# The Role of Leukocytes from L-PRP/L-PRF in Wound Healing and Immune Defense: New Perspectives

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**Abstract:** Platelet concentrates for topical use are innovative tools of regenerative medicine and their effects in various therapeutical situations are hotly debated. Unfortunately, this field of research mainly focused on the platelet growth factors, and the fibrin architecture and the leukocyte content of these products are too often neglected. In the four families of platelet concentrates, 2 families contain significant concentrations of leukocytes: L-PRP (Leukocyte- and Platelet-Rich Plasma) and L-PRF (Leukocyte- and Platelet-Rich Fibrin). The presence of leukocytes has a great impact on the biology of these products, not only because of their immune and antibacterial properties, but also because they are turntables of the wound healing process and the local factor regulation. In this article, the various kinds of leukocytes present in a platelet concentrate are described (particularly the various populations of granulocytes and lymphocytes), and we insist on the large diversity of factors and pathways that these cells can use to defend the wound site against infections and to regulate the healing process. Finally, the impact of these cells in the healing properties of the L-PRP and L-PRF is also discussed: if antimicrobial properties were already pointed out, effects in the regulation of cell proliferation and differentiation were also hypothesized. Leukocytes are key actors of many platelet concentrates, and a better understanding of their effects is an important issue for the development of these technologies.

**Keywords:** Blood platelet, fibrin, growth factors, leukocytes, platelet-rich fibrin (PRF), platelet-rich plasma (PRP), regenerative medicine, wound healing.

## 1. INTRODUCTION

Platelet concentrates for topical use are nowadays often used as surgical adjuvants or suspensions for regenerative medicine [1]. Since the beginning of the craze for these products [2, 3], platelet concentrates were often called PRP (Platelet-Rich Plasma), and the objectives of these technologies was first to concentrate platelets and to force them to release their growth factor contents on a wounded site. However, these various platelet concentrate technologies also concentrate many other actors, sometimes more important than platelet growth factors: this is particularly true for the fibrin matrix and the leukocytes [1, 4]. Several authors have already pointed out that these preparations were often platelet-leukocytes fibrin gels [5, 6], and that leukocytes probably had a very strong impact in the healing properties of these products [7], particularly for their antimicrobial effects [8-10] and the regulation of immune reactions [11, 12]. The lack of proper terminology and classification is often the source of misunderstandings in science [13], and a complete classification system of the platelet concentrate technologies was only proposed [1] and reinforced [4] recently, based on the fibrin and leukocyte content of the products. In the 4 defined families of platelet concentrates, 2 families contain significant amounts of leukocytes [1]: L-PRP (Leukocyte- and Platelet-Rich Plasma) and L-PRF (Leukocyte- and Platelet-Rich Fibrin). These 2 families of products are the most often used technologies in this field, but little is known about the impact of leukocytes in these preparations. Therefore, it is now very important to highlight the incredible number of properties, factors and pathways that these cells can use to influence their environment, to regulate the tissue reactions in a wounded site and finally to promote a better healing.

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## 2. THE VARIOUS POPULATIONS OF LEUKOCYTES

The word leukocyte origins from Greek leukos – white and kytos – cell, thus the white blood cell term is in common use. These cells develop from the haemopoietic cell line. Haemopoietic stem cells (HSCs) can be found in bone marrow in adults, and also in minor amount in the blood [14]. These cells can be obtained directly by iliac crest harvesting or from the blood, following pre-treatment with cytokines; G-CSF (granulocyte colony-stimulating factor) induces cells to be released from the bone marrow compartment [15]. HSCs mature in several differentiation pathways: myeloblast into progranulocyte and finally granulocyte, lymphoblast into lymphocyte and monoblast into monocyte [16].

Leukocytes are divided into granular cells with granules in their cytoplasm – granulocytes, and agranular cells with relatively clear cytoplasm – lymphocytes and monocytes. The first group consists of neutrophils, eosinophils and basophils Fig. (**1**). The name neutrophil derives from staining characteristics of histological hematoxylin and eosin (H&E) preparations. In this procedure, basophils stain dark blue,

