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# Should granulocyte transfusion therapy for septic neutropenic neonates be resurrected?

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ABSTRACT

Nearly half a century ago, granulocyte transfusions were trialed in critically ill, septic, neutropenic neonates and showed improved survival when used concurrently with antimicrobials. Benefits were particularly noteworthy for Gram-negative and fungal infections. The introduction of granulocyte colony-stimulating factor into clinical medicine in 1991 and inherent problems associated with granulocyte procurement for transfusion caused granulocyte transfusions to become nearly extinct for this patient population. Simultaneous technological and clinical management advancements have enabled the survival of younger neonates, who are at the highest risk for neutropenia and neonatal sepsis. These infants have well-documented developmental deficiencies in the number and functional capabilities of their neutrophils compared to older patients. A continued surge in antimicrobial resistance and an increasing number of Gram-negative infections have created an urgent need for clinicians to rethink old therapies and consider new ones. This review details the evolution of granulocyte transfusions and whether they should be resurrected in neonatal patients.

## 1. Neutrophils of neonates

Neutrophils, the first cells to defend against and respond to bacterial, viral, and fungal infections, are vital to innate immunity [1]. However, well-known phenotypic and functional differences exist for neonatal compared to adult neutrophils [1]. Neonates also exhibit greatly diminished neutrophil bone marrow storage pools, per kilogram of body weight, which limits their ability to rapidly increase the number of circulating neutrophils early during an infection, potentially leading to life-threatening neutropenia [1]. These disparities directly relate to the infant's gestational age (GA) and clinical condition after birth. Therefore, the youngest and most critically ill neonates have the fewest and least functional neutrophils, placing them at the highest risk for infectious disease-mediated morbidity and mortality [1,2].

Granulocyte transfusions (GTX) were first introduced into clinical practice in the 1960s to treat neutropenic adult patients with serious infections or neutrophil dysfunction after *in vivo* experiments in canines demonstrated the ability of donor neutrophils to circulate and migrate to sites of inflammation [3]. GTX investigations in neonates commenced in the 1980s [4] but were short-lived, with the last randomized, controlled trial in neutropenic septic neonatal patients published in 1992 [5]. A meta-analysis in 1989 of six controlled trials reported improved survival in septic neonates who received GTX in addition to antibiotics [6]. Conversely, a 2011 Cochrane Review [4] concluded that GTX *vs* placebo or no intervention demonstrated no difference in all-cause mortality during the infant's hospital stay, even though cumulative subject numbers were small (44 neonates). Data also suggested improved survival of neutropenic neonates with early-onset sepsis (EOS; sepsis within the first 72h of life) or Gram-negative bacterial sepsis following treatment with GTXs, particularly if dosed with $\geq$0.5 X $10^9$ cells/kg.

Sepsis-related mortality in neonates significantly declined from 1979 to 2000 associated with clinical and technological advancements in the treatment of critically ill neonates and the implementation of the Centers for Disease Control and Prevention (CDC) guidelines for the prevention of perinatal Group B *Streptococcus* (GBS) transmission [7]. However, neonatal mortality from bacterial sepsis has stagnated over recent decades, and medical management remains mostly unchanged. This trend continues despite a surge in antimicrobial resistance and a notable increase in the virulence of isolated pathogens [2,8]. An urgent need exists to identify effective therapeutics to combat infectious diseases. Technological advancements improving the safety and efficiency of granulocyte harvesting provide a compelling case to reexamine GTX as a promising therapeutic. This review will entertain the possibility of reintroducing GTX as an adjunctive treatment for neonates with sepsis and neutropenia and will detail advancements in granulocyte harvesting.

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## 2. Infectious risks of neonates and neutrophil function

The neonatal period (first 28 days of life) has the highest lifetime risk of sepsis [2], which is indirectly correlated with GA at birth [8]. The global impact of neonatal sepsis is staggering, with nearly 5 million cases and 800,000 deaths occurring every year [2]. Survivors may have substantial long-term morbidities, including neurodevelopmental impairments [9]. Notably, infectious disease is the second leading cause of neonatal mortality worldwide, surpassed only by complications related to prematurity [2].

