Chapter 7

Neutrophils Plasticity: The Regulatory Interface in Various Pathological Conditions

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Additional information is available at the end of the chapter

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Abstract

It is now known that neutrophils make up a population of complex cells with great plasticity, challenging the old view of neutrophil association with tissue damage and early phases of infection. Here, we discuss different contexts in which these cells can induce anti-inflammatory responses. Although distinct surface markers and cytokines profiles were shown, the most reliable characterization of suppressor neutrophil subtypes relies on their functional characteristics. One important example of inhibitory neutrophils generation comes from in vivo treatment with G-CSF, for 5 days, as for hematopoietic-stem-cell-transplantation (HSCT). In this case,donor blood is enriched in degranulated granulocytes harboring a functional regulatory phenotype, characterized by IL-10 production. These cells, when transferred together with HSCT, are able to reduce graft-versus-host-disease, being influenced by Treg cells and influencing them back. Importantly, this protection is long lasting and specific, keeping immunocompetence to other antigens. This regulation is paramount in HSCT, and represents a simple approach to be applied in humans. In summary, we discuss the interaction of neutrophils with other cell types and its consequence in immunomodulation. We believe these features confer an important bridge between innate and adaptive immune system, building a new knowledge for an underestimated cell type.

Keywords: regulatory neutrophils, neutrophils subtypes, T cell inhibition, Cytokines, G-CSF, GVHD

1. Introduction: Regulatory neutrophils and their profile

1.1. New technologies changing the concept of a short half-life

Besides the known features of neutrophils as fast migrating pro-inflammatory cells, the literature has shown that this is not a homogeneous population. In fact, several different subtypes of neutrophils have been well described regarding its characteristics and mechanisms of action. However, there are still some subtypes not so well understood.

The classical, and popular, concept of neutrophils says that they are the first cells to arrive and accumulate at the site of infections where they rapidly release several toxic molecules and undergo apoptosis [1]. In the meantime, they clear the infected area through phagocytosis or "neutrophils extracellular traps" (NETs) that occur when activated neutrophils release their uncondensed chromatin and granule contents. These molecules bind to pathogens causing death, contributing to fight against infections, and causing important tissue damage. Macrophages come over after this, clean up cell debris, and amplify the response [2, 3].

However, since different and modern techniques started to be accessible to research, some functions and concepts assigned to neutrophils have changed. The paradigm of the short half-life fell with the improvement of cellular techniques that enable better stains for different phenotypic markers or gene expressions. A variety of different neutrophils subtypes that are different in their phenotype, function, package of cytokines produced, degree of maturation, and site of action have been identified. So, a number of different types of neutrophils are being described including the ones with regulatory properties (hereafter referred as regulatory neutrophils—RN) that we will look closer in this chapter [4, 5].

How can these new findings interfere in the classic view of these cells?

Previously, a half-life of ~24 hours for human neutrophils and ~8 hours for murine neutrophils was believed. However, recently, using *in vivo* labeling techniques with $^{2}H_{2}O$, a stable isotope, it was shown that human neutrophils in homeostatic conditions present an average 5.4 days of half-life [6]. The discrepancy with previous studies is believed to be due to *ex-vivo* manipulation and i.v. injection of neutrophils, which affects cells viability and *in vivo* distribution. Neutrophils longer half-life allows the conditions to develop phenotypic and functional alterations, including synthesis of a great number of cytokines, ability to recirculate and alter or influence other immune cells [7].

Neutrophils half-life can be dictated by several factors as inflammatory conditions, cytokines, cell interactions, PAMPS (pathogen-associated molecular pattern), and DAMPS (danger-associated molecular pattern), which might inhibit apoptosis and prolong the cell life span [8]. Then, this longer half-life associated with all sort of stimuli paves the way for new regulatory subtypes to emerge.

1.2. Neutrophil subtypes generated in specific conditions

The classical murine neutrophils can be identified by $Ly6G^{+}$ expression, while human neutrophils have to accomplish the expression of CD14, CD15, and CD16, always associated with

a visual inspection that must identify a band or hypersegmented nucleus with a light pink cytoplasm, full of granules [4].

Although the existence of different neutrophils subtypes is currently accepted, the basis for their classification remains obscure. Distinct surface markers or new cytokines are common characteristics used to define neutrophils, but heterogeneity can also be explained by a stage of differential activation of neutrophil subpopulations [3, 7].

In some autoimmune diseases, such as systemic vasculitis associated (ASV) with anti-neutrophil cytoplasmic autoantibody or systemic lupus erythematous (SLE), circulating neutrophils display an increased expression of the specific surface marker, CD177. This is a molecule compartmentalized in secondary (specific) granule, that is co-expressed with its membrane ligand proteinase 3 (mPR3) in neutrophils from ASV patients. mPR3 is one of the main targets of ANCA autoantibodies, and, in this case, CD177 is important to mPR3 expression influencing the potential of neutrophils to be activated by ANCAs that usually target mPR3. However, levels of CD177 expression in patients and their influence on ANCA have not been defined as disease biomarkers yet, the $CD177^{+}$ neutrophils (NB1 in humans) represent indeed a new subset [9, 10].

Importantly in SLE, where the response is driven against nuclear antigens in various target organs such as the skin, kidney, and joints, neutrophils have been described as the source of DNA antigens due to its extravasation of nuclear content when forming NETs. Besides the $CD177^{+}$ NB1 neutrophils, low-density granulocytes (LDGs) are another subpopulation, which has been described in SLE. Specifically in this case, LDGs can assume a highly inflammatory profile, including the release of NETs, which amplifies disease physiopathology [11, 12]. The same LDG subtype was described in rheumatoid arthritis (RA). In RA, they show a low expression of TNFR, potentially affecting TNFi (inhibitor) treatment [13]. Beyond that, LDGs were also reported in mycobacterial infections being associated with disease severity [14]. This probably happens because it suppresses the immune response allowing mycobacterium growth. In severe asthma [15] and in interstitial lung disease in dermatomyositis [16], similar to what was described in autoimmune diseases like SLE and RA, the LDG acts worsening the pathologic condition.

The above features evidence the complex behavior of neutrophils requesting a lot more to be described about the plasticity of neutrophils in disease pathogenesis.

Apart from the uncommon profile of neutrophils in autoimmune diseases, we can highlight the phenotype of aged or senescent neutrophils. This particular subset expresses CXCR4 in high densities ($CXCR4^{hi}$). CXCR4 mediates cell retention in the bone marrow along with low expression of CD62L ($CD62L^{low}$). They express high CD11b and have hypersegmented nuclei [17]. Recently, it was described that ageing neutrophils are regulated by the microbiota in a toll-like receptor (TLR)-dependent way. The microbiota is the community of microorganisms that lives within the body and in harmony with it. The most studied group in this regard is bacteria from the human gastrointestinal (GI) tract that harbors an estimated $\sim 10^{14}$ individuals from about 1000 species in a single individual. Close to ~15,000 species of bacteria have already been identified from human GI samples [18]. These commensal bacteria can influence the immune system inducing a pro-inflammatory or a suppressor response,

which depends on the bacteria quality and the milieu of activation. When the microbiota is depleted with the use of antibiotics, the number of aged neutrophils (that are highly activated cells) decreases, and the pathogenesis of sickle-cell disease or endotoxin-induced septic shock improves, showing that aged neutrophils have an important role in inflammatory diseases [19].

