediated TRALI.[125,126] Observational studies demonstrating the presence of lipids with neutrophil priming activity in the plasma of TRALI

patients provide additional supportive evidence of the link between bioactive lipids and non-antibody mediated TRALI.[127] The extent to which bioactive lipids may contribute to systemic inflammation or modulation of immune function outside of TRALI remains unclear and is a topic deserving of further study.

Extracellular vesicles

Extracellular vesicle count and profile in blood products

The term extracellular vesicle (EV) broadly encompasses larger microvesicles (200-1200 nm), exosomes (30-150 nm) and apoptotic bodies (50-500 nm).[128-130] For over a decade, it has been appreciated that plasma from healthy subjects contains EVs, including exosomes, derived from leukocytes, platelets, RBCs and endothelial cells.[131-133]

EV counts in RBC products increase with storage duration.[86,134] Storage-related morphological changes to RBCs are accompanied by shedding and release of RBC-derived EVs, while residual platelets and leukocytes contribute to platelet-derived and leukocyte-derived EVs.[135-138] Tracking EV cell of origin reveals that RBC-derived EVs increase continuously during storage, while platelet-derived EV counts peak at 3-4 weeks of storage.[86,139] EV release and accumulation are significantly influenced by component manufacture processes and storage conditions such that individual products may have very different EV profiles despite similar storage duration.[140,141]

***In vitro* evidence for EV TRIM effects**

Though once considered debris without bioactivity and discounted as artifact, EVs are increasingly recognized as playing a central role in the body's complex network of intercellular signaling, both in normal physiology and in disease.[142] EVs derived from stored platelets bind to and activate neutrophils *in vitro,* and have anti-inflammatory or pro-inflammatory effects on monocytes and macrophages.[135, 143,144] Neutrophil and RBC-derived EVs are also capable of suppressing inflammatory responses.[130,145] Similar to the variability in effects of EVs from various cell types, EVs isolated from plasma have dual pro-inflammatory and immunosuppressive effects.[139,146] The proposed mechanism of action of blood-derived EVs varies, with immunosuppressive effects potentially mediated by FasL expression by EVs, and inflammatory effects resulting from direct activation of monocytes and other antigen-presenting cells after EV uptake by these cells.[139,146]

***In vivo* evidence for EV TRIM effects**

Given the incomplete understanding of how EVs from different cells of origin might act, it is not surprising that *in vivo* evidence of an EV-based role in TRIM is scant. The circulating half-life of EVs appears to be fairly short, less than 15-20 minutes in a rat model.[86] However, the biologic activity of EVs is likely related to EV uptake by target cells rather than plasma concentration. For example, injected EVs are rapidly and widely distributed to the spleen, liver, kidneys, and lungs in mice.[147] Donor dendritic cell-derived EV uptake by dendritic cells in a recipient mouse can activate responding T cells in an antigen-specific manner.[148] This property has been exploited by several groups as a potential vaccine delivery approach.[149-151] Additionally, adoptive transfer of CD154 (CD40L)-expressing platelet-derived EVs is sufficient to stimulate IgG production and germinal center formation in mice after adenovirus vaccination, indicating that exogenous EVs can modulate a nascent immune response[152]. The significance of

the immunomodulatory effects of EVs found in blood products transfusion recipients remains an open question and an area of active research. Better understanding EV interaction with the human immune system would allow manipulation of this pathway, both in the context of transfusion-related immunomodulation and in the context of immune perturbation seen in many hospitalized patients.

FUTURE DIRECTIONS

Ample evidence exists that RBC products are capable of interacting with and modulating immune cell function through a variety of mechanisms and mediators; however, conclusive clinical evidence of TRIM effects in transfused patients remains elusive. Given recent clinical studies that fail to demonstrate benefit to fresh RBC transfusion compared to longer stored products, one might conclude that RBC TRIM does not exist in the era of pre-storage leukoreduced blood products or that RBC storage duration does not contribute to TRIM mechanisms.[87,115,153,154] However, emerging evidence suggests that the concentrations of potentially immunomodulatory mediators vary not only with storage duration, but also with donor characteristics, manufacturer, storage solution, and other processing factors.[88,155-158] We are only beginning to understand the complex interplay between storage duration, processing methods, RBC unit contents, and subsequent potential TRIM effects. Similarly, a patient's underlying state of inflammation and/or immune suppression at the time of transfusion likely influences the immunologic response to transfusion. Critically ill patients, in particular, exhibit both exaggerated systemic inflammation and immune suppression that fluctuate over time.[159-164] In this context, one would expect that immunologic effects of RBC transfusion might vary

widely based on the underlying state of the recipient's immunologic response. However, most studies to date have failed to sufficiently characterize or account for individual differences in pre-transfusion immune function. Additionally, patients who are transfused with RBCs often also receive other blood products, which may have different or additive TRIM effects.[14,165] Overall, much work remains to understand interactions between individual blood product characteristics and patient-specific risk factors with respect to clinical consequences of TRIM.