In the US, an estimated 380,000 neonates, or 1 in 10 newborns, are born preterm [10]. Although the overall incidence of EOS is around 1.08 (95 % confidence interval [CI], 0.95–1.23) cases per 1000 live births, this incidence is 18.47 (95 % CI, 14.57–23.38) cases per 1000 for infants born at the youngest GA (22–28 weeks) [8]. While GBS is the primary pathogen isolated from term infants, premature infants are most likely to be infected with *Escherichia coli*. Alarmingly, in very low-birth-weight infants (VLBW; 401–1500 g at birth), the incidence of *E. coli* sepsis has been increasing over the last two decades [8]. GBS isolates are universally sensitive to first-line empiric antibiotics (i.e., ampicillin and gentamicin), whereas almost 8 % of *E. coli* isolates are resistant to this drug combination [8]. Although death is unlikely to result from EOS in term infants, one-third of infants <37 weeks' GA will die from EOS-related complications [8]. Globally, low-to-middle-income countries experience a profoundly different microbial epidemiology with a predominance of Gram-negative bacteria and a staggering rate of resistance to common first-line empirical antibiotic therapy [2].

Similar trends are observed for late-onset sepsis (LOS or infection ≥72h of life). Even though the overall incidence of LOS is 88.5 per 1000 live births (99 % CI 86.4–90.7), infants born ≤23 weeks GA experience a sharp rise at 322 per 1000 (99 % CI 306.3–338.1)[11]. Whereas half of offending pathogens are Gram-negative bacteria and fungal organisms in neonates born at ≤ 23 weeks' GA [11], epidemiologic data shows fungal pathogens are four times more likely to cause infection in infants born ≤23 than those ≥28 weeks' GA [11].

Innate immunity provides an initial host defense against pathogen invasion and consists of physical barriers, antimicrobial peptides, soluble mediators, and effector cells. Neutrophils are considered the "police force" of the immune system because they are the first cells to respond to and combat pathogenic microbes, especially bacteria and fungi [1]. During fetal development, neutrophil production and maturation progress similarly to other major organ systems. Primordial myeloid cells first appear in the peripheral blood around 14–16 weeks of gestation [12]. Neutropoiesis, or neutrophil production, progresses from the primitive yolk sac, to the liver and spleen around 7–8 weeks [13], and ultimately to the bone marrow around 7 months [1]. In contrast to healthy adults, neonates are more vulnerable to infection due to considerably low absolute neutrophil cell mass per gram body weight (1/4 adult levels) [14]. This vulnerability is even more pronounced in infants <32 weeks' GA [1].

The number of neutrophil progenitors in the marrow, per kilogram body weight, is also lower in neonates than in adults. The proliferative neutrophil pool in a healthy human adult contains 4 to 5 X $10^9$ cells/kg body weight, while neonates have only 10 % adult values [1,12]. During the early phases of infection, bone marrow reserves of mature neutrophils can be rapidly depleted as cells are released into the circulation. They are, therefore, more likely to develop neutropenia (absolute neutrophil count [ANC] of <1000/mL). This phenomenon significantly increases sepsis-associated morbidity and mortality [15]. By contrast, adults maintain a substantial bone marrow reserve of near-mature and mature neutrophils that can be quickly mobilized in early proinflammatory responses (~20 times that found in the bloodstream) [16]. They also have a sizable reserve of quiescent neutrophil progenitors that can be rapidly recruited into the cell cycle during infectious or inflammatory processes to quickly surge the number of circulating neutrophils [1,15].

Small for gestational age (SGA) infants (birthweight <10th percentile) have high rates of neutropenia at birth compared to non-SGA infants, with an incidence of 6 % vs. 1 %, respectively [17]. Notably, extremely low-birth-weight (ELBW; <1000 g at birth) infants have the highest rates of neutropenia without an identified cause, but this finding is not associated with an elevated mortality risk compared to those born with normal values [18]. Neonatal neutrophils also have differences in cell membrane receptors, correlated with fetal maturation, that may lead to diminished chemotaxis [19], impairments in slow rolling and adhesion [20], inability to transmigrate through the vascular endothelium [21], and reduced capacity to phagocytose and kill intracellular pathogens due to respiratory burst suppression [1,21,22]. These functional deficiencies are further exacerbated in physiologically stressed preterm and term neonates [1,22].