Another neutrophil subtype extremely relevant for immunology are the tumor-associated neutrophils (TAN), that are subdivided into type 1 (N1), with anti-tumor activity, and type 2 (N2), that fulfills a pro-tumor activity and will be discussed in the next subsection [20]. They have high relevance in the prognostic of some types of tumors, as colorectal [21], non-small cell lung [22], and breast where ratio of neutrophils/lymphocyte (NLR) is used as a toll to correlate with a better or poor prognostic [23].

Plasticity of TANs depends on many factors such as cytokines, chemokines, and adhesion molecules. These factors can be secreted by other immune cells or by the tumor itself [24]. The anti-inflammatory cytokine TGF-β has an important role in this scenario: in its presence, TANs can be directed to a pro-tumor N2 phenotype, and in its absence (using blocking antibodies) TANs are driven to an anti-tumor N1 phenotype [20]. On the other hand, these neutrophils can also interfere with the tumor microenvironment through the release of cytokines (TNF-α, IL-1β, IL-12), chemokines (CCL2, CCL3 and CCL5), reactive oxygen species (ROS), and growth factors, creating a diverse niche amplifying or down-regulating the inflammatory response [5, 25, 26]. Phenotypically, the N1 type presents a hypersegmentated nuclei with high expression of FAS, ICAM, and TNF-α production, making them able to activate TCD8+ lymphocytes, helping to eliminate the tumor cells [20].

These ambiguous characteristics evidence how the plasticity of neutrophils can impact in health and in pathological conditions. Until now, we covered how neutrophils can assume different subtypes and contribute to a pro-inflammatory milieu, amplifying the immune responses in autoimmune diseases as well in highly activated conditions, as the aged neutrophils in inflammatory infections. In tumor setting, the ratio neutrophil/lymphocyte can even predict the patient prognostic highlighting the importance of neutrophils in disease outcome.

In Section 1.3, we describe the profile of RN, the conditions in which they were described their mechanism of action, and the possibility to manipulate them for therapeutic usage.

1.3. Regulatory neutrophil phenotypes

As stated above, nowadays, the literature accepts that neutrophils can assume different subtypes. It is important to point here that the term "regulatory" or "suppressor" indicates the capacity of these cells to induce an anti-inflammatory response, either by interacting directly with other cells or by secreting molecules that induce polarization of other cell types.

The classification of RN subtypes is still unclear. The most reliable characterization of the suppressor neutrophil subtypes remains being their functional characteristics. Although there are some markers, such as $CD62L^{low}/CD11b^{low}$ highly associated with suppressor phenotypes and others such as CD244, CD115, CD11c, CD32, CD35, CD45, and CD66b, which can be up-regulated, there is no consensus for a specific combination of markers for suppressor neutrophils. Their phenotype heterogeneity is probably because they are modulated

according to individual conditions [3, 4]. So, in this section, we discuss the literature on the different subtypes (or phenotypes) of RN.

Among the subtypes described, we can highlight the granulocytic myeloid-derived suppressor cells (G-MDSCs), which are an important sub-population of circulating neutrophils [27]. MDSCs are a heterogeneous population of immature and mature cells, of myeloid origin, first described in tumor-bearing mice and comprise two groups of cells identified regarding their morphology and phenotype. Monocyte-MDSCs (M-MDSCs) are $CD11b^{+}Ly6C^{high}Ly6G^{-}$ and have a typical monocyte morphology, and cells $CD11b^{+}Ly6C^{low}Ly6G^{+}$ with typical granulocytic morphology are G-MDSC [28, 29]. The G-MDSC phenotype is characterized mainly by large amounts of ROS expression and low amounts of nitric oxide synthase (NOS). The opposite is true for the M-MDSC phenotype that acts mainly expressing arginase-1 (Arg1) and NOS. MDSCs can inhibit T cell responses in many ways. After being generated as a consequence of intense inflammatory environment in the presence of factors like GM-CSF (granulocyte-macrophage colony stimulating factor), G-CSF (granulocyte colony stimulating factor), VEGF, IL-6, MDSCs are recruited to the site of the primary tumor and secondary lymphoid organs (lymph nodes, spleen) by chemokines such as CCL2, CXCL12, and CXCL5 [30]. Upon arrival in the specific site, they can modify the microenvironment by secreting NOS, ARG, and/or ROS.

Although described as harmful in autoimmune diseases, it is also known that mouse and human LDGs are a heterogeneous population composed of mature and immature neutrophils with suppressive capacity. Neutrophils are classified as LDG or low-density neutrophils (LDNs) and high-density granulocytes (HDNs) depending on their density. In general, LDG or LDN cells co-purify with PBMC at the low-density layer in a ficoll gradient, rather than with the high-density layer, which is the usual for the classic neutrophils [31, 32].

It was shown in a tumor model that LDN comprises at least two different populations: one with a segmented nucleus (mature) and another with banded or ring-shaped nucleus (immature) that resembles the G-MDSC phenotype. Both can be generated from HDN in a TGF-β-dependent way. In this case, a new nomenclature was suggested to circulating mature neutrophils. The HDN that are pro-inflammatory with anti-tumor profile would be called Nc1 and its counterpart mature LDN, which shows a pro-tumor activity would be Nc2. The Nc2 has reduced expression of inflammatory molecules and inhibit $TCD8^{+}$ proliferation *in vitro* evidencing more than one type of RN [32].

During pregnancy, where immunosuppressive state is required to allow implantation and growth of the fetus, an important population of LDG producing arginase-1 was identified in PBMC and placentae of pregnant women and in the cord blood. Besides, these neutrophils were described as cells that released specific granules (once they increase expression of CD66b), and the azurophilic granules, where arginase-1 is stored (once CD63 is expressed). These phenotypical markers associated with others mean that these cells have been activated and are degranulated. Presence of arginase-1 collaborates to impair T cell responses once the L-arginine deprivation induced by release of arginase contributes to T cell hyporesponsiveness and immune privilege at the materno-fetal interface [33, 34].

In HIV infection, LDGs act to inhibit the immune system, worsening the condition. PBMCs from HIV-infected patients are rich in high arginase LDGs, suggesting that they are activated neutrophils that had degranulated [35].

Some years ago, our group observed that LDG was increased in the peripheral blood of G-CSF-treated donors of peripheral blood stem cells. These cells were capable to inhibit T cells IL-4 and IFN-γ production in a hydrogen peroxide (H_2O_2)-dependent way [36]. In a murine model of graft versus host disease (GVHD), the main limitation of stem cell transplantation, LDGs prevented 100% mortality [31]. These cells were better characterized recently [37] and will be described at the end of this chapter.