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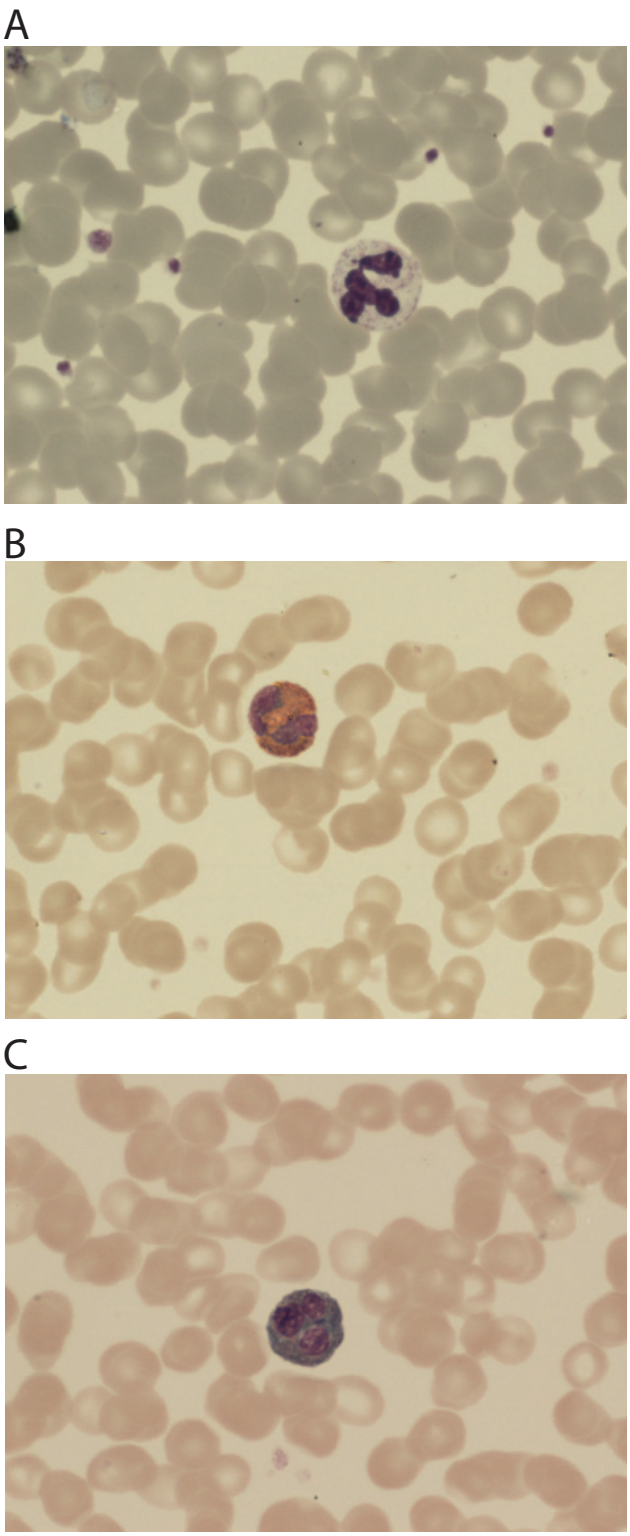


**Fig. (1).** Cells with granules in their cytoplasm; **A** – neutrophil, **B** – eosinophil, C – basophil.

eosinophilic white blood cells stain bright red, while neutrophils stain neutral pink [16, 17]. Granulocytes (polymorphonuclear leukocytes; PMN; PML) are central cellular effectors of the innate immune system. Neutrophils are formed in the bone marrow where their cytoplasmic granules are synthesized in an orderly progression: primary (or azurophilic) granules, then secondary (or specific) granules [18]. Nucleotide acids and polyribosomes are localized in large amount in neutrophils. Thus, many different enzymes and other active peptides can be produced, e.g. the antimicrobial proteins and peptides appear to be largely confined to the primary and secondary granules [18]. Eosinophils contain at least 2 granule populations; the primary granules, containing the Charcot-Leyden crystal protein, and the secondary granules that carry cytotoxic proteins [18]. Their nucleus usually has only two lobes. As the term "eosinophil" indicates, these granules are not neutral but stain red or pink when eosin or a similar dye is used in the staining process. Basophilic granulocytes have a 2 or 3 lobed nucleus. The lobes are usually not as well defined as in neutrophilic granulocytes and the nucleus may appear S-shaped. The specific granules of basophils are stained deeply bluish or reddish-violet. The granules are not as numerous as those in eosinophils [16-18].

The second group consists of lymphocytes and monocytes Fig. (**2**). Lymphocytes are divided into B- and T-cells. These cells are very variable in size, ranging between 5 and 15 μm in diameter. In circulating blood the small lymphocytes occur mainly, and their nucleus may appear to fill the entire cell. Large lymphocytes have a wider rim of cytoplasm which surrounds the nucleus. Both the nucleus and the cytoplasm stain blue. Monocytes diameter size can range between 12 and 18 μm. Their cytoplasm does not contain any structures that would be visible in the light microscope using most traditional stains. They circulate for a few days in the blood and migrate into tissues, where they mature and turn into macrophages [16, 17].

## 3. LEUKOCYTES IN HEALING PROCESSES

The soft tissue and bone healing are one of the most important biological processes with a very large number of cell types and substances taking part in. The role of platelet-derived growth factors was reported widely. However, they are only one of the many actors that interplay during healing. The healing is an interactive process that involves soluble mediators, extracellular matrix components, resident cells including platelets, and infiltrating leukocyte subtypes, which participate in different manners in the classically defined three phases of wound healing: inflammation, tissue formation, and tissue remodelling [19]. Some authors distinguish the hematoma creation period as a first phase. However, most of them include this phase into the inflammation part [20].

After the tissue damage, the blood flows out from the vessels and the clotting process is activated with platelets: they are the first cells recruited at the site of injury and involved to prevent the blood loss and to engage the healing cascade. Platelets have generally been considered to be important in the wound healing [19]. However, in Szpaderska *et al.* investigation, mice dosed with anti-platelet antisera to induce thrombocytopenia exhibited significantly altered wound inflammation in comparison with control group [21]. Wounds of thrombocytopenic mice contained significantly more macrophages and T cells and no delay in the reparative aspects of wound healing was observed. The rate of wound reepithelialization, collagen synthesis, and angiogenesis was

nearly identical for thrombocytopenic and control mice. Moreover, analysis of vascular endothelial growth factor (VEGF), fibroblast growth factor 2 (FGF-2), transforming growth factor beta1 (TGFβ1), keratinocyte growth factor (KGF), and epidermal growth factor (EGF) revealed no difference in the levels of these growth factors in the wounds of control and thrombocytopenic mice. These results suggest that platelets presence is not obligatory to proper wound closure, and other elements have to take over their functions in each phase of the healing process [21].