## 3. Investigating granulocyte transfusions in septic, neutropenic neonates

In the USA, granulocytes are typically collected *via* leukapheresis, a process where white blood cells (WBCs) are separated and stored in leukopaks, and the remaining red blood cells (RBCs) and plasma are returned to the donor [3]. This granulocyte concentrate, which contains variable platelets, RBCs, and WBCs, is then irradiated to prevent transfusion-associated graft versus host disease [3]. The retained neutrophils, being very sensitive, may become activated by minimal stimulation, leading to degranulation of noxious granular proteins, formation of reactive oxygen species, NETosis, and cell death through apoptosis [1]. It is crucial to commence transfusions as soon as possible after collection to preserve granulocyte function and avoid cell death [23]. Granulocyte concentrates can be stored at room temperature for a maximum of 24h, preserving acceptable neutrophil viability and function [3].

Donor availability depends on the size of the donor pool and the patient requirements, such as cytomegalovirus (CMV) serology status or HLA (human leukocyte antigens) match, and often requires a one-day lead time to identify a suitable donor [3]. Granulocytes are retrieved by blood donation centers from healthy donors, usually following co-stimulation with dexamethasone and granulocyte-colony-stimulating factor (G-CSF) to trigger a rapid rise in circulating neutrophils available for harvesting. A standard, single steroid dose can double or triple a donor's ANC over 4–24 h[24], while a single dose of G-CSF can increase the count 7- to 10-fold [23], resulting in GTX doses of 3–5 X $10^{10}$ cells [24]. Steroids have been shown to enhance neutrophil survival while inhibiting granulocyte mobility, adhesion, and microbial killing [25]. Exposure to G-CSF improved neutrophil chemotaxis, endothelial adhesion, phagocytosis, and oxidase production [26]. When used together, these drugs cause a 10- to 13.5-fold increase in the neutrophil donor's ANC [24] and produce normally functioning neutrophils [26].

Granulocyte concentrates for transfusion are not currently recognized as a licensed blood component by the Food and Drug Administration [3]. Historically, GTXs are administered to adult patients who have deficient neutrophil production resulting from chemotherapy [27] or refractory bacterial or fungal infections despite targeted antimicrobial treatment. Alternatively, adult patients may receive GTXs due to aplastic anemia [28] or neutrophil dysfunction, such as in chronic granulomatous disease (CGD) [29], although these indications are less common [3]. Transfused cells have been shown to migrate to sites of infection [3] and maintain their phagocytic capabilities [30]. Presently, widely accepted indications for GTX include: (1) neutropenic sepsis (ANC ≤500 cells/mL), (2) bacterial or fungal infection with a poor antimicrobial response after 24–48h, (3) neonatal sepsis, (4) neutrophil function disorder, and (5) a reasonably favorable expectation for neutrophil recovery [23].

Unlike adult patients treated with GTX, septic neonates produce neutrophils but their supply of mature cells is quickly depleted by an accelerated rate of utilization [31]. One of the first published studies by

Laurenti and colleagues [32] investigated the use of GTX in septic neonates, most of whom had antibiotic-resistant *Klebsiella* infection. Mortality was significantly reduced in the group who received granulocyte transfusions compared to those who did not (mortality 10 % versus 72 %), an effect that was most prominent for VLBW infants (mortality 10 % versus 91 %). Another study by Christensen and colleagues [31] prospectively investigated neonatal GTXs after bone marrow examination to assess neutrophil storage reserves. Seven infants with severe depletion (<7 %) were eligible for GTX and received a volume of 10–15 ml/kg over 45 min to provide about 0.7 X $10^9$ neutrophils/kg (range 0.2–1.0 X $10^9$). Six of seven infants experienced a rise in blood neutrophil counts immediately following the transfusion, but all seven had values that exceeded the lower limit of normal for age by 12–18 h following the transfusion. All seven infants survived, and no adverse events were recorded. Conversely, only one of nine nontransfused, neutrophil-depleted infants with sepsis survived ($p < 0.01$). In another trial, thirty-five neutropenic and septic infants on antibiotics were randomized to GTX or intravenous immunoglobin (IVIG) therapy [5]. Survival was significantly higher in infants who received GTX versus IVIG (100 % vs. 64 %; $P < 0.03$), and no participants experienced adverse events.