On the other hand, in infection with methicillin-resistant *Staphylococcus aureus* strain, three different subtypes of mouse neutrophils with different susceptibilities to infection have been described. Besides the normal PMN-N (polymorphonuclear neutrophils), there are at least two distinct PMN subtypes (PMN-I and PMN-II). The suppressor subtype (PMN-II) can express TLR2/TLR4/TLR7/TLR9 and has low levels of MPO (myeloperoxidases). PMN-II is involved with the generation of alternatively activated macrophages (M2), through IL-10- and CCL2-dependent mechanisms. These M2 macrophages have anti-inflammatory properties and induce a Th2 response [38], modulating the adaptive immune response at the expense of neutrophils.

Regarding cytokines production, RN IL-10$^+$ producing cells have been described. The IL-10 is an important cytokine, which can be produced by many different cell types, as B cells, mast cells, eosinophils, macrophages, DCs, and a large number of T cell subtypes that act regulating the synthesis of pro-inflammatory chemokines and cytokines, such as IL-1, IL-6, TNF-α, as well as nitric oxide (NO), collagenase, and gelatinase [3, 39, 40].

In murine models, several studies have shown that neutrophils produce IL-10 in response to a variety of infections, such as *S. aureus* [38], *Candida albicans* [40], *Trypanssoma cruzi* [41], and in inflammatory conditions, such as post-burn [42] and after G-CSF treatment [37]. However, these data are still a matter of conflict in the literature since just a few studies show the same phenotype in humans [43–45] and others were incapable to reproduce it [46, 47].

These differences in IL-10 production between mouse and human neutrophils may result from different factors, such as culture conditions, contaminating cells, or post-transcriptional regulation of IL-10 gene expression [47, 48].

Moreover, the cytokine IL-22 has also been described as being produced by neutrophils, besides being produced by many different cells, including Th17, Th22, NK cells, Tγδ, and ILC (innate-like lymphocytes). RN IL-22$^+$ is mainly important for intestinal barrier maintenance exerting a local modulation and keeping the integrity of the intestinal mucosa, generating a protective response against certain extracellular pathogenic bacteria [49, 50]. IL-22 has the ability to synergize with other cytokines to induce gene expression of antimicrobial peptides, chemokines, matrix metalloproteinase, cytokines, and epithelial acute phase proteins in the skin, liver, lung, and intestine [51, 52]. Of note, it was described that neutrophil-producing IL-22 has an important role in intestinal protection in a model of colitis. The adoptive transfer of IL-22-producing neutrophils to IL-22-deficient animals was protective for dextran-induced colitis inducing the release of antimicrobial peptides RegIIIβ and S100A8 by colonic cells, protecting the intestinal barrier from microbes and helping the resolution of disease [53].

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At last, as stated before, in tumor settings, neutrophils can assume ambiguous features being supportive or inhibiting the tumor growth. We described above the TAN-N1 pro-inflammatory neutrophils, protecting from tumor, but it is also important to highlight the TAN-N2 neutrophils that are suppressive and, in this case, harmful for the patient. N2 TANs are tumor resident neutrophils that influence the establishment, development, and spread of cancers. They can be generated in a TGF-β milieu and also by G-CSF produced by tumor cells, among others [20]. Under G-CSF stimuli, neutrophils are generated and expand, creating a pro-tumorigenic niche, being able to favor metastatic microenviroment [54]. TAN-N2 cells express arginase, contributing to inhibition of T cell responses. They release chemokines such as CCL2 and CCL5 that favor the recruitment of other cell types, including regulatory T cells (Tregs). Also, they can produce oncostatin-M that works promoting angiogenesis and neovascularization favoring tumor growth [20, 55, 56]. Depletion of neutrophils from tumor-bearing mice shows an increase in $TCD8^{+}$ cells, supporting the concept that N2 acts in a suppressive way being an RN [20]. As stated above, the ratio between lymphocytes and neutrophils (NLR) is used to predict the patient prognosis in cancer patients. In breast tumors, a high NLR is associated with a poor prognostic and a shorter overall survival [57].

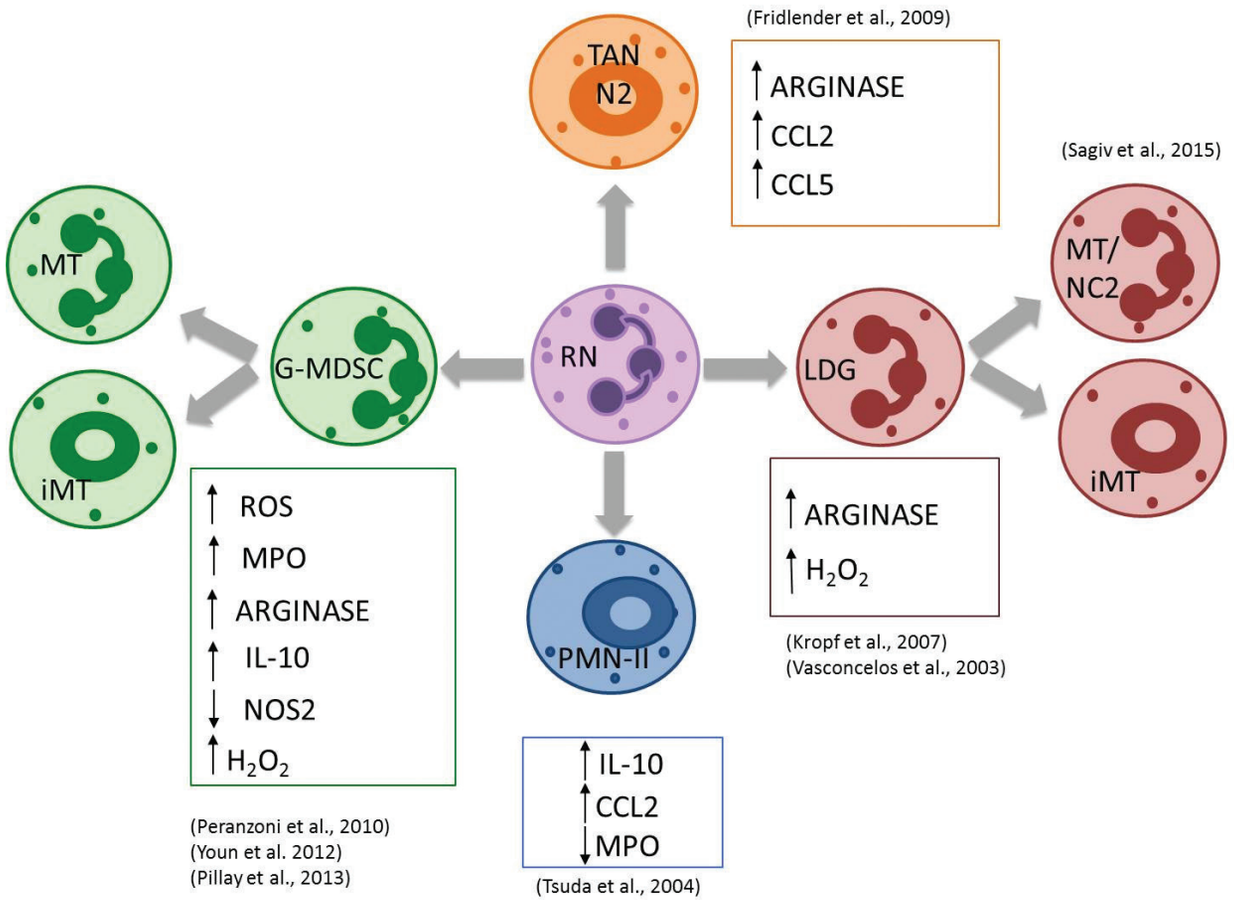

Figure 1. Illustration of the most well-described regulatory neutrophils (RN) subtypes and their main mechanisms of action. TAN-N2 (Tumor associated neutrophil type 2); G-MDSC (Granulocytic myeloid derived suppressor cell); PMN-II (Polymorphonuclear type II); LDG (low-density granulocyte); NC2 (circulating neutrophils type 2); MT (mature); iMT (immature).

