Opinion TRENDS in Immunology Vol.28 No.8

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Neutrophil granules: a library of innate immunity proteins

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Gene expression profiling has revealed that circulating neutrophils rest between two major bursts of transcriptional and protein synthetic activities. The first occurs in the bone marrow. This equips the neutrophil with stocks of innate defense armory that are packaged into different granule subsets. The second burst occurs when the neutrophil exits circulation and migrates into tissues to find, capture and phagocytose microorganisms. This burst results in the synthesis and secretion of cytokines and chemokines that support resolution of inflammation and healing of damaged tissue. Gene expression profiling has revealed that neutrophils express a variety of innate immunity proteins, known previously only to be expressed in other cells. Likewise, it has become clear that some proteins previously thought to be specific to the neutrophil are expressed in epithelial cells during inflammation.

Overturning the traditional view of neutrophils

Neutrophil constituents are traditionally considered as potent antimicrobial peptides and proteolytic enzymes, specific to the neutrophil. These proteins assist in the killing and digestion of microorganisms but are potentially harmful to the host if released inappropriately. This perception of granule proteins needs changing to (i) accommodate the importance of granule membrane proteins for the ability of neutrophils to perceive signals from the environment; (ii) appreciate the vast heterogeneity of granules that allow the neutrophil to grade its release of granule proteins in a qualitative manner that minimizes damage while preserving functionality; (iii) integrate the results obtained by microarray techniques and sensitive proteomics that have greatly expanded the number of proteins known to be localized in neutrophil granules, and, conversely, have revealed that proteins, previously believed to be specific to neutrophils, can be expressed in a variety of cell types, primarily in epithelial cells. The latter can largely be epitomized by viewing neutrophils and epithelial cells as cells sharing much the same antimicrobial armory. The important difference is that epithelial cells make these only on request, that is, when an infection or inflammation is established, whereas neutrophils have learned to activate a specific set of transcription factors that ensure production of stocks of antibacterial and proteolytic proteins that are stored in granules ready for use as and when the need arises, thus covering the delay before epithelial cells start their own production.

Secretory vesicles and granule membranes as stores of receptors and other functional membrane-integrated proteins that provide communication with the environment

With the introduction of monoclonal antibodies and flow cytometry, it became clear that circulating neutrophils express only a few receptors on their surface, and therefore do not respond very well to signals from the environment. This was most clearly demonstrated by analyzing the surface expression of the $\alpha_M\beta_2$-integrin [1] but has been expanded further [2].

The discovery of a novel, highly secretory compartment termed the 'secretory vesicle', which is triggered to fuse with the plasma membrane in response to minor elevations of intracellular Ca^{2+}, provided a structural basis for understanding the transition of the neutrophil from a cell with few receptors on its surface and thus mininal responsiveness to soluble inflammatory mediators and extracellular matrix, to a highly responsive cell [3–7]. Secretory vesicles have been shown to be the main source of a variety of receptors (Table 1). L-selectin and the binding partner of the endothelial P-selectin are both expressed on the tips of microvilli of circulating neutrophils [8]. Ligation of these, as occurs when circulating neutrophils are captured by the selectins presented by activated endothelium, will generate the stimulatory signals sufficient to trigger fusion of secretory vesicle membranes [9] with the plasma membrane, and cause immediate upregulation of neutrophil β_2-integrins and chemotactic receptors. This tunes the neutrophil for firm adhesion to activated endothelium and for subsequent chemotactic-directed migration. As secretory vesicles are endocytic in origin, the fusion of secretory vesicle membrane into the plasma membrane at this critical step in inflammation does not result in the release of proteolytic enzymes but only of plasma proteins that form the matrix of secretory vesicles [10].