Transfused neutrophils can be found in the recipient's bloodstream for up to 24h [33,34]. In adults, ≥1 X $10^9$ granulocytes/kg body weight in daily transfusions are required to increase a neutropenic adult's circulating granulocyte count to normal values [35]. Failure to achieve a therapeutic granulocyte dose may result in an absent or subclinical response and may not appropriately treat or prevent infection in affected patients [36]. This dosing issue is more problematic in adult patients because the donor and recipient are usually around the same size. Because the typical granulocyte concentrate contains $10^{10}$ neutrophils, a 70-kg recipient would receive ~1 to 1.5 X $10^8$ neutrophils/kg, while the neonate could receive 2 to 10 times this number [31].

Although GTXs have been shown to benefit septic neonatal patients with severe neutropenia, the practice of administering GTXs in this population has become nearly obsolete in the USA [35]. This shift is due to the difficulties associated with procuring and storing granulocytes (i.e., short half-life), adverse transfusion reactions, and alternative treatments (i.e., G-CSF) [35]. GTX-associated transmission of cytomegalovirus to a critically ill neonate is a risk of this treatment option, so donors are usually CMV-seronegative [35]. Other commonly observed reactions include fever, chills, and marginal arterial oxygen desaturations [3,35]. These host responses occur in about 20 %–40 % of adult patients due to cytokine release, either from transfused WBCs or the patient's cells in response to recognition of the donor HLA or HNA (human neutrophil antigens) antibodies in the granulocyte product [23].

In neonatal patients, pulmonary complications were the only reported harmful side effects in GTX trials but were only observed when buffy coat methods were used for collection [4]. One case report in 1988 details a near-fatal pulmonary reaction to presumed WBC antibodies in a neonate with Rh hemolytic disease being treated with exchange transfusions [37]. This neonate developed bacterial sepsis on day of life two, for which GTXs were started. Although the first transfusion was uneventful, almost immediately after the second GTX started, the neonate developed severe respiratory distress, bradycardia, hypotension, cyanosis, and acidosis, so the transfusion was stopped. No WBC antibodies were detected in either the infants or maternal serum. In this case, granulocytotoxic antibodies were discovered in the parous donor who provided plasma for the exchange transfusion administered just before the second GTX. The infant made a full recovery.

Studies support the safety of GTX within the first three weeks of life in terms of long-term immunologic health. Investigations found an absence of impairments in humoral, cell-mediated, and phagocytic immunity at 6–23 months [4]. Therefore, key factors to consider before embarking on this treatment course are the patient's neutrophil count, including peripheral and bone marrow stores, the likelihood of bone marrow recovery, the type and severity of infection being treated, and the recipient alloimmunization [35].

## 4. Recent improvements in donor granulocytes

Since the zeniths of neonatal GTX therapy in the 1980s and early 1990s, much has been learned regarding hematopoietic stem cells (HSC) and lineage expansion of myeloid cells, including neutrophils (i.e., neutropoiesis). *Ex vivo* expansion of immortalized HSCs (iHSCs) and pluripotent progenitor cells (i.e., common myeloid progenitor [CMP] and granulocyte-macrophage progenitor [GMP] cells) are being explored to facilitate off-the-shelf products [38,39]. This emerging technology addresses key hurdles of apheresis-derived granulocytes while simultaneously promoting desirable traits, including:

(1) Procurement of vast quantities of neutrophils from a single donor progenitor cell;
(2) Genetic engineering for universal antigenic profiling to minimize alloimmunization [40];
(3) Genomic editing could enhance antimicrobial-specific cellular properties [41];
(4) Cryopreservation of immortalized progenitor cells, addressing timely availability and long-term storage challenges [39],
(5) Standardization of the resulting product composition, attenuating the risk for bloodborne infections [42],
(6) Transient engraftment of transfused cells with neutrophil expansion and differentiation within the recipient. This feature effectively enhances cell effector functions and longevity [39], while attenuating the need for multiple GTXs due to the short half-life of terminally differentiated neutrophils. It also carries a low risk for malignant transformation [38].