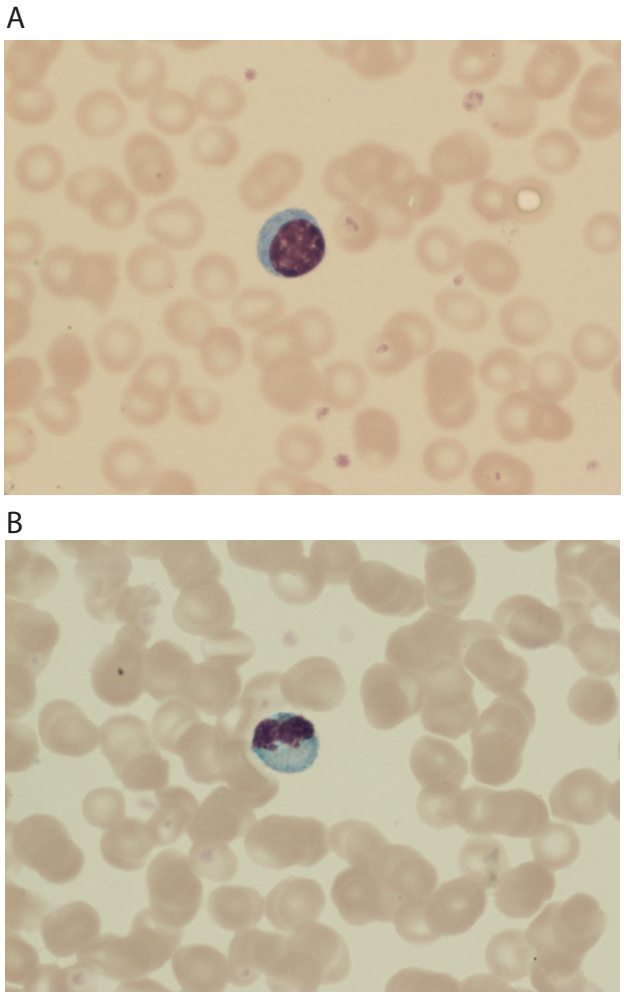


**Fig. (2).** Agranular cells; **A** – lymphocyte, **B** – monocyte.

These duties are executed by leukocytes. After the "passive" neutrophils' extravasation at the injury site, they are captured in the fibrin net and then migrate to the wound together with additional, actively recruited neutrophils from adjacent blood vessels to form a dense barrier against invading pathogens and counteract against flare-up infection [22]. Their main role is to produce inflammatory cytokines and a battery of growth factors. At day 1 after tissue injury, nearly 50% of all cells at the wound site are neutrophils [20]. The studies suggest that the peak neutrophil infiltration in the damaged soft tissue occurs within 24 hours post-injury and is associated with both maximum fibre tearing and maximum oxidant production [23-25]. Under physiological wound healing conditions, monocytes/macrophages invade the wound area concomitantly and they play a central role in wound repair [26, 27]. They exhibit immunological functions as antigen-presenting cells and phagocytes and are in particular an important source of growth factors [19, 28]. Macrophages also secrete collagenase, which stimulates the process of cleaning the wound, excrete transforming growth factors (TGF) to stimulate the keratinocytes, as well as platelet-derived growth factors (PDGF), which formerly was considered to be specific of thrombocytes [29, 30]. They also release interleukin-1 (IL-1), fibroblast growth factor (FGF) and tumor necrosis factor (TNF), which are substances that stimulate fibroblasts to produce collagen, and improve angiogenesis [31]. Granulocytes and macrophages engage the production of inflammatory mediators such as leukotriene B4 and platelet activating factor (PAF), stimulating the expansion and the increased permeability of blood vessels, and stimulating the production of inflammatory cytokines and proteolytic enzymes [32]. These factors act on endothelial cells of blood vessels, stimulating the adhesion of neutrophils and lymphocytes and their migration outside the vessel [33]. Lymphocytes also produce growth factors, and they may contribute to tissue remodelling during the late phase of wound healing [34, 35]. Toulon *et al.* reported that human epidermal T cells are able to produce insulin-like growth factor 1 (IGF-1) upon activation and promote wound healing in a skin organ culture model [36]. However, an analysis of the functional capabilities of T cells isolated from chronic versus acute wounds revealed a striking difference. Both $\alpha\beta^+$ and $V\delta1^+$ T cells isolated from acute wounds actively produced IGF-1, demonstrating that they are activated during tissue damage to participate in wound repair. But these cells isolated from nonhealing chronic skin wounds did not produce IGF-1. No significant differences were observed in CD69 expression on $\alpha\beta^+$ and $V\delta1^+$ T cells from patients with chronic wounds compared with normal epidermis. This indicates that, although present, T cells in chronic wounds are functionally impaired and are unable to produce IGF-1 during the tissue repair process [36]. These studies suggest that the sources of essential healing substances are descended not only from platelets, but also from leukocytes. Szpaderska *et al.* noted in the wound of thrombocytopenic mice the increased number of macrophages and T cells [21], what may be an answer to the absence of platelets.