Human neutrophil granule heterogeneity

Traditionally, neutrophil granules are subdivided into peroxidase-positive granules based on the presence or absence of myeloperoxidase. The peroxidase-positive granules are also called primary or azurophil granules, and peroxidase-negative granules are termed specific or secondary granules [11,12]. However, granules are much more heterogeneous both with regard to structure, that

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Available online 12 July 2007.

www.sciencedirect.com 1471-4906/$ – see front matter doi:10.1016/j.it.2007.06.002

Table 1. Neutrophil granule proteins[a,b]

Azurophil granules	Specific granules	Gelatinase granules	Secretory vesicles
Membrane proteins			
N.a.	CD11b/CD18, CD66, CD67	CD11b/CD18, CD67	CD11b/CD18, CD67
N.a.	Gp91phox/p22phox	Gp91phox/p22phox	Gp91phox/p22phox
N.a.		MMP25	MMP25
N.a.	TNFR[c], uPAR	TNFR[c]	LIR1–4,-6,-7,-9[c]; CD35; CD16; C1q-R; IFN-αR1 and IFN-αR2[c];IFN-γR1 and IFN-γR2[c]; TNFR1 and TNFR2[c]; IL-(1,4,6,10,13,17,18)R[c]; TGF-βR2[c]; CXCR-1[c]; CXCR-2[c]; CXCR-4[c]; CCR-1, -2, -3[c]; Ig(G,A,E)FcR[c]; TLR-1, -2, -4, -6, -8[c]; CD14; MyD88[c]; MD-2[c]; fMLPR; TREM1[c]
CD63, CD68, presenilin	SNAP-23, VAMP-2, Stomatin, PGLYRP[c]	SNAP-23, VAMP-2, Nramp1	SNAP-23, VAMP-2, Nramp1, alkaline phosphatase, DAF, CD10, CD13
Matrix Proteins			
Elastase, cathepsin G, proteinase 3	Collagenase, Gelatinase, uPA, cystatin C[c], cystatin F[c]	Gelatinase, arginase 1	Plasma proteins
Defensins, BPI, MPO, lysozyme	hCAP18, NGAL, B12BP, lysozyme, lactoferrin, haptoglobin, pentraxin 3, prodefensin	Lysozyme	N.a.
Sialidase, Azurocidin, β-glucoronidase, azurocidin	α1-anti-trypsin[c], SLPI, orosomucoid, heparanase, β2-microglobulin, CRISP3	β2-microglobulin, CRISP3	N.a.

[a]Abbreviations: B_{12}BP, vitamin B12 binding protein; CRISP, cysteine-rich secretory protein; DAF, decay-accelerating factor; Gp, granule protein; LIR, immunoglobulin-like receptor; n.a., not applicable; uPA, urokinase plasminogen activator.

[b]Localization of proteins in the matrix and the membrane of neutrophil granules and secretory vesicles. The list is not completely exhaustive but illustrates the major classes of proteins found in the various types of neutrophil granules. Yellow, adhesion molecules; blue, receptors; orange, antibacterial proteins; green, proteases; colorless, other functional classes of proteins.

[c]The localization is inferred from the gene expression profile according to the targeting-by-timing hypothesis but has not been confirmed at the protein level.