Despite those RN that share some similarities regarding their mechanism of action based on arginase production, H_2O_2, Treg induction by IL-10 secretion, and M2 generation, there are still some differences among the cell types that prevent them from being placed in the same general group of RN. Many authors have been trying to establish a nomenclature to these RN; however, new subsets keep being described as well as new features which make this a hard task and fill the literature with different names, many times for the same described cell. Some of the subsets are well defined as MDSC, like high-density mature, low-density mature or immature, as can be seen in **Figure 1**. In this regard, these cells can express different markers, be sensitive to diverse stimuli, and influence different cell types showing important consequences in amplification of suppressive immune response. The crosstalk of neutrophils with other cell types and the maintenance of the suppressor "tonus" will be explored in more details in Section 2 (**Figure 1**).

2. Changes in the immune response under neutrophils influence

As mentioned earlier, neutrophils are recruited to different tissues after injury or infection and, in this sites, encounter resident and/or recruited leukocytes, which promote interactions that influence each other mutually. Under neutrophil influence, other cell types acquire regulatory properties worsening or improving the host condition. On the other hand, neutrophils can suffer influences that polarize them to a suppressor phenotype.

2.1. Neutrophil influence in macrophage polarization

In *Nippostrongylus brasiliensis* infection, neutrophils acquire a N2 phenotype that secrete high amount of IL-13 that in turn is essential to provide helper functions to promote alternatively activated M2 macrophage polarization with long-lived profile. The M2 macrophages mediate parasitic larval damage during recall responses in the lung and are essential to nematode damage and clearance [58]. The presence of these M2 macrophages also impairs host antibacterial resistance against sepsis. In thermally injured mice, PMN-II displays an immunosuppressive phenotype with production of CCL2 and IL-10. These induce macrophages conversion to M2 favoring *Enterococcus faecalis* translocation [59]. The same mechanism was also described in humans with severe burn injuries [60].

2.2. NK, NKT, and ILCs suppression by neutrophils

Neutrophils are also able to inhibit NK cell activity. *In vitro* co-culture assay demonstrated that in the presence of neutrophils and G-MDSCs, there was a significant decrease in NKp30 expression that led to a reduced NK cell cytotoxicity against *Aspergillus fumigatus*. Moreover, activation markers CD69 and CD137 expression and secretion of the effector molecule IFN-γ were also decreased in NK cells incubated with neutrophils or G-MDSCs before the infection with *A. fumigatus* [61]. In vaccinia virus infection, the G-MDSC subset was responsible for the NK function and proliferation inhibition mediated by ROS [62]. A crucial role of primary tumor-mobilized neutrophils and NK crosstalk in the establishment of lung pre-metastatic niches was described. In this case, they are able to inhibit NK cell-mediated clearance of

metastatic cells and simultaneously promoting intraluminal survival and extravasation at the metastatic site. *In vitro* functional assay of lung NK cells obtained from tumor-bearing mice showed that these cells were significantly less responsive to the NKG2D or NKp46, NK-activating receptors (measured by expression of CD107 and IFN-γ) than the naïve mice [63]. Reciprocally, human NK cells (resting or activated by IL-12) were able to induce neutrophil apoptosis dependent on cell-cell contact and caspases, which overcome the antiapoptotic effect of GM-CSF. Involvement of the activating NK cell receptor NKp46 and the Fas pathway was observed in this process [64]. This regulatory effect of NK cells on neutrophils was also observed in the DSS-induced colitis model. However, in this model, NK cells significantly lowered the percentage of apoptotic neutrophils in co-cultured assay. The regulatory effect is dependent on down-regulation of the inflammatory neutrophil functions (decrease in IL-6 and increase in IL-10 production) and is largely dependent on direct NK cell-neutrophil contact, via their inhibitory receptor NKG2A [65].

Mouse and human invariant NKT (iNKT) cells were also inhibited by contact with live neutrophils. iNKT cells from mice with acute inflammatory neutrophilia (as in peritonitis) display decrease in T-bx21 and GATA3 expression and diminished cytokine production compared with those from control mice. *In vitro* assay demonstrated that cell-cell contact between iNKT and neutrophils is required for the inhibitory effect and that this encounter impairs the cytotoxicity capacity of iNKT cells [66].

The relationship between neutrophils and innate lymphoid cells (ILC3) was shown in human decidua during pregnancy. The ILCs are important effectors of innate immunity present in small amounts in lymphoid tissues and enriched at barrier surfaces, such as the skin, lung, intestine, and mucosal-associated lymphoid tissues. Characterized by the absence of recombination activating gene (RAG)-dependent rearranged antigen receptors, lack of myeloid cell and dendritic cell phenotypical markers and lymphoid morphology, ILCs undergo neither clonal selection nor expansion when stimulated. These cells reflect the phenotypes and functions of T lymphocytes and NK cells. There are at least three subtypes of ILCs, which are named ILC1, ILC2, and ILC3. They represent the innate counterparts of $CD4^+$ Th1, Th2, and Th17, respectively [67–69]. It has been observed that the numbers of Natural Cytotoxic Receptors positive ILC 3 (NCR^+ ILC3) infiltrating decidual tissues positively correlate with those of infiltrating neutrophils. Neutrophils are present in human decidua during the first trimester of normal pregnancy but not in spontaneous miscarriages decidua. *In vitro* assays show that decidual NCR^+ ILC3 release CXCL8 and GM-CSF and can induce neutrophil migration and survival, respectively. Moreover, NCR^+ ILC3-derived GM-CSF induces expression of HB-EGF and of IL1rα in neutrophils that have anti-inflammatory activity and helps to mediate trophoblast invasion, pointing out a possible role of these cells in the early phases of pregnancy [70].

2.3. Dendritic cells on the neutrophils target

The crosstalk between neutrophils and dendritic cells (DC) can be deleterious for DC functions in *Leishmania major* infection. When neutrophils from ear dermis of C57BL/6-infected mice were cultured with bone marrow-derived dendritic cells, they were engulfed by the DC. These DCs show a significant reduction in expression of MHC class II, CD40, and CD86, as well as

an inhibited capacity to stimulate T $CD8^+$ lymphocytes proliferation and IFN-γ production. These effects were mediated by the tyrosine kinase receptor "Mer" expressed on DCs [71].