In contrary, the leukocytes are not also essential for tissue repair. Although neonatal PU.1 null mice do not possess neutrophils or macrophages for recruitment to the sites of tissue damage, the skin wounds can efficiently heal. Indeed, the repair in the PU.1 null mice results in less indication of fibrosis and an altered cytokine and growth-factor profile compared to wildtype, but the final outcomes are comparable [37]. Only in one aspect of the repair and remodelling process are neonatal PU.1-null mice obviously retarded – they are much slower at clearance of cell and matrix debris at the wound site as they are missing their professional phagocytes [37, 38].

## 4. LEUKOCYTES IN IMMUNE HOST DEFENSE

### 4.1. Neutrophils

Neutrophils are one of essential elements of the immune system and they play an important role in healing processes. The primary granules of neutrophils contain a diversity of

The primary granules of neutrophils contain a diversity of antimicrobial peptides and enzymes, and are largely degranulated into the phagosome thereby exposing ingested microorganisms to high concentrations of granule contents. Secondary granules contain distinct antimicrobial proteins and peptides, and are deployed toward the leading edge of the chemotaxing neutrophil from which they are readily degranulated extracellularly [18]. Activated neutrophils migrate to the infection site where granule-associated active substances are released. Some antimicrobial neutrophil's peptides were particularly investigated: lactoferrin, defensins, bactericidal/permeability-increasing protein (BPI), azurocidin/heparin-binding protein, cathelicidins, phospholipases $A_2$, calprotectin. Antimicrobial peptides are usually organized structurally to have hydrophobic and hydrophilic sides. This enables them to interact in both the aqueous environment and the lipid-rich membrane. These peptides can act together with other proteins with antimicrobial activity to increase their potency [39-42].

#### *4.1.1. Lactoferrin*

Lactoferrin belongs to the transferrin family proteins. Its molecular mass is 80 kDa. Lactoferrin is produced in the secondary granules of neutrophils and also by many exocrine glands [18]. Lactoferrin acts as an iron-chelator, which may contribute to its antimicrobial activity by depriving microorganism nutrient, but it can also exert a directly microbicidal effect via membrane disruption [18]. Moreover, lactoferrin has effects on cell growth and differentiation, embryonic development, myelopoiesis, endothelial cell adhesion, cytokine and chemokine production, regulation of the immune system, and modulation of the inflammatory response [43]. In Naot *et al.*'s investigation, the effects and mechanism of action of lactoferrin on bone cells *in vitro* and in a local injection model *in vivo* was examined, and anabolic reaction was noted, which induced proliferative, differentiating and anti-apoptotic effects in osteoblasts and their ability to inhibit osteoclastogenesis [44].

#### *4.1.2. Bactericidal/Permeability-Increasing Protein (BPI)*

BPI is a 55- to 60-kDa protein mainly found in the granules of neutrophils and, in a lesser extent, in eosinophils. BPI was also detected on the surface of neutrophils and monocytes [45]. BPI selectively exerts multiple activities against gram-negative bacteria via sequential effects on outer and inner lipid membranes, opsonization to enhance phagocytosis by neutrophils, and neutralization of gram-negative bacterial lipopolysaccharide (LPS) or endotoxin [46]. During and after phagocytosis, the lipid A region within bacterial endotoxin is partially de-acylated by the host enzyme acyloxyacyl hydrolase. Endotoxin associated with intact bacteria and bacterial remnants is also partially de-acylated in the extracellular inflammatory fluid, but in a much slower rate. Therefore, a more rapid detoxification mechanism may be needed in inflammatory fluids and could be mediated by locally mobilized endotoxin binding proteins, such as BPI [45].

#### *4.1.3. Azurocidin (also Known as CAP37/Heparin-Binding Protein-HBP)*

CAP37, azurocidin, and HBP are the same protein. However, some authors refer to the protein as azurocidin only [47]. The PMN (polymorphonuclear neutrophilic granulocytes) granule proteins HBP, LL-37, and cathepsin G are potent multifunctional mediators of immune reactions and were first identified and isolated by Shafer *et al.* in 1984 [48]. All 3 proteins were previously shown to have monocyte-activating properties. Soehnlein demonstrated that HBP activates monocytes via 2-integrins, leading to $Ca^{2+}$ mobilization, monocyte adhesion, and the release of cytokines [47].

Azurocidin is stored in azurophilic granules and secretory vesicles, and demonstrates direct microbicidal activity against *Escherichia coli*, *Streptococcus faecalis*, and *Candida albicans* [18]. A double-loop mutant of azurocidin in which all eight basic residues were replaced with glutamines demonstrates decreased ability to bind heparin and to kill *Escherichia coli* and *Candida albicans* [49]. Azurocidin acts in synergy with neutrophil elastase and cathepsin G to kill *Capnocytophaga sputigena* and has opsonic activity toward *Staphylococcus aureus* [18, 49]. Antimicrobial activity against gram-negative bacteria such as *Salmonella typhimurium*, *Escherichia coli*, and *Pseudomonas aeroginosa*, may be a result of the binding of azurocidin to lipid A on the surface of these bacteria, and gram-positive bacteria such as *Staphylococcus aureus* are resistant to azurocidin. However, some studies reported, that azurocidin may destroy gram-positive bacteria directly by IgG-opsonizing and increase the expression of FcγRs, resulting in increased phagocytosis and bacterial clearance [46, 48].

#### *4.1.4. Cathelicidins*

Cathelicidin family components have been found in all the mammalian species that were investigated including humans, monkeys, mice, rats, rabbits, guinea pigs, pigs, cattle, sheep, goats, and horses [18]. Each species, however, contains a different set of related genes, which reveal the presence of differential selective pressures [18]. In the human genome, the cathelicidin exons 1-4 are found on chromosome 3p21. These are transcribed as a single gene, *CAMP* (cathelicidin antimicrobial peptide), which translates to a 18 kDa proprotein referred to as "hCAP18" (human cationic antimicrobial protein 18 kDa). The other nomenclature commonly used to describe the protein is "hCAP18/LL-37" because LL-37 was the first isolated mature peptide dominantly expressed in neutrophils [50, 51]. Generally, the cathelicidins are a structurally diverse group of antimicrobial peptides that are expressed at the C-terminus of 11- to 20-kDa inactive proforms in the neutrophil secondary granules of mammalian species [18]; for humans hCAP-18/LL-37. However, cathelicidins are produced not only by neutrophils, but also by natural killers (NKs), T- and B-cells, monocytes and mast cells. Several studies suggest that hCAP-18/ LL-37 participates in the immune defense. For instance, the hCAP-18 gene is up-regulated in skin in response to cutaneous infection or injury and in inflammatory disorders of the skin, and LL-37 is involved in prevention of bacterial invasion i.e. *Shigella* infections [52]. Indirect evidence that endogenous expression of LL-37 protects from skin infections, comes from the demonstration that mice null for the CRAMP cathelicidin gene are much more susceptible to skin infection caused by the group A *Streptococcus* than wild-type mice are [53]. Moreover, LL-37 is involved in the repair of damaged tissue and the wound closure, by promoting wound angio-

genesis and re-epithelialization ; LL-37 is lacking in chronic ulcer epithelium. The evidence that this peptide acts in re-epithelialization of healing skin epithelium is provided by the ability of LL-37-specific antibodies to inhibit this process in the *ex vivo* wound healing model [54]. The precise mechanism of this activity has not been clarified, yet.

#### *4.1.5. Phospholipases A2*

Phospholipases A2 (PLA2s) form a superfamily that currently contains 15 separate, identifiable groups and numerous subgroups of PLA2. These enzymes are characterized by their ability to specifically hydrolyze the sn-2 ester bond of phospholipid substrate. There are five main categories of PLA2: the secreted small molecular weight sPLA2s, the larger cytosolic $Ca^{2+}$-dependent cPLA2s, the $Ca^{2+}$-independent iPLA2s, the PAF acetylhydrolases, and the lysosomal PLA2's [55]. Group II PLA2, which are structurally defined by a unique disulfide arrangement, are found in neutrophil granules and are also secreted by the liver into plasma as acute-phase reactants [46]. Many different studies have examined the role the secreted PLA2s play in eicosanoid release, and these studies have been inconclusive. They show that the up-regulation of groups IIA, V, and X caused a cytosolic group IVA (GIVA) PLA2 dependent increase in eicosanoids [55]. Some authors focused on selective direct bactericidal action of PLA2 against gram-positive bacteria including *Staphylococcus aureus*, *Listeria monocytogenes*, and vancomycin-resistant Enterococcus. PLA2 is found for example in human tear fluid, and antimicrobial activity was noted against gram-positive bacteria [46].

#### *4.1.6. Calprotectin*

Calprotectin was originally discovered as an antimicrobial protein that was present in the cytoplasm of neutrophil granulocytes by Dale in 1983 [56]. The name calprotectin comes from the fact that it binds to calcium and it has antimicrobial properties. It represents 50-60% of neutrophilic cytosolic protein [57]. Calprotectin is secreted extracellularly from stimulated neutrophils [58] and monocytes [59], or is released as a result of cell disruption or death [60]. Calprotectin inhibits the microbial growth through competition for zinc. Zinc chelation that is mediated by histidine-rich regions of calprotectin represents an important antimicrobial mechanism in host defense [61]. Calprotectin concentrations of 50-250 μg/ml were found to inhibit growth of *Escherichia coli*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, lower concentrations (4-32 μg/ml) are sufficient to inhibit growth of *Candida albicans*. Cells expressing calprotectin are able to resist invasion by *Listeria monocytogenes* and *Salmonella enterica serovar Typhimurium* [61].

#### *4.1.7. Defensins*

The defensins are a group of small cationic peptides (4-kDa) and are categorized in three subfamilies, α, β-, and circular θ-defensins. α- and β-defensins show a broad antibacterial activity against gram-positive and negative bacteria, and have antifungal activity [39]. Whereas classical (α-) defensins are characterized by 6 invariant cysteine residues forming 3 disulfide bonds, β-defensins contain a distinct disulfide arrangement. Humans express α-defensins in neutrophils and β-defensins in intestinal Paneth cells, as well as pulmonary and reproductive epithelia [46]. Binding of the positively charged defensin with the negatively charged bacterial membrane precedes membrane permeabilization and is thought to be the mechanism of bacterial killing by defensins. Defensins also have antiviral properties against adenovirus, papilloma virus, human immunodeficiency virus (HIV), and herpes simplex virus (HSV)[39]. Defensins also induce the secretion of cytokines and other molecules from host cells. For example, α-defensins up-regulate the expression of TNF-α and IL-1β in monocytes activated with *Staphylococcus aureus* [39].