is, their content, and with regards to function, that is, their propensity for exocytosis or fusion with the phagocytic vacuole. Granules can be viewed as a continuum where subsets are defined based on a selection of marker proteins. This heterogeneity of granules is best explained by the targeting-by-timing hypothesis, which states that granules are formed during all stages of neutrophil development in the bone marrow, from the early promyelocyte until the segmented neutrophil. When formed, the granules are packed with proteins that are diverted from the constitutive secretory pathway to storage granules, with no sorting amongst individual storage granules [13]. Granule heterogeneity arises because the profile of granule proteins that are synthesized changes as neutrophil precursors mature (Figure 1). What diverts granule proteins from the constitutive secretory pathway is not known, except that the proteoglycan serglycin is involved in the sorting of elastase to azurophil granules, but serglycin does not seem to play a role for sorting other granule proteins [14]. Thus, azurophil granule proteins are synthesized at the promyelocytic stage only. Specific granule proteins are synthesized at the myelocyte stage only. Granules with a high content of gelatinase are formed at the metamyelocyte stage and band cell stage, after which granule formation ceases. Secretory vesicles are then formed by endocytosis. Although this explains the structural heterogeneity of granules, it does not at first sight explain their functional heterogeneity, that is, their propensity for exocytosis. This, however, is also a continuum where the granules formed at the early stages of neutrophil maturation have little capacity for exocytosis, and those formed at the latest stage, namely, gelatinase granules and secretory vesicles, are exocytosed most readily [5,6,15,16]. The propensity for exocytosis is reflected in the density of vesicle-associated membrane protein (VAMP-2), a fusogenic protein associated with the granule membrane. This is highest in secretory vesicles and decreases in gelatinase granules, decreasing further in specific granules [17]. Thus, exocytosis can be viewed as a stochastic event, where an exocytotic trigger, such as a rise in intracellular Ca^{2+} (a so-called Ca^{2+} transient), will cause exocytosis primarily of the granules that have the highest concentration of v-SNARES such as VAMP-2. The concentration of v-SNARES associated with the granule and vesicle membranes could be the result of increased synthesis of VAMP-2 and other members of the soluble *N*-methylmaleimide-sensitive factor attachment protein (SNAP)–SNAP receptor (SNARE) family of proteins as the cells mature. Although this has not been verified, the mRNA profile of VAMP-2 increases dramatically during myelopoiesis as cells mature from promyelocytes to bands and segmented neutrophils [18]. Certainly, this view offers a mechanistic way of understanding how the neutrophil can grade its response according to the situation. It explains how the neutrophil can make firm contact with activated endothelium, release high concentrations of gelatinase [also known as metalloprotease 9 (MMP9)] [6] and expose leukolysin (also known as MMP25) on its surface [19] when passing through the basement membrane, without releasing tissue-destructive serine proteases.