The immunosuppressive effect of apoptotic and necrotic neutrophils was also observed in humans' DC. *In vitro*, DC phagocytes apoptotic/necrotic neutrophils and display up-regulation of CD83 and MHC II. However, a decrease of important molecules that stimulate T lymphocytes as CD40, CD80, and CD86 was observed showing that apoptotic/necrotic neutrophils are able to induce a suppressor immune response through DC modulation [72].

DCs infected with *Mycobacterium bovis* (BCG) produced high levels of CXCL1 and CXCL2 that attract neutrophils. In this process, the close contact mediated by CD11b between DCs and neutrophils induces the production of large amounts of the immunosuppressive cytokine IL-10 via MyD88 and Syk pathways in neutrophils. These IL-10^+ neutrophils specifically shut down IL-17A production by Th17 cells. This mechanism could break IL-17A production and avoid exacerbated neutrophil recruitment modulating inflammation [73].

MPO is an enzyme found in neutrophils azurophilic granules and is important for intracellular pathogen killing. It was demonstrated that MPO is deposited by neutrophils in lymph nodes, where it interacts with DCs (by catalytic activity through various ROS and DC Mac-1). In this way, MPO is involved in DC changes during the induction of adaptive immunity. DC display reduced activation, defect in uptake/processing antigens, and inhibited migration to LNs by reduced expression of CCR7, leading to reduced adaptive immune response [74].

Neutrophil elastase is a serine proteinase stored in neutrophils azurophilic granules that can damage endothelial cells and cleave endothelial cell-associated adhesion molecules. As MPO, elastase shows modulating effect on DCs. In presence of elastase, immature DC increases the expression of TGFβ-1 and decreases the IL-6, as well their ability to allostimulate T cells. Elastase-treated dendritic cells not only inhibit the proliferation of allogeneic T cells but also increase TGF-β1 expression inducing the differentiation higher number of $CD4^+$ $Foxp3^+$ Treg cells in MLR cultures. Together, these data suggest mechanisms by which tolerogenic DCs generated by neutrophils elastase exposure contribute to immune regulation [75, 76].

2.4. Neutrophils and B cells crosstalk

During *S. aureus* infection, neutrophils infiltrate the draining lymph nodes, occupying the medulla and interfollicular areas. These cells form transient and long-lived interactions with B lymphocytes and plasma cells inducing a decrease in B cell IgM production in a TGF- β1 dependent manner [77].

Site-specific splenic neutrophils function as professional helper cells for marginal zone B cells, specialized area in T cell-independent responses to circulating antigen, leading to the generation of affinity-matured antibodies. Neutrophils colonize the marginal zone of the spleen after postnatal mucosal colonization by microbiota. In the spleen, these neutrophils interact with local macrophages that produce IL-10, splenic sinusoidal endothelial cells, that in response to the microbial TLR ligands secrete IL-10 and neutrophil-attracting chemokines, and other STAT3-activating stromal factors, which induce modification of neutrophils phenotype, acquiring a "B cell-helper phenotype." These neutrophils B-cell helper (NBH) are

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divided into two subpopulation according to their molecule profile expression. NBH1 cells had intermediate expression of CD15 and CD16 and NBH2 cells had low expression of CD15 and CD16. Despite their morphological and ultrastructural similarity to NBH2 cells, NBH1 cells were more activated than NBH2 cells, as they had higher expression of CD27, CD40L, CD86, CD95, and HLA-II but lower expression of CD24. Relative to genes expression (mRNA abundance) compared with "conventional neutrophils," NBH1 and NBH2 cells had more abundant mRNA immunoregulatory molecules, such as IL-10, IL-10 receptor, arginase-1, RALDH1, iNOS, IDO, SOCS1, progranulin, and SLPI, suggesting a skew toward a regulatory profile. In fact, these neutrophils could suppress CD4 proliferation in a contact-independent way. Thus, NBH cells could function as professional MZ B cell helper cells and may suppress T cells to induce immunoglobulin responses in a T-independent manner. These NBH cells were specially characterized by their higher expression of CD40L and surface BAFF and released high amounts of BAFF, APRIL and IL-21, crucial molecules for the B cell functions [78].

2.5. T cell activities under neutrophils control

Finally, we will describe one of the most important crosstalk of neutrophils, which can have long-term consequences: interactions with T cells. The mechanisms involved in the T cells suppression by neutrophils can be achieved by depletion of essential amino acids from the microenvironment, such as L-arginine, generation of ROS, or through cell-cell contact. Neutrophils produce large amounts of arginase-1 that are stocked in gelatinase granules. Release of arginase-1 requires cellular activation and degranulation of gelatinase and azurophilic granules. Arginase is an enzyme that metabolizes L-arginine into L-ornithine and urea. L-arginine is crucial to T cell proliferation, in the absence of it T lymphocytes are arrested in the G0–G1 phase of the cell cycle. Also, in the absence of L-arginine, expression of TCRζ (CD3 zeta chain) is down-regulated and cofilin dephosphorylation is impaired affecting F-actin remodeling, which is essential for T cell effector function [79–82].

Another important mediator of neutrophil-T cell inter-talk is the ROS, which are membrane-permeable and act on neighboring cells. Peroxide (H_2O_2) can suppress lymphocyte proliferation by decreasing NF-κB activation, down-regulating TCRζ and oxidating cofilin. H_2O_2 has a short half-life and is degraded by many endogenous anti-oxidants. Thus, a close contact between neutrophils and T lymphocyte is required for the suppressor effect. The cell-to-cell contact is mediated by expression of the integrin CD11b/CD18 in neutrophil [4, 83, 84].

The cell-cell contact mediated immunosuppression on T cells can be through a PD-L1-PD1 pathway. PD1 is a negative co-stimulatory receptor expressed primarily on activated T cells. Its main role is to limit the effector functions of T cells during inflammatory response. When engaged by one of its ligands, PD1 inhibits kinases, which reduces cytokine production and suppresses T cell proliferation [85, 86]. In volunteers who participated in a human endotoxemia clinical trial, submitted to LPS inoculums, was observed an accumulation of suppressive neutrophils ($CD16^{hi}$ $CD62L^{lo}$) that exhibit an increased expression of PD-L1 gene and membrane-bound molecule, which was attributed a exposure and stimulation of these cells with IFN-γ and to a lesser extend IFN-α or IFN-β. These IFN-γ-treated neutrophils were able to inhibit proliferation of polyclonal-activated T cells in PD-L1 and cell-cell contact-dependent mechanism [87]. The same phenomenon was observed in murine model of sepsis [88].

In HIV-infected patients, neutrophils play an unappreciated role contributing to the chronic state of immunosuppression leading to opportunistic infection. Low-density neutrophils (which display the same phenotype of G-MDSC) from the peripheral blood of HIV-1 viremic patients express high level of PD-L1. The PD-L1 expression on neutrophils was regulated by the interaction of these cells with inactivated HIV-1 virions, IFN-α, and TLR-7 and TLR-8 ligands. These neutrophils suppress T cell function via PD-L1/PD-1 interaction and production of ROS [89]. The same suppressive function was also observed in *Burkholderia pseudomallei* infected neutrophils, that up-regulated expression of PD-L1 and was able to inhibit $CD4^+$ T cell proliferation and IFN-γ production in response to polyclonal activators, mediated by the PD-L1/PD-1 pathway [90].