Defining immunomodulatory mediators found within blood products, and understanding how these mediators may modulate recipient immunity is essential to identify potential TRIM effects at the bedside. A bench to bedside approach must carefully attempt to define these mediators in context of host immune function. Next, guided by an enhanced understanding of TRIM biology, observational studies will be necessary to determine patient-specific risk factors for specific TRIM effects and related clinical consequences. Moreover, delineation of the effects of RBC donor, product processing and storage conditions upon accumulation of immunomodulatory mediators can then inform future prospective and interventional trials aimed at defining and ameliorating TRIM effects for those patients most at risk.

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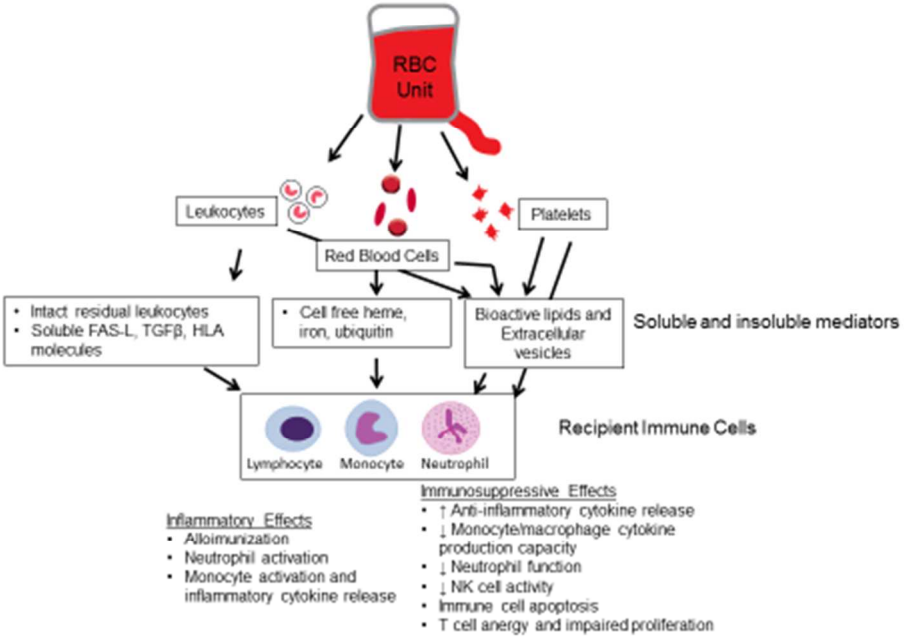
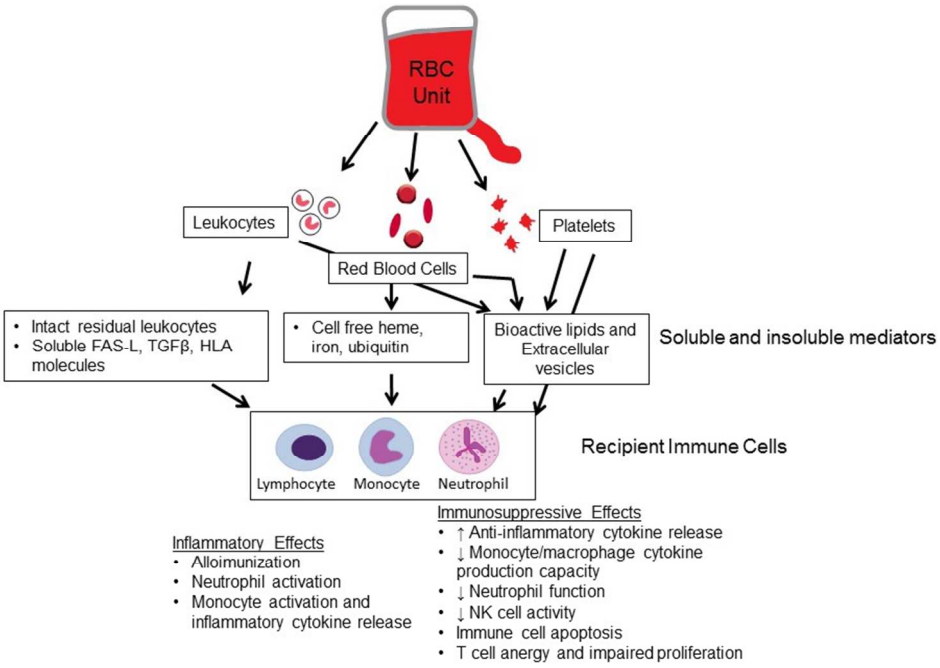
Figure 1

Red blood cell (RBC) units contain multiple immunomodulatory mediators, including leukocyte-derived, red blood cell-derived, platelet-derived, and lipid and microvesicle-derived factors. Effects of these mediators on immune cell function vary and include both inflammatory and immunosuppressive changes. As such, the sum total immunomodulatory effects of RBC transfusion on recipient immune function will likely vary based on individual unit and recipient characteristics.

Figure 1: Proposed Mechanisms of RBC Transfusion Related Immune Modulation





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