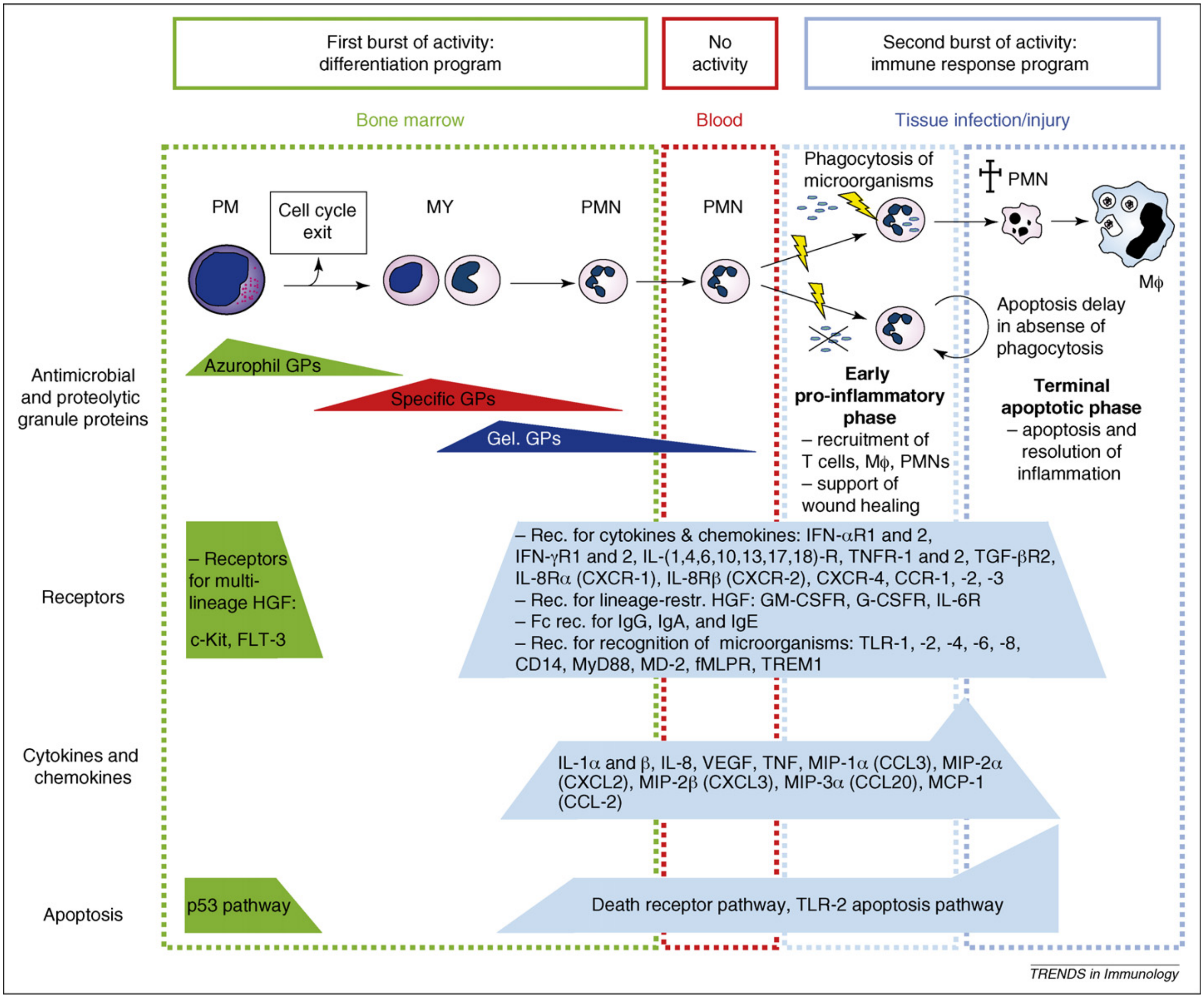

Figure 1. Neutrophil gene expression profile during myelopoieses and diapedesis. Conventionally thought to be relatively quiescent, the complexity of neutrophil transcriptional activity can be revealed by examining these cells at different stages of their life cycle. The figure depicts the sequential mRNA expression profiles for neutrophil proteins in relation to maturation. Green indicates expression in myeloblasts and promyelocytes; red indicates expression in myelocytes and metamyelocytes. Dark blue indicates expression in late metamyelocytes and early band cells. Light blue indicates expression in segmented neutrophils in bone marrow, blood, exudates and during phagocytosis. Abbreviations: FLT, FMS-like tyrosine kinase; G-CSF, granulocyte stimulating factor; GM-CSF, granulocyte-macrophage stimulating factor; HGF, hepatocytes growth factor; IL, interleukin; IFN, interferon; MCP, monocytes chemoattractant protein; MD, myeloid differentiation; Mϕ, macrophage; MIP, macrophage-inflammatory protein; MY, myelocyte; PM, promyelocyte; PMN, polymorphonuclear granulocyte; TGF, transforming growth factor; TLR, Toll-like receptor; TNF, tumour necrosis factor; TREM, triggering receptor expressed on myeloid cells.

Gene expression profiling of neutrophils and their precursors

With the targeting-by-timing hypothesis as theoretical background, we performed microarray profiling of blood neutrophils and neutrophil precursors isolated from normal human bone marrow that were separated into three major stages of maturation: myeloblasts and promyelocytes as one population, myelocytes and metamyelocytes as another, and bands and segmented neutrophils as the third [18]. First, this gives a view of how many genes it takes to make a neutrophil from a myeloblast. Out of the 17 020 genes present on the Affymetrix HG-U133A and HG-U133B chip, an impressive number of 11 300 genes are active at one stage or another during neutrophil maturation, but perhaps more impressive is the finding that the profile of transcribed genes changes dramatically as the cells mature. This allows us to test the validity of the targeting-by-timing hypothesis, assuming that the onset and level of mRNA expression correlates with protein synthesis.