Thus, as can be seen in **Figure 2**, the subtypes of RN as well as their diverse interactions with other cell types perform an amplification of the immune response that may help or hinder the host. In Section 3, we describe in details the mechanism of action of an important regulatory neutrophil subtype in GVHD control (**Figure 2**).

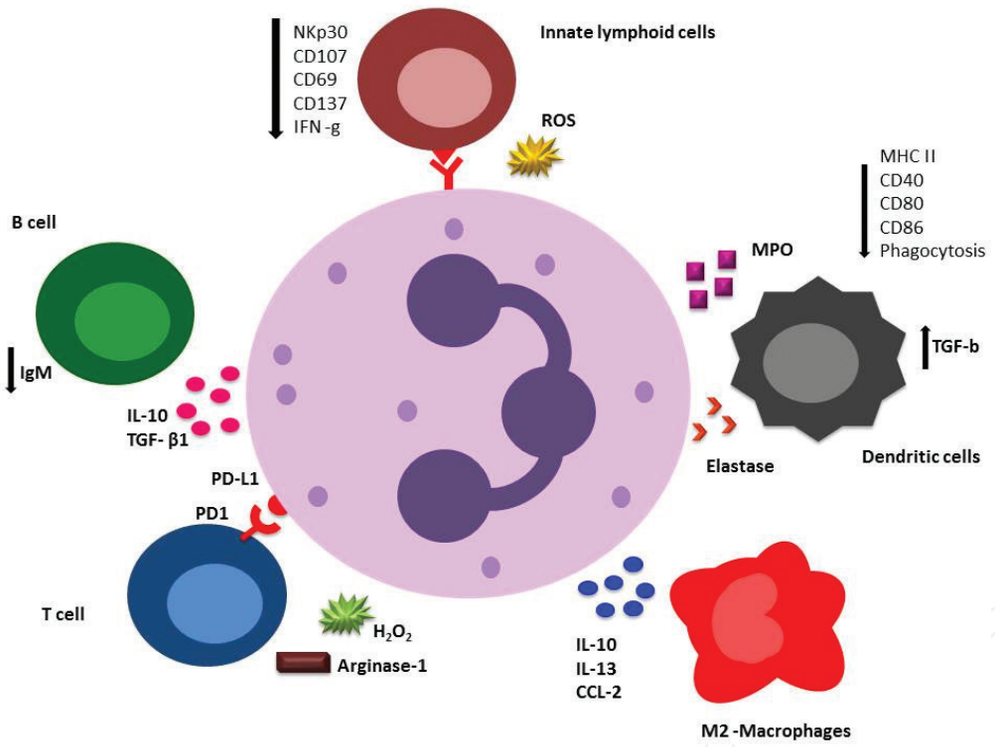

Figure 2. Regulatory neutrophils act on immune cells playing immunosuppressive role. Neutrophil MPO and elastase induce decrease in uptake/antigens processing by DC, inhibit migration to the lymph nodes, and their ability to stimulate T cells are impaired. Also, elastase induces TGF-β1 production by DC. Moreover, the uptake of apoptotic neutrophils down-modulates expression of MHCII, CD40, CD80, and CD86. The secretion cytokines IL-10, IL-13, and chemokine CCL-2 are implicated in the ability of neutrophils to induce changes in macrophages phenotypes to the "M2" anti-inflammatory kind. The immunossuppression of neutrophils over T cells is mediated by the production of ROS, ARG-1, and co-inhibitory molecule PD-L1. Moreover, neutrophils secret IL-10 that exert suppression of T cells functions. TGB-β1 secretion by neutrophils directly influences humoral response by decreasing IgM production. Splenic neutrophils display a particular profile, which produces high amounts of soluble factors essential to the B cells maintenance and also express IL-10. ROS production by neutrophils and cell-cell contact between ILC and neutrophils decreases cytotoxicity, reduces ILC responsiveness to activator receptor, and down-modulates expression of NKp30, CD69, CD137, CD107, and of IFN-γ.

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3. Role of regulatory neutrophils in GVHD protection

GVHD is characterized by a robust adaptive immune response caused by donor T cells (present in the incoming graft) after hematopoietic stem cells transplantation (HSCT), a frequent treatment for hematopoietic disorders, and leukemia. In order to be transplanted, the receptor patient undergoes a conditioning regimen that consists of radio/chemotherapy that eliminates the disease and creates the niche for the new incoming bone marrow cells. However, the conditioning regimen also damages the epithelial cells of the patient, mainly the gastrointestinal mucosa, with barrier breakdown and microbiota extravasation. In this case, when donor cells arrive, they find an inflammatory milieu and the T cells are activated by host antigen-presenting cells (APCs) in an inflammatory context and migrate to organs such as the gut, skin, liver, and lungs. The result is the development of GVHD, which has high morbidity and mortality rates, and is the most important limitation of HSCT [91, 92]. Although it is well known that GVHD is mediated and dependent on T cells, elimination of T cells from the graft does eliminate the GVHD. However, it brings other undesired consequences as the lack of anti-leukemia response and deficient hematopoiesis with bone marrow failure [92].

As mentioned before, LDGs have been found in PBMC of stem cell donors treated with G-CSF. These cells were described to be able to inhibit IFN-γ and IL-4 production by T cells in a H_2O_2-dependent way [36]. In the mouse model for GVHD, the same suppressor phenotype was found, and these LDGs were able to inhibit experimental GVHD [31].

Recently, we extended these results showing, among other things, that $Ly6G^+$ RN mediates disease inhibition. In a mouse model of hematopoietic stem cell transplantation (HSCT), after G-CSF treatment, donor spleen cells are enriched in neutrophils (from ˜2 to ˜20%) and, as related in the literature by others, Treg cells were also increased [93, 94]. However, depleting Treg cells from the graft does not alter the GVHD outcome while depleting neutrophils $Ly6G^+$ causes a huge detriment to clinical scores and survival rates. In terms of GVHD, it is important to point that protection was long-lasting and specific, keeping the immunocompetence to reject the skin grafts from third-party mice and rejecting the leukemic cells, keeping the GVL effect. These results show that neutrophils instruct T cells toward a specific tolerant state [37].

So, looking closer to RN, it is important to note that treatment with G-CSF increased the $Ly6G^+Ly6C^-$ population but not the $Ly6G^-Ly6C^+$ or $Ly6G^+Ly6C^+$ population consistent with the enrichment of neutrophils but not macrophages or MDSC [95, 96]. The RN has a reduction in the expression of some surface molecules, such as MHC-II, CD62L, and co-stimulatory (CD80, CD86, CD40), increased phagocytic capacity and produce large amounts of H_2O_2 molecules. Under stimuli in culture, they produce low IFN-γ, TNF-α, IL-17F, IL-2, IL-12 while increasing IL-22 and the suppressor cytokine IL-10. Also, they have low arginase-1 expression and high NOS, associated with low levels of MPO, which justifies the high amount of H_2O_2. Altogether, these features encompass a different subtype of suppressor neutrophils.