Sykes and colleagues [39] modified an estrogen receptor-Hoxb8 (ERHoxb8) fusion protein, keeping the transformed myelocytes in a perpetual and conditionally immortalized state of self-renewal in the presence of estrogen. Removal of estrogen inactivates Hoxb8 activity, and the GMPs resume their normal and synchronized differentiation processes, leading to mature neutrophils. Successful studies in adult murine models of bacterial and *Aspergillus fumigatus* infection showed transfusion of CMP/GMPs resulted in a rise in the absolute number of donor myeloid cells in the recipient, with improved survival and reduced pathogen load in infected tissues [39]. Moreover, transfused GMPs homed to the host's bone marrow and spleen and continued lineage expansion, ultimately producing fully functional mature circulating neutrophils [39]. Alternatively, Trump and colleagues [43] investigated the use of reprogrammed somatic cells to generate induced pluripotent stem cells (iPSC) to serve as a limitless renewable source of large quantities of granulocytes. Unlike GMPs, iPSCs can self-renew indefinitely and produce differentiated progeny from each of the three embryonic germ layers [43]. This method for producing fully functional neutrophils requires *ex vivo* differentiation of iPSC to excess activating forms of AKT. Genetically modified iPSCs can also generate variable cell lineages with specific antigenic profiles that could prevent or significantly reduce the frequency of granulocyte transfusion-associated alloimmunization [38,43].

Clinical trials of universal allogeneic myeloid progenitor cells (MPC) derived from HSC are ongoing. An open-label phase II prospective, randomized, controlled trial of Romyelocel-L (Cellera Therapeutics, Inc.) was published by Desai and colleagues in 2021 [40]. In this trial, a single dose of Romyelocel-L was used with or without G-CSF therapy in patients receiving induction chemotherapy for acute myeloid leukemia. Study patients receiving Romyelocel-L had functional neutrophils six days after drug administration, with cell migration to peripheral tissues and organs, leading to decreased incidence of infections, antimicrobial use, and hospitalization.

Donor $CD34^+$ HSPs used to develop this technology can be sourced from cord blood, apheresis products, or bone marrow [38]. Early (≤15)

passages of cultured iPSCs can lead to a 20-fold expansion per passage of one iPSC, or a rise of ~$10^{19}$ cells. This proliferation equates to over 650 million therapy units of 5 X $10^{10}$ granulocytes, without consideration of additional amplification during recipient bone marrow proliferation and differentiation. Therefore, this therapeutic enables an unlimited source of neutrophils [38].

The major disadvantage of the technologies outlined here is the inability to quickly obtain a vast quantity of mature neutrophils that allow for effective, timely, and safe GTXs to neutropenic patients in the initial stages of severe infection. Thus, these therapeutics have been targeted toward patients with prolonged periods of bone marrow dormancy, such as cancer patients receiving induction chemotherapy or those with neutrophil dysfunction. However, the earliest neonatal GTXs completed at the University of Utah in the 1980s by Christensen and colleagues often had a turnaround time of a day, beginning from the decision to order a neonatal GTX until those neutrophils were harvested and fully transfused [31]. The most significant delays resulted from donor identification and procedures surrounding the collection and processing of the leukapheresis product. Given the small number of storage neutrophil pools in critically ill preterm infants and their elevated risk for neonatal sepsis, this product might be beneficial to prevent sepsis or fungal infections, but investigational studies are lacking.

The infrequent use of GTXs in the broader medical community hinders allocation of financial support for the development and optimization of granulocyte products. Difficulties experienced by the investigative team of the Resolving Infection in Neutropenia with Granulocytes (RING) trial expose additional limitations [44]. This trial is the most recent study to evaluate standard antimicrobial therapy or routine therapy plus G-CSF and dexamethasone-stimulated apheresis granulocytes at a target daily dose of 4 X $10^{10}$ cells per transfusion in neutropenic adult patients with proven or probable bacterial or fungal infections [44]. This study struggled with patient recruitment and the ability to achieve the desired granulocyte dose for transfusion. Results showed no difference in the composite outcome of survival and resolution of infection between the treatment and control groups (42 % and 43 %; $p > 0.99$) [44].