The gene expression profiles accurately predicted the localization of the known granule proteins but also identified mRNA for several proteins not previously known to be expressed in neutrophil precursors. The list of such proteins includes pattern recognition molecules such as Ficolin-1, and pentraxin 3, members of the immunoglobulin-like receptor family (LIR 1–4, -6, -7 and -9), the acute phase proteins haptoglobin and orosomucoid, and finally protease inhibitors like secretory leukocyte protease inhibitor (SLPI), cystatin C and cystatins F (Table 1). Thus, the neutrophil might be able to reduce tissue destruction by secreting inhibitors of its own proteases. The microarray analysis also provides a basis for correcting previously reported localizations of arginase 1 [20,21], α_1-antitrypsin and FcRn [22], and for excluding proteins that have been incorrectly listed as neutrophil granule proteins, such as C6 and C7 [23].

The gene expression profiling demonstrated a shift in apoptosis pathways from pathways responding to DNA damage in promyelocytes toward pathways that are activated by extracellular signals through surface receptors that become expressed in band cells and segmented neutrophils (Figure 1). This means that dividing neutrophil precursors do not respond to extracellular death signals while differentiating toward mature neutrophils, and are thus not wiped out by inflammatory mediators such as death receptor ligands. Instead, they undergo apoptosis if DNA damage occurs during cell division. The late upregulation of death receptors during neutrophil development corresponds to their localization within secretory vesicles. Activated neutrophils thus become responsive to death receptor ligands. Hence, inflammatory mediators not only induce neutrophil migration to sites of infection and killing of microorganisms, but could also initiate an apoptotic program. This is most crucial because apoptosis of neutrophils facilitates resolution of inflammation, and prevents tissue damage caused by necrotic cell lysis and the release of cytotoxic granule proteins. Diapedesis as such seems to induce an antiapoptotic state in neutrophils [24], while phagocytosis seems to promote apoptosis [25,26]

We compared gene expression profiles from mature neutrophils from the bone marrow, circulating neutrophils, and neutrophils that have migrated into tissues and are collected in a skin window chamber, the latter being a model for tissue neutrophils [18,24]. This showed that whereas no major change in gene expression is associated with the release of neutrophils from the bone marrow to the circulation, major changes occur when neutrophils migrate into tissues. In particular, the tissue neutrophil turns on genes to generate a variety of proteins that might play a role in signaling to other effector cells of the immune system (Figure 1). Certainly, it would not make sense for the neutrophil to start generating antimicrobial proteins on arrival at the site of infection. Instead, antibacterial proteins are carried in stock ready for use, whereas arrival at sites of inflammation initiates the secretion of inflammatory chemokines and cytokines to attract activated monocytes, other neutrophils and T cells, and also to support wound healing.

Although these microarray studies have taught us that the neutrophil contains many more proteins than we anticipated, the full functional significance of this still needs to be determined. Analysis of gene expression profiles of epithelial cells engaged in inflammation has revealed that proteins previously thought to be specific to neutrophils are indeed also generated by epithelial cells. These include the cathelicidin human cationic antimicrobial protein of 18 kDa (hCAP18) [27–29], neutrophil gelatinase-associated lipocalin (NGAL) [30–32] and bactericidal/permeability-increasing protein (BPI) [33]. It is also appropriate to refer to defensins as granule proteins that become expressed in epithelial cells during inflammation. Defensins are small antibiotic peptides with six conserved cysteins. α-Defensins are major constituents of azurophil granules of neutrophils. Epithelial cells produce β-defensins during inflammation and wound healing. β-Defensins differ from α-defensins in their size and the pairing of the cysteines, but they evolved from the same ancestral gene (Figure 2).