Confirming that IL-10 production is particularly important to GVHD suppression, transference of a G-CSF-induced neutrophil from IL-10-deficient mice in a HSCT context, abolished GVHD protection showing high mortality rates and poor clinical scores. This evidences that $IL\text{-}10^+$ neutrophils are the agents of protection [37].

Nevertheless, the long-lasting and specific protection cannot be explained only by neutrophils suppression as it lasts for several months and the half-life of neutrophils does not exceed 6 days. When analyzing the amount of Treg cells, in spleen and mesenteric lymph nodes (mLN) of the hosts, it was found that 4 days after HSCT, Tregs were increased on mLN but not on spleens. Moreover, 25 days after HSCT, both spleen and MLN show Treg increase, suggesting a systemic increase in Treg. In this way, when Treg cells are depleted early after the transplantation, the GVHD protection is abolished, showing Treg induction within the host right after transplantation. These specific suppressor effects are compatible with the antigen-specific suppression previously observed with Treg cells [97, 98]. The ability of neutrophils to suppress T cell activation is reinforced by the fact that Tregs are less sensitive than conventional T cells to H_2O_2 suppression [99].

Indeed, it's known that G-CSF treatment increases the number of Treg cells in the donor, and that these cells produce IL-10 [93]. Treg cells stimulated with LPS are able to induce IL-10 and TGFβ-producing neutrophils. It means that Treg cells have anti-inflammatory properties that influence other cells, including neutrophils, which upon contact with LPS-stimulated Tregs, may prevent the induction of the Th17 profile [100] (**Figure 3**).

Given these various functions of the $Ly6G^+$ $IL\text{-}10^+$ neutrophils described herein, at least three possibilities, which may act together, can be suggested to explain the specific suppression by RN in GVHD model.

At first, peroxide production can act by inhibiting T cell activation because its production is carried out with L-arginine consumption and because it acts by inhibiting TCRζ chain phosphorylation. Second, the high phagocytic capacity may contribute to the clearance of translocated gut bacteria after the conditioning regimen. The diminished bacterial load and translocation will contribute to diminished activation of allogeneic T cells leading to a mild GVHD. Third, Tregs generated in the donor after G-CSF treatment influence the generation and modulation of spleen neutrophils to an $IL\text{-}10^+$-producing suppressor subtype, which expresses low levels of MHC II and co-stimulation (as can be seen in **Figure 4**). After HSCT, when in contact with T cells, neutrophils with low levels of co-stimulatory molecules associated with high secretion of IL-10 favor the generation of Treg cells within the host that suppresses GVHD and maintains other functions of the immune system while maintaining immunocompetence and the GVL effect (**Figure 4**).

In addition to this, we believe that the high phagocytic capacity found in these neutrophils, coupled with the high production of H_2O_2, is related to the limitation of bacterial extravasation that occurs as a result of the barrier breaking [101]. It was recently described that neutrophils can colonize intestinal tissues 3 days after transplantation, so the $Ly6G^+$ cell, besides polarizing T cells, could maintain the intestinal integrity [102]. In fact, it has already been described that G-CSF decreases the effects of intestinal barrier breakage showing low levels of endotoxin in the blood and lower numbers of translocated bacteria observed in the spleen, liver, and mesenteric lymph nodes compared to those not treated with G-CSF [103]. Bacterial elimination is of utmost importance for the control of GVHD, so much that it has been described that germ-free animals develop an attenuated and very late form of GVHD [104]. Besides this, in our view, in this early phase after HSCT neutrophils polarize T cells in

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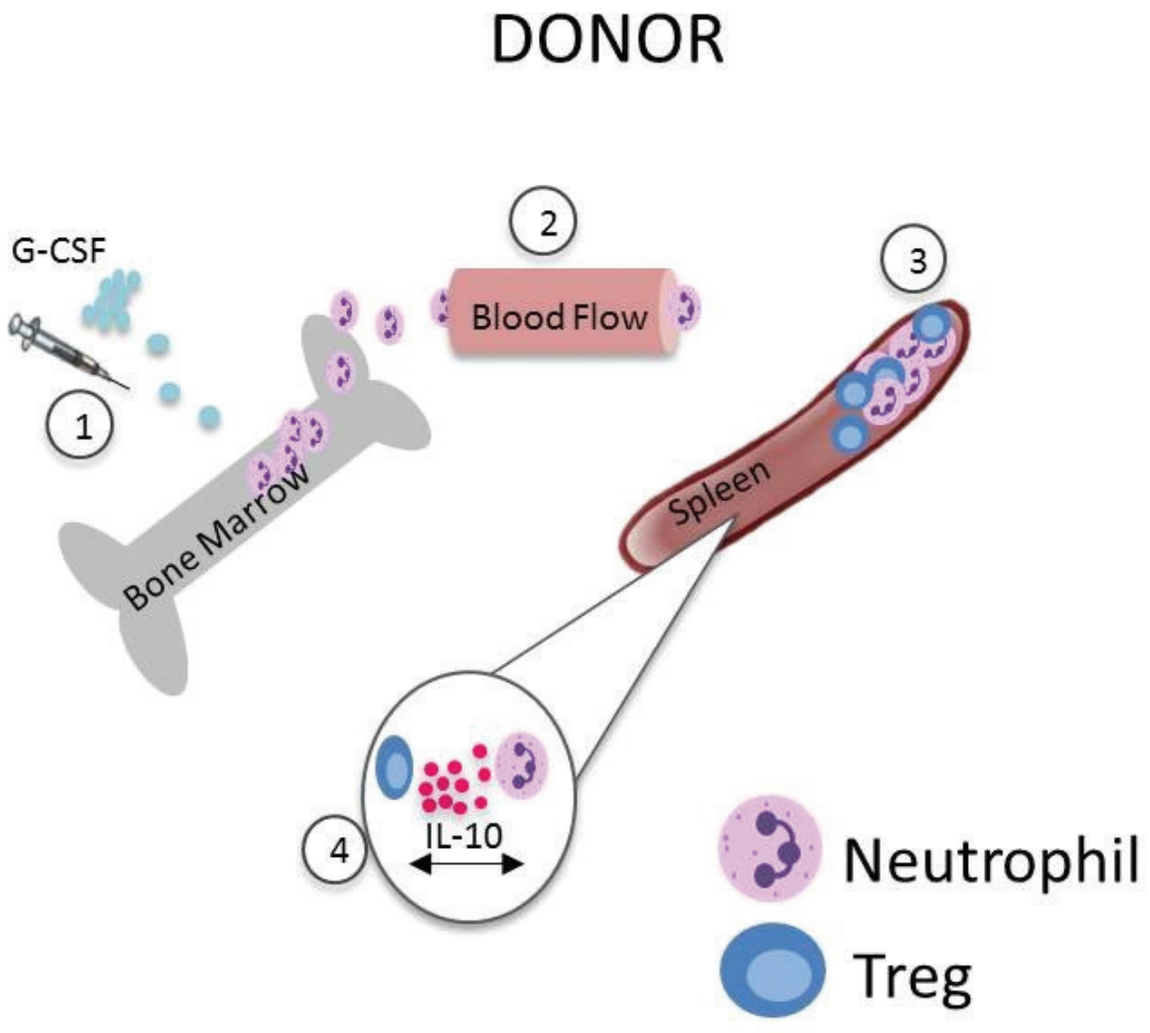

Figure 3. Regulatory neutrophils IL10^{+} neutrophils generation. (1) G-CSF treatment of HSCT donors mobilizes neutrophils from the bone marrow to the periphery. (2) and (3) Neutrophils migrate to spleen where they found Treg cells also G-CSF stimulated. (4) In the spleen, neutrophils and T cells undergo mutual influence and polarize each other to a regulatory phenotype.

the mLN towards a regulatory phenotype, and these regulatory cells spread to GVHD target organs, as we suggest in **Figure 4**.