### 4.2. Eosinophils

Eosinophilic leukocytes are mobilized from the bone marrow to the blood and tissues in response to Th2 stimuli characteristic of allergic inflammation and parasitic helminth infection [62]. The granules are the storage sites for numerous mediators, including the four major cationic granule proteins: EPO, major basic protein (MBP), eosinophil cationic protein (ECP), and eosinophil-derived neurotoxin (EDN)[63]. MBP demonstrates antihelminthic (*Trichinella spiralis, Schistosoma mansoni, Brugia* species), antiprotozoal (*Trypanosoma cruzi*), and antibacterial (*E. coli*, *S. aureus*) activities and is deposited on the surface of the fungus *Paracoccidioidomycosis brasiliensis* in biopsy samples from patients [46]. Yousefi *et al.* identified a remarkable phenomenon of catapult-like ejection of mitochondrial DNA by eosinophils with the potential to contribute to antibacterial defense [64]. The authors then showed that, when blood eosinophils were primed *in vitro* with IL-5 or interferon γ (IFN-γ) and subsequently stimulated with LPS, C5a, or the eosinophil chemokine eotaxin, they would release mitochondrial (but not nuclear) DNA, thus forming extracellular traps containing antibacterial eosinophilic cationic protein and major basic protein [64, 65].

### 4.3. Lymphocytes

Lymphocytes are small white blood cells that bear the major responsibility for carrying out the activities of the immune system. The two major classes of lymphocytes are: B cells, which grow to maturity independent of the thymus, and T cells, which are processed in the thymus. Both B cells and T cells recognize specific antigen targets. The studies support the concept that regulatory T lymphocytes (CD4, CD25 regulatory and NK, T cells) play a key role in the control of immune responses and are affected by injury and sepsis. This may be related to their capacity to interact with components of the innate and adaptive immune responses and to their ability to be activated non-specifically by bacterial products and/or cytokines and to regulate through direct cell-cell and/or soluble mediators [66].

Because the majority of facultative intracellular bacteria are trapped in the liver immediately after systemic infection, T cells that reside in and/or infiltrate the liver should play a decisive role in the following course of infection [67]. The liver is a rich provenance of unconventional T cells, called natural killer (NK) T cells. The invariant natural killer (NK) T cells represent a unique subset of T lymphocytes which express the Vα14 chain of the T cell receptor (TCR), that recognizes glycolipid antigens presented by the nonpoly-

morphic major histocompatibility complex (MHC) class I-like antigen presentation molecule CD1d, and they participate in the protection against some microbial pathogens [67]. NKT cells have been shown to play a role in various clinical conditions, including autoimmunity, allergy, and cancer [66]. Many studies have suggested that an important function for NKT cells might be in serving as a protective brake (particularly in vital organs such as the liver) on the damaging effects of the local inflammatory immune response [66]. Alternatively, because of their ability to undergo activation rapidly after antigenic exposure, NKT cells may also play a major role in the first line of defense against invading pathogens [66]. Indeed, a role in pathogen clearance has been described for NKT cells in bacterial, viral, fungal, as well as parasitic infections [66].

### 4.4. Macrophages

Blood monocytes migrate into the tissues of the body and there differentiate into macrophages. Therefore, macrophages are not present in platelet concentrates.

## 5. PRP ENRICHED IN LEUKOCYTES: THE L-PRP

### 5.1. Leukocyte Populations in PRP

In the literature about platelet-rich plasma, most authors focus only on platelets. However, several reports have indicated that the classical 2-stages PRP preparation protocol, which consists of 2 centrifugations, concentrates a substantial amount of leukocytes. This manual procedure was used in Cieslik-Bielecka's study in rats [68]. She identified these cells through flow cytometry after labeling T lymphocytes for CD3, CD4, CD8, CD27, CD28, TCRab, TCRgd and their active form for CD25, RT1B ; B lymphocytes for CD45R, CD45RA, CD90 ; NK for CD161a ; monocytes and granulocytes for CD18, CD11bc Fig. (**3**).

In gravitational platelet separation systems developed by companies, where automatic partition of the buffy coat from the red blood cells and the acellular plasma layer is reached, a huge number of leukocytes can also be obtained. Their phenotype was identified through flow cytometry after labeling leukocytes for CD45: monocytes (CD14+/CD33+), granulocytes (CD15+), lymphocytes B (CD19+), lymphocytes T (CD3+), T helper cells (CD3+/CD4+), suppressor and cytotoxic T cells (CD3+/CD8+), cytotoxic T cells (CD3+/CD16+/CD56+), NK (CD45+/CD3-/CD16+/CD56+) and their progenitor form (CD34+), with fluorochrome-conjugated antibodies [69]. Therefore, the proper term to use as a nomenclature for these products is clearly "leukocyte- and platelet-rich plasma (L-PRP)".