## 5. Conclusions

Gram-negative resistance to carbapenems increased more than any other antibiotic class between 1990 and 2021, with nearly 1 million associated deaths reported in 2021, globally. In children <5 years of age, *Klebsiella pneumoniae*, *Streptococcus pneumoniae*, and *E. coli* were the most common pathogens attributable to AMR-related deaths. The study findings suggest these children are developing more severe infections, which are increasingly difficult to treat due to limited access to adequate antibiotics [45].

A recent report [45] concluded the effective development of new pharmaceuticals that target Gram-negative organisms could avert nearly 11 million deaths forecasted between 2025 and 2050. The World Health Organization (WHO) has published an update on the number of antimicrobial drugs currently in the pipeline [46]. According to their report, as of December 2023, a total of 97 products (57 antibiotics and 40 nontraditional antibacterials) were in clinical development, three of which are in the pre-registration phase. Thirty-two antibiotics with new chemical entities and 30 nontraditional antibacterials will target WHO bacterial priority pathogens [46]. While this WHO report offers hope, it is noted that since the late 2010s, only eight new antibiotics have been approved with activity against multi-drug resistant Gram-negative bacteria [47]. Echinocandins and oral glucan synthase inhibitors are the only new antifungal classes developed in the last twenty years [47].

The introduction of G-CSF into clinical practice in the early 1990s provided an alternative to GTX with notable success in rapidly increasing the concentration of circulating neutrophils in neonatal patients. However, early trials showed disappointing results in preterm infants with suspected or proven sepsis who also received concurrent antibiotic therapy, with no difference in mortality observed within 14 days from the start of therapy [48]. The PROGRAMS trial, which specifically studied the prophylactic use of granulocyte-macrophage colony-stimulating factor (GM-CSF) in SGA preterm infants in the first 5 days of life, also found no benefit in sepsis-free survival to day 14 from trial entry compared to the control group [1]. However, a subgroup analysis showed a significant decrease in mortality by day 14 in those with both neutropenia and systemic infection at the time of enrollment [RR 0.34 (95 % CI 0.12–0.92); NNT 6 (95 % CI 3–33)][48], indicating that further appropriately powered studies should be untaken to determine the efficacy of these drugs in this specific patient population [1].

Interest in novel therapies to combat the rise in AMR infections is rising. Preterm infants have the highest risk of neutropenia and sepsis-related mortality. As with other organ systems, postnatal neutrophil deficits are exacerbated in the most immature neonates, resulting in a 10-fold greater risk for early infection compared to term infants and a 30 % mortality rate in those infected [1,8]. Therefore, transfusing neutrophil precursors that will produce fully functional, mature neutrophils (i.e., Romyelocel-L) might be beneficial to combat virulent pathogens, given age-related phenotypic and functional disparities between cells in the youngest, smallest neonates.

Currently, there is inconclusive evidence to support or refute the use of GTX in neutropenic, septic neonates [4]. Continued engineering breakthroughs that exploit *in vivo* neutropoietic pathways for *ex vivo* neutrophil generation might encourage new neonatal investigations. The rise in AMR and difficulty providing adequate antimicrobial therapies may also pique interest in this therapeutic, as survivors of neonatal infections have a higher adjusted risk of technology-dependent, chronic morbidities, including home oxygen, tracheostomy, and gastrostomy [11]. These comorbidities are associated with a significant health burden and resource utilization, including hospital readmissions in the first year after birth [11]. Bringing back neonatal GTX for septic. neutropenic neonates might attenuate this risk if aggressive early interventions could shorten the host's exposure to toxic proinflammatory mediators. However, clinical trials would be required and securing financial and investigative resources in an era of waning community interests would probably prove difficult.

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## Declaration of competing interest

Shelly M. Lawrence does not have any conflicts of interest to disclose.

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