As we can conclude, the initial idea that neutrophils act only by responding rapidly to inflammatory stimuli and subsequently enter apoptosis causing tissue damage has been modified by the finding that activated neutrophils can perform many other functions. Studies in this regard challenge the classical view of neutrophils as fully differentiated cells and raise the question that these cells may exhibit extraordinary plasticity. In GVHD, prevention strategies that spare the recipient or that decrease tissue damage are of extreme importance [100]. In this case, the generation of a new subtype of neutrophils, capable of inhibiting GVHD maintaining the immunological competence, is paramount for the control of the disease and represents a simple approach to be applied in humans.

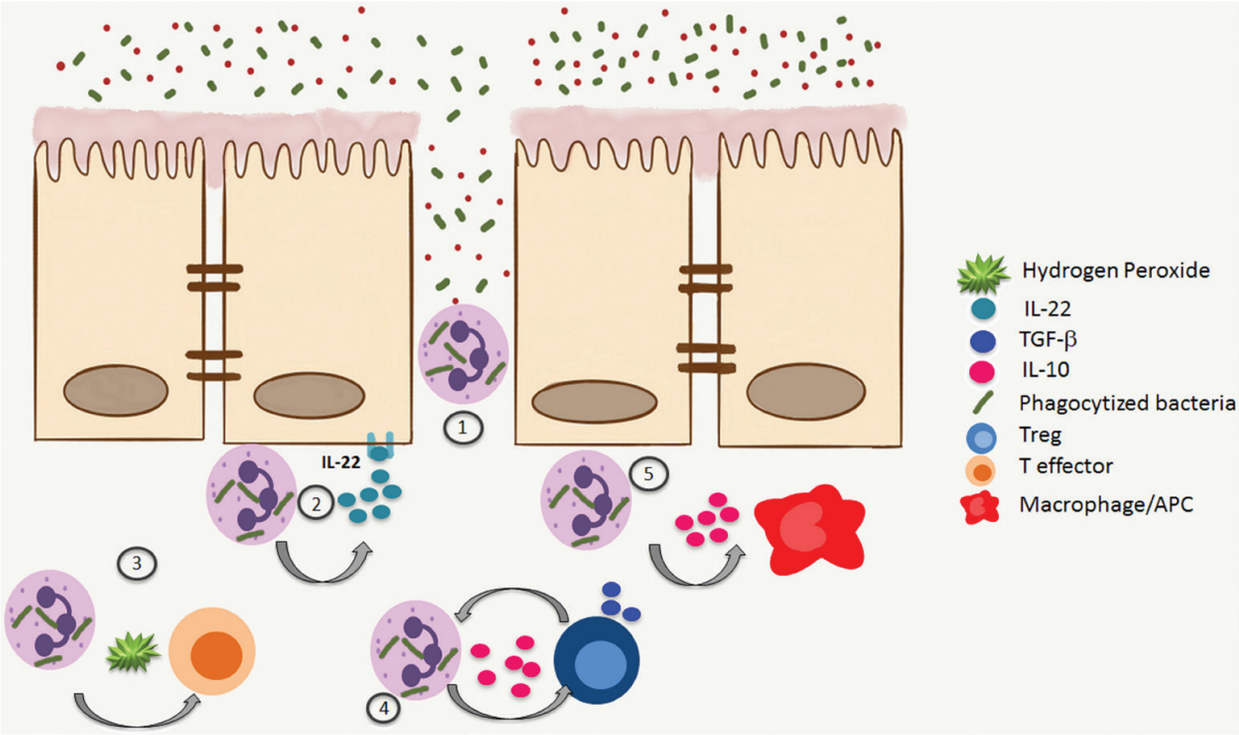

Figure 4. Potential role of regulatory neutrophils in GVHD onset. (1) After HSCT, regulatory neutrophils limit the bacterial translocation from the intestinal barrier breakdown, once they have high phagocytic capacity. (2) Production of the cytokine IL-22 contributes to epithelial healing. (3) High levels of hydrogen peroxide inhibit T effect or functions protecting the host from clonal expansion of alloreactive T cells. (4) The IL10 derived from neutrophils, polarize host Tregs and this last produces IL-10 and TGF-β comprising a mutual cycle. (5) Alternative macrophage (M2) differentiation can be influenced by the regulatory milieu.

4. Final considerations

In mouse model, treatment with G-CSF generates in the HSCT donors an increase of Treg cells, which may be responsible for the generation of suppressor neutrophils. When transferred to the HSCT receptors, these neutrophils are able to suppress the GVHD by reducing the clinical and histopathological signs of the disease. On the other hand, these protected individuals retain the anti-leukemia effect, which is important to prevent relapses, and reject allogeneic skin grafts, showing that the protection of GVHD is due to a specific suppression and not a systemic immunosuppression of the recipient, which claims for a T cell function.

The type of neutrophil generated after G-CSF treatment is obviously different from the classical neutrophil, known as inflammatory. Although activated and mature, they express low levels of MHC-II and co-stimulatory molecules, low levels of MPO, and are degranulated. On the other hand, they are producers of the suppressive cytokine IL-10 and also show an increase in IL-4 and IL-22. Together, these characteristics make this cell a potential suppressor, since several mechanisms able to modulate T cells are present.

We believe that after transfer to the recipient, these neutrophils (which have a longer half-life, as recently reported in the literature) rapidly colonize the sites activated by the conditioning

regimen. It is known that in 3 days there is neutrophil colonization in mLN. Although reports in the literature associate this early neutrophil colonization with the increased damage caused by GVHD, this neutrophil is of a distinct subtype, generated after treatment with G-CSF. Thus, in these activated sites, neutrophils can act to control local inflammation, generating a regulatory environment, favoring the generation of new Treg cells that amplify the specific protection observed in our study.

We conclude that neutrophils function goes beyond the microbicidal function. The classical view is too narrow to explain the many features acquired by these cells. Many different and complex subtypes of regulatory neutrophils have been recently described. Although their characterization is not precise, regulatory neutrophils can be grouped together based on their functional profile. Also, the interaction between these neutrophils and other cell types, such as the ones described here (macrophages, NK, NKT, ILC, DC, B and T cells), potentiate the regulatory response in different conditions. So, the regulatory neutrophils confer an important bridge between innate and adaptive immune system in many different conditions, building a new role for an underestimated cell.

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