It is evident that the expression of these innate immunity proteins must be controlled by fundamentally different mechanisms in the different tissues. One mechanism operating in the bone marrow ensures the constitutive expression of granule proteins at a specific stage of maturation of neutrophil precursors, while the mechanisms that control expression in epithelial cells only are activated on demand. The constitutive expression in bone marrow cells is largely determined by the transcription factor CAAT enhancer-binding protein ε (C/EBPε), in concert with purine-rich box 1 (PU.1), both of which are expressed constitutively in a sequential manner, but none of these are active in epithelial cells. Here, expression can be induced directly by NF-κB following stimulation with cytokines, as observed for NGAL [32]. Direct activators of NF-κB, such as IL-1 and TNF-α, do not induce hCAP18 expression in keratinocytes. By contrast, the expression of hCAP18 in keratinocytes is induced by insulin-like growth factor-1 (IGF-1) [31], vitamin D [34–36] or short chain fatty acids [37] in an NF-κB-independent manner. Thus, even though the expression of proteins that localize to specific granule proteins is regulated in a similar manner in the bone marrow, the expression of the same proteins is controlled by distinct mechanisms in epithelial cells.

Role of novel neutrophil proteins

NGAL, a protein belonging to the lipocalin family and also termed lcn2 or siderocalin, has been ascribed a role as a siderophore-binding protein that prevents microorganisms from siderophore-mediated acquisition of iron (siderophores are the strongest iron chelators known. They are produced by bacteria when the availability of Fe^{3+} is limiting bacterial growth. The bacteria take up the iron–siderophore complex through specific receptors). NGAL has been shown to be bacteriostatic for microorganisms that generate catecholate-based siderophores, such as enterochalin generated by most *Escherichia coli* strains. In addition, NGAL binds to the siderophore produced by *Mycobacterium tuberculosis* with very high affinity, but direct inhibition of growth of *M. tuberculosis* has not been examined [38,39]. The role of NGAL in host defense has been confirmed by elegant studies in mice with knockout of the NGAL gene. These mice succomb rapidly after intraperitoneal injection of enterochalin-producing *E. coli*, whereas their wild-type littermates survive [40]. The function of NGAL as a siderophore-binding protein might infer a role for a receptor for NGAL on the host cells to prevent the destruction of NGAL by bacterial proteases and to divert iron away from microorganisms [41]. NGAL has also been ascribed a role as an inducer of apoptosis in myeloid and lymphoid cells [42]. This has not generally been confirmed. Myeloid and lymphoid development appears to be normal in NGAL knockout mice [43], and exposure of normal myeloid precursors to NGAL does not induce apoptosis [44].

LL-37, the 37 amino acid long C-terminal peptide, generated by physiological cleavage of hCAP18, the only

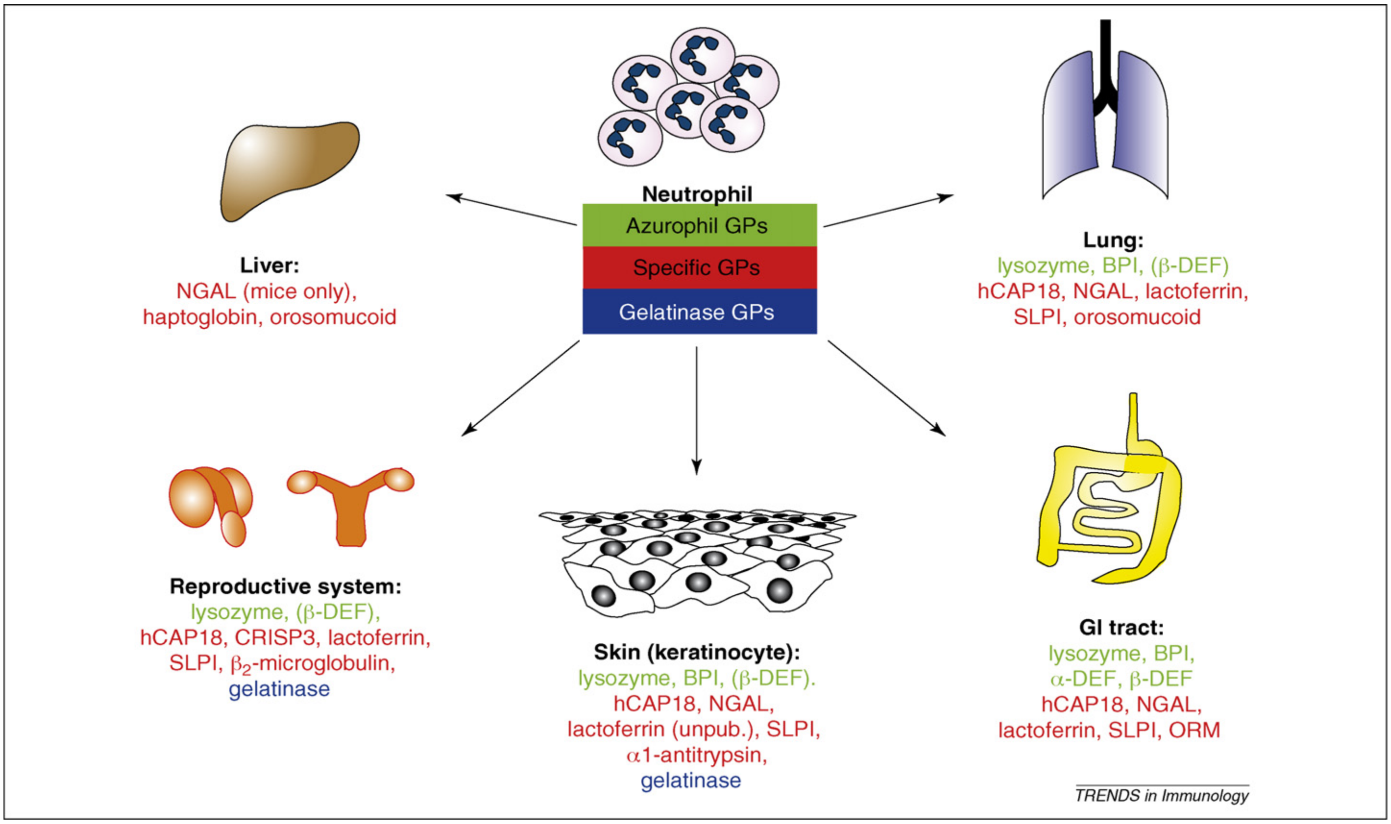

Figure 2. Expression of neutrophil granule proteins in other tissues. The figure highlights proteins that are found in neutrophil granules and can also be expressed in other tissues, either constitutively or in response to stimulation by proinflammatory mediators. Green, proteins found in azurophil granules of neutrophils; red, proteins found in specific granules of neutrophils; blue, proteins found in gelatinase granules of neutrophils. Abbreviations: DEF, defensin; GI, gastrointestinal; ORM, orosomucoid.

human member of the cathelicidin family of antibiotic peptides, seems to have both anti- and pro-apoptotic effects dependent on the cell type. LL-37 is apoptotic to various epithelial cells from the skin and lung [45–47] in addition to smooth muscle cells [48]. However, LL-37 has anti-apoptotic effects on neutrophils mediated by activation of the receptor for f-Met-Leu-Phe and the $P2X_7$ nucleotide receptor [45,49]. In endothelial cells, LL-37 seems to have a direct growth-promoting effect and the ability to induce angiogenesis [50].

Conclusion

These studies have taught us that cells once considered transcriptionally static, such as epithelial cells and neutrophils, are indeed quite dynamic and must be examined in the appropriate context. Studies now indicate that neutrophils play a major role in orchestrating the inflammatory response, in the resolution of inflammation, and in wound healing, in addition to simply killing microorganisms. Further studies will surely reveal hitherto unappreciated complexity in neutrophil function.

Acknowledgements

This work was supported by the Danish Medical Research Council (N.B. and K.T-M.), the Novo Nordic Foundation (K.T-M. and O.E.S.) and the Swedish Research Council (O.E.S.).

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