### 5.2. The Effects of L-PRP Against Infections

Bielecki *et al.* performed a microbiological examination of L-PRP gel on 20 healthy volunteers [8]. In this *in vitro* study, they demonstrated a strong activity comparable to gentamicin and oxacillin for L-PRP gel against *methicillin susceptible Staphylococcus aureus* (MSSA). L-PRP gel also inhibited the growth of *methicillin resistant Staphylococcus aureus* (MRSA) and *Escherichia coli*, but no activity was observed against *Enterococcus faecalis*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*. Moreover, in the plate

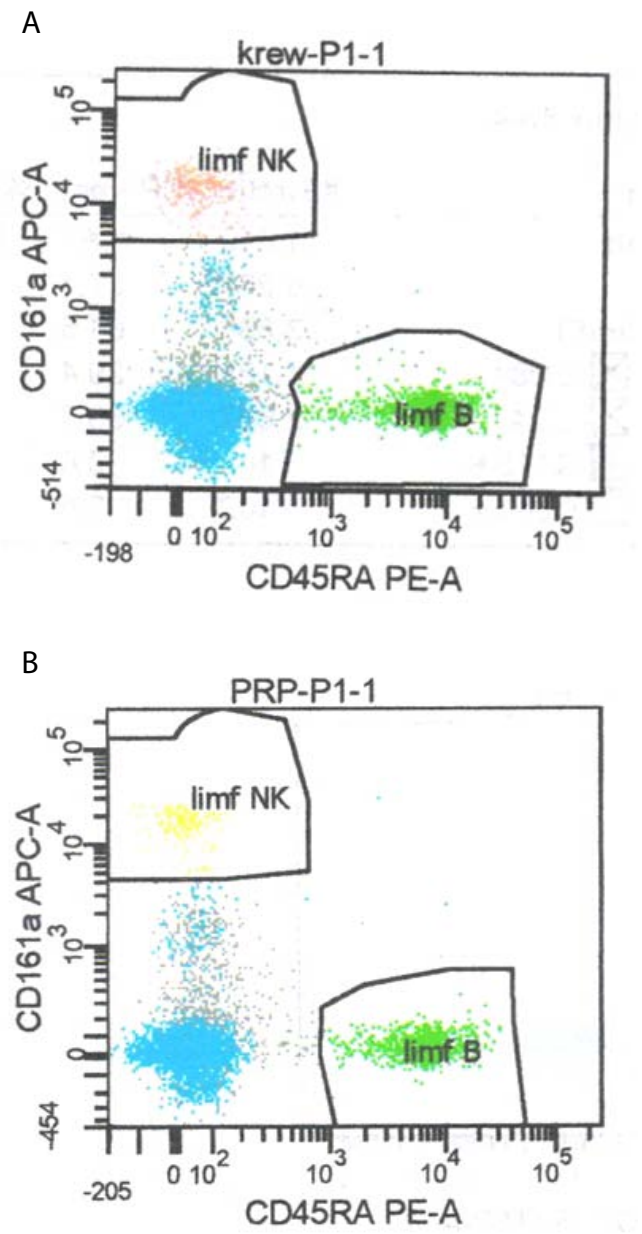


**Fig. (3).** The analysis of NK and B-cells in blood (**A**) and L-PRP (**B**) by flow cytometry in rats.

coated with *Pseudomonas aeruginosa*, the authors observed the growth of bacteria under the disc containing L-PRP gel. These *in vitro* tests suggested that L-PRP treatment of chronic ulcers with coexisting *Pseudomonas aeruginosa* infection might be contraindicated. The authors concluded that the mechanisms of the antibacterial effects of L-PRP gel were not yet clearly described and should be carefully investigated, and this work illustrated the complexity of the leukocyte and platelet role in antimicrobial properties of L-PRP [8]. In another unpublished investigation on 20 healthy volunteers with another L-PRP protocol, we showed a slightly weaker antimicrobial activity of L-PRP gel against MRSA and MSSA in comparison with the previous study; however, this second L-PRP gel presented a strong activity against

*Enterococcus faecalis* and *Pseudomonas aeruginosa*. The statistical analysis demonstrated a significant correlation between leukocytes and growth inhibition zone in 3 strains; MRSA (p=0.013), MSSA (p=0.015) and *Pseudomonas aeruginosa* (p=0.019). The leukocyte concentration had no influence on inhibition of *Enterococcus faecalis* growth, but a strong correlation of this effect with the amount of active T-lymphocytes (CD3+/CD16+/CD56+ with CD25+) was found (p=0.046). No relationship between the platelet counts and microbial inhibition was noted.

The investigations of Everts and collaborators reported the strong *in vitro* activity of L-PRP gel against *Staphylococcus aureus* [70]. This antimicrobial activity was maximum during the first hours after the application and, according to the authors, this L-PRP might be useful for infection prophylaxis but maybe not for infection treatment. They mentioned also that L-PRP gel application can be the supplement to surgical debridement and might reduce the number of remaining bacteria after wound curettage, enough to prevent reinfection. Moojen *et al.* also measured the concentration of myeloperoxidase (MPO), an enzyme produced mainly by neutrophils and monocytes, which is a potent bactericidal oxidant and is toxic to micro-organisms and fungi. However, MPO release and activity showed no significant correlation with the observed bacterial killing for any of the L-PRP products [70].

Jia *et al.* injected gelling L-PRP into the rabbit tibial canal after MSSA inoculation and the prophylactic efficiency was evaluated by microbiological, radiological and histopathological examinations. The authors used also cefazolin and control group, where no treatment was introduced. The results confirmed that gelling L-PRP reduces the infection rate and number of viable bacteria in comparison with control group [71].

Some authors also reported clinical observations about a decreasing number of infections and the stimulation of the healing processes after L-PRP gel usage in orthopaedic and cardiac surgery. Yuan *et al.* published a case report describing the evaluation of infection after intramedullary nailing [72]. In this case, many kinds of treatments had been previously applied, but were not effective and the patient refused further open operation under anaesthesia. They finally attempted to use L-PRP gel, and the wound healed after L-PRP gel application. Another case report described a 42-year-old man, who experienced femoral and crural fracture with large skin defect after accident in a coalmine [10]. This was associated with an injury of popliteal vessels, so the transplantation of a vein from the undamaged leg was necessary to restore the arterial blood circulation. Since the venous circulation system was insufficient, the patient underwent fasciotomy. During operation the necrotic tissues were cut out and the wound was covered with platelet-leukocyte rich gel. Subsequently, they observed instant angiogenesis and epithelialization processes. Therefore, non-healing skin defect was covered with L-PRP gel on two other occasions 10 and 20 days after operation without wound debridement. Repeated antimicrobial examinations were performed in order to detect the residual bacteria from the wound between the 1$^{st}$ and 6$^{th}$ day after L-PRP gel application. The final outcome was very positive. Finally, in Khalafi's study, 1028 patients underwent coronary artery bypass grafting procedures [73]. In the L-PRP gel group, one incidence of sternal infection occured (0.18%) in comparison with 11 cases (1.98%) in the control group. There were 3 cases (0.53%) of notable drainage from the sternum in the L-PRP gel group compared to 30 cases (5.39%) in the control group. For the leg vein harvest site, the L-PRP gel group had no reported infections and there were 61 (10.89%) incidences of excessive drainage compared to 3 (0.66%) surgical site infections and also 212 (48.4%) cases of excessive leg drainage in the control group. Following propensity scoring, they concluded that L-PRP gel application reduced the risks of chest wound infection, chest drainage and leg wound drainage

All these studies illustrate the key role of leukocytes in the clinical applications of L-PRP gels. It also proves that all the L-PRP gels are not equal, because their leukocyte formula can be very different. This aspect was almost never discussed in the literature, and requires further investigation.

## 6. PRF ENRICHED WITH LEUKOCYTES: THE L-PRF.

The main difference between the L-PRP and L-PRF families is the architecture of the fibrin matrix [1]. The L-PRF presents a stronger fibrin polymerization and a denser fibrin network [74-76]. This parameter is very important, because the matrix architecture also influences the biology of the material [77] and the cells trapped inside, particularly the various families of leukocytes. Moreover, fibrin itself has a strong general influence on the healing processes [78, 79], particularly through the promotion of neoangiogenesis [80, 81] (and therefore the drainage of new leukocytes through new vessels). Therefore, in a L-PRF, the effects of the leukocytes and the fibrin matrix are deeply interconnected.

In the literature about L-PRF, the leukocyte populations have been pointed out several times [76, 82-85]. The L-PRF clot and membrane contains at least 50% of the leukocytes from the initial blood harvest, and these cells are enmeshed in the fibrin matrix following a specific three-dimensional cell distribution [76]. The lymphocytes are more concentrated than the other leukocytes. A PRF membrane releases significant amounts of growth factors and matrix proteins during more than 7 days [83, 86-89], and the leukocytes seem to be the source of the overproduction of some of these growth factors (particularly Vascular Endothelial Growth Factors (VEGF) and Transforming Growth Factors β1 (TGFβ1))[83]. However, the main *in vitro* discovery was that cell cultures of osteoblasts and bone mesenchymal stem cells with L-PRF were in fact cocultures with leukocytes [84, 85, 90]: the leukocytes seem to regulate directly the proliferation and differentiation patterns of the various cell types in culture. The role of leukocytes as regulation turntables is very important to understand the biology of a complex biomaterial like the L-PRF, but this is also a key issue for potential *in vitro* tissue engineering with L-PRF.

L-PRF was mainly tested in oral [91] and maxillofacial surgery [92-96], implant dentistry [97-103] and periodontology [104]. The role of the leukocytes in all these applications has not yet been fully investigated, even if a beneficial impact on wound healing and antibacterial properties were often hypothesized. The overpopulation of lymphocytes in this

technique is of particular interest [76], because lymphocytes are turntables of the local regulation during healing, and this may explain why the L-PRF membrane can continue to produce large quantities of growth factors during a long period. The perspectives in terms of tissue engineering and regenerative medicine for this leukocyte enriched fibrin matrix are outstanding and require many investigations in the future.

## 7. CONCLUSIONS

The leukocytes have a great impact on the intrinsic biology and the properties of the platelet concentrates, not only because of their immune and antibacterial potential but also because these cells are turntables of the wound healing process. Unfortunately, their impact has been almost completely neglected in the literature on the topic. For example, some of the antimicrobial factors and pathways described in this article were never cited in an article about platelet concentrates for topical use, and this illustrates the strong lack in the literature and researches on these technologies. In this article, we have pointed out the main actors and some of their neglected mechanisms when used in a platelet concentrate. However, this is only a first step: the role of leukocytes in L-PRP and L-PRF requires further investigations, but, first of all, the presence of these cells must not be neglected (or sometimes forgotten) anymore in this field.

## DISCLOSURE OF INTEREST

The authors declare no competing financial interests.

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