Supplying the demand for granulocytes: function and gene expression profile of mobilized neutrophils
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Chapter 9

Summary and general discussion
Neutrophils are the most abundant cells of the human immune system, specialized in the destruction of invading microorganisms such as bacteria and fungi. To fulfill its duty successfully, the neutrophil is equipped with unique features, unmatched by other cells in the immune system. Neutrophils are able to migrate to sites of infection at high pace, following the chemotactic signals sent by host tissue or by bacterial products. Furthermore, neutrophils are professional phagocytes, able to ingest and subsequently kill large numbers of bacteria. They are also indispensable in fighting invasive mold infections. Their excellent killing ability results from a special range of antimicrobial ‘weapons’, including a system to produce reactive oxygen species (ROS), proteolytic enzymes and antimicrobial peptides. Additionally, neutrophils are also able to shape immune responses by a specific “cytokine cross-talk” with other cell types of the immune system.

The importance of neutrophils in the defense of the body is underscored by the high infection rate observed in patients with congenital or chemotherapy-acquired neutropenia, or those with inherited neutrophil function defects, such as chronic granulomatous disease (CGD) or leukocyte adhesion deficiency (LAD). Even in the era of modern, highly effective antibiotics and anti-fungal agents, frequent infections in these patients are demonstrated by hospital admissions, organ damage and significant number of deaths. Clinical experience and data from animal studies suggest that control of infections in these patients requires recovery of bone marrow neutrophil production. Therefore, strategies that aim to increase the number of neutrophils in the circulation (especially in case of suspected prolonged neutropenia), such as granulocyte transfusion (GTX), could play an important role in the management of this life-threatening infectious condition.

The first attempts to replace missing granulocytes in neutropenic patients were undertaken already in 1934, when Strumia injected neutrophils intramuscularly into neutropenic patients, in the hope that neutrophil breakdown products would stimulate the remaining endogenous neutrophil functions. Later, granulocytes from patients with chronic myeloid leukemia or healthy donors were used for transfusion. The first source was soon dropped for ethical reasons. Transfusion of granulocytes from healthy donors also failed to prove effective. This was attributed mainly to the fact that healthy donors do not posses sufficient numbers of circulating neutrophils to provide large enough doses for effective granulocyte transfusions (GTX).

A new era in transfusion medicine began with the availability of recombinant hematopoietic growth factors to treat neutropenic patients and to mobilize neutrophils from
the bone marrow in normal donors for GTX therapy. The use of G-CSF alone for donor stimulation permits collection of approximately $40 \times 10^9$ granulocytes, and the combination of G-CSF (600 µg subcutaneously) with dexamethasone (8 mg orally in a single dose) doubles this number.\(^5\)

Although the use of G-CSF and dexamethasone for mobilization of granulocytes provides sufficient numbers for transfusion, the clinical efficacy of granulocyte transfusions in improving the patient conditions and resolving the infections still remains to be proven. As discussed in Chapter 2, granulocyte transfusions from mobilized donors have been used now for several decades, for clearing bacterial and fungal infections unresponsive to the standard antimicrobial therapy. Most studies reviewed in this chapter suggest that GTX are beneficial, especially when adequate numbers of neutrophils are administered. The frequency of the transfusion as well as the duration of infection before the start of GTX could be additional factors determining the efficacy. Unfortunately, the majority of these reports are retrospective studies or case-control studies without proper control groups. All studies mentioned indicate the need for a well-designed, large-scale, randomized trial to finally prove or disprove the efficacy of GTX. On the other hand, it is not easy to enroll patients with life-threatening infections into randomized trials. The main concern is that such an approach would deprive some patients of treatment that is potentially life-saving, in a clinical setting in which treatment failure often results in patient death. Nonetheless, a phase-III randomized, controlled clinical trial of high-dose granulocyte transfusions has been opened in the United States and is currently recruiting participants. This may be an opportunity to finally discard or prove the benefits of GTX.

Neutrophils collected from normal donors stimulated with G-CSF plus dexamethasone have been reported to display normal or near normal functions \textit{in vitro}.

As we show in Chapter 3, GTX neutrophils display an altered phenotype. Decreased levels of CD16 and CD62L were attributed previously to the stimulation with G-CSF, which also causes an increased expression of CD64 and CD177. Because these molecules are important for neutrophil functions, these alterations could possibly affect the behavior of the neutrophils. For example, L-selectin (CD62L) is a key molecule involved in leukocyte interaction with vascular endothelial cells, crucial for the first steps of neutrophil extravasation. This was initially indicated by the inhibitory effect of the anti–L-selectin mAb MEL-14 on neutrophil migration from the blood into sites of acute inflammation in the skin.\(^6\) The involvement of L-selectin in
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Neutrophil migration was further indicated by the finding that significantly fewer neutrophils are recruited into the inflamed peritoneum of L-selectin–deficient (L-selectin−/−) mice than into that from wild-type mice. Moreover, in vitro, cytokine-activated human endothelial cells can bind human neutrophils, and this binding is blocked by an anti–L-selectin mAb. Taking this into account, downregulation of L-selectin in GTX granulocytes might impair their ability to bind to endothelial cells and prevent their extravasation. However, subsequent in vitro experiments showed that in our hands GTX neutrophils bind to and further migrate through a layer of endothelial cells by following a chemotactic gradient, at a pace comparable to that of control cells, regardless of a low L-selectin expression. One of possible explanations of this effect would be that the loss of L-selectin is compensated by the induced or increased expression of other protein(s) involved in this process. Indeed, GTX neutrophils display a highly increased expression of neutrophil-specific antigen (NB1, CD177), one of the ligands for platelet-endothelial cell adhesion molecule-1 (PECAM-1), which is also expressed on activated endothelial cells. Additionally, pretreatment of neutrophils with anti-CD177 blocking antibodies strongly inhibits their transendothelial migration, proving the importance of this interaction in the migration process.

Another important alteration in the phenotype of mobilized cells, in the context of their functional capacities, is the increase in expression of CD64 (FcαRI), the high-affinity Fcα receptor for IgG, whereas CD16 (FcαRIII) is partly lost from the surface. A similar phenotype has been observed in patients with severe bacterial infections. It is possible that neutrophils upon contact with increased plasma levels of G-CSF, which also occurs during infections, switch their Fc-receptor repertoire from low to high affinity, thereby providing potentially better binding and subsequent elimination of IgG-opsonized pathogens. As shown in mice, CD64 deficiency impaired their protection from bacterial infections. On the other hand, genetic deficiency of either CD16 or CD64 did not result in a specific human phenotype, suggesting functional redundancy between the Fcα receptors. Accordingly, G-CSF/dexamethasone-mobilized neutrophils did not show any alterations in the recognition and subsequent killing of opsonized bacteria, yeast or fungi in in vitro assays.

With all the functional properties being mostly unaffected, G-CSF/dexamethasone-mobilized granulocytes are consider safe for therapeutic use against life-threatening infections in immune-compromised patients.

Although some of the major functional characteristics relevant for host defense seem well preserved in mobilized granulocytes, the extent to which other rele-
vant properties of these cells are altered by the *in vivo* pre-activation with G-CSF/dexa was not known. Several previous studies had shown that repeated G-CSF administration, as is practiced for stem cell mobilization, strongly affects the transcription of various genes in the immune cells (mainly PBMC), leading to an increased transcription of genes important for innate immune responses, while simultaneously decreasing the transcription of various genes involved in the adaptive part of the immune response.\textsuperscript{14} An additional study in an animal model showed that single administration of dexamethasone strongly alters the transcription of various genes in bovine neutrophils, with potential consequences to the functional behavior of these cells.\textsuperscript{15} Therefore, in Chapter 4 we investigated to which extent a single administration of G-CSF in combination with dexamethasone alters the neutrophil transcriptome, and how those changes can possibly affect the functional aspects of the neutrophils. Using a microarray technique we identified $\sim$1000 genes that were differentially transcribed in mobilized neutrophils when compared to neutrophils isolated from the same donors just before G-CSF/dexa administration. This indicates that although comparable in basic functional assays, mobilized neutrophils represent a relatively different cell type from those observed in the circulation under steady-state conditions. The specific differences may be rather subtle and difficult to trace, but nonetheless crucial when it comes to clearing the infection. The detailed analysis of the modified mRNA pool, based on the putative function of the proteins, allowed their classification into various gene ontology (GO) groups. Most of the differentially transcribed genes could be placed in six main gene ontology categories, such as: 1) gene transcription and regulation of gene transcription, 2) signal transduction, 3) immune responses, 4) cell survival and apoptosis, 5) cell adhesion and motility, and 6) cell metabolism. Differential transcription of various genes, and subsequently differential expression of proteins, involved in such crucial processes as gene transcription, signal transduction or cell metabolism, implies that mobilized neutrophils can possibly react in a different way when encountering various stimuli, e.g. cytokines, microbial derivatives or invading microbes, when compared to ‘normal’ neutrophils. However, despite extensive knowledge currently available on the function of various genes, it is still difficult to predict whether and what kind of effect a change in the transcription of a certain gene would have on the behavior of this particular cell in a specific physiological situation. Therefore, it would be of a great interest to be able to connect the change in gene transcription to specific ‘alterations’ in neutrophil behavior.

G-CSF, administered either *in vivo* or *in vitro*, is one of the best known pro-survival factors for neutrophils. Moreover, also dexamethasone increases the neutro-
phil life-span. Indeed, as shown further on in Chapter 4, mobilized granulocytes display a prolonged life-span when cultured in vitro, similar to the situation when non-mobilized neutrophils are cultured in vitro in the presence of G-CSF and dexa-methasone.

Previous studies in our lab have indicated that G-CSF primarily inhibits apoptosis by preventing the activation of the executioner of apoptosis, the cysteine protease caspase-3. However, the exact mechanism of this action remains unclear. Therefore, we focused on changes in the transcription of genes that could possess a potential role in the apoptotic process. From the pool of potentially relevant genes we focused in particular on the CAST gene, encoding calpastatin—the endogenous inhibitor of calpains. Calpains are Ca\(^{2+}\)-activated cysteine proteases. It has been previously shown that neutrophils from COPD patients, with a strongly prolonged life-span, possess highly increased calpastatin levels. In addition, calpains have been implied in the regulation (acceleration) of spontaneous neutrophil death.

We showed that stimulation of neutrophils either in vitro or in vivo with G-CSF/dexa increases the calpastatin mRNA level, and that a similar upregulation can be observed on the protein level. Upon neutrophil culture, the cells went into apoptosis, which correlated with the decrease and finally a total loss of calpastatin. This loss was delayed by either G-CSF or proteases inhibitors such as zVAD (caspase inhibitor) or CI3 (calpain inhibitor). This suggests that the degradation of calpastatin during neutrophil culture is probably due to cleavage by either calpains that overcame the inhibitory activity of calpastatin, or caspases, shown previously to be able the cleave calpastatin. Further study on the role of calpains in neutrophil apoptosis and their possible modulation by G-CSF is described in Chapter 5. We discovered that G-CSF delays the increase in intracellular Ca\(^{2+}\) during neutrophil apoptosis and hence the activation of calpains. Active calpains were shown to be responsible for the degradation of XIAP, and therefore for activation of caspase-9 and -3, and acceleration of apoptosis. Thus, G-CSF seems to regulate neutrophil apoptosis by regulation of both the calcium levels within the cells, which prevents calpain activation, and the expression of the calpain inhibitor calpastatin.

Obviously, also other genes implied to be involved in the regulation of programmed cell death, with altered transcription in mobilized granulocytes, could play an important role in the prolonged life-span of mobilized neutrophils.

Neutrophils serve within the body as a first, main line of defense: professional phagocytes that are able to quickly recognize and kill invading pathogens. These cells
further produce various cell signaling molecules such as chemokines and cytokines, which are able to attract and activate other immune cell types.

To properly recognize pathogens neutrophils are equipped with various receptors that allow them to distinguish between “self” and “non-self”, called pattern-recognition receptors (PRRs). The recognition of microbial ligands by various PRRs triggers the underlying signaling cascades leading to the activation of different neutrophil functions and the subsequent elimination of the pathogen. Any alteration in either expression of PRRs or any protein crucial for the signal transduction induced by ligand binding to those receptors could possibly influence neutrophil effector functions and either impair or enhance neutrophil defense mechanisms. Up-to-now, various deficiencies in the expression of PRRs or components of their signaling pathways, and their consequences have been described. For example the lack of Dectin-1 – a C-type lectin receptor crucial for fungal recognition, or CARD9 – an adaptor protein acting downstream of Dectin-1, increases the susceptibility to fungal infections. On the other hand, the lack of MyD88, or IRAK-4, two components of Toll-like receptor (TLR) signaling pathways, results in recurrent bacterial infections, especially in early childhood, underscoring the importance of innate immune responses in this phase of life.

In Chapter 6 we show how the loss of IRAK-4 protein affects neutrophil functions, and subsequently the defense mechanisms against bacterial pathogens. Human neutrophils were shown to express all TLRs except TLR-3 and -7. Their stimulation with appropriate ligands results in the induction of several neutrophil functions, such as priming for NADPH oxidase activity, adhesion, or cytokine production. We showed a complete unresponsiveness of IRAK-4-deficient neutrophils to stimulation with TLR ligands. We also observed that in vitro killing of different pathogens by IRAK-4-deficient neutrophils was not altered, essentially demonstrating that the intracellular killing of bacteria and fungi by neutrophils is independent of recognition of these pathogens by surface-expressed TLRs. Nonetheless, TLR signalling seems to be crucial for the initial recognition of bacteria and for fine-tuning the immune response against them. Its importance is underlined by the rather high mortality range of IRAK-4-deficient patients, despite the use of antibiotics. Since the clinical manifestations of IRAK-4 deficiency become less severe with age, a strict TLR dependence of pathogen recognition by innate immune cells probably occurs primarily in young individuals. Later on in life it can probably be compensated by other recognition systems, such as specific antibody generation.
GTX are given to patients with severe life-threatening infections, either bacterial or fungal. It is crucial then that transfused neutrophils are fully capable of recognizing the pathogens and properly reacting to their presence, thereby substituting missing immune responses of the patient. Chapter 7 continues with the detailed analysis of the gene transcription profile of mobilized granulocytes obtained by the microarray technique, paying special attention to the genes encoding proteins involved in the immune response. GTX neutrophils show remarkably increased transcription of several TLRs. Their stimulation with various TLR ligands showed normal priming of the NADPH oxidase activity, also with suboptimal doses of the stimuli. This suggests that the increased TLR gene transcription does not simply translate into transmission of stronger signals, indicating that the TLR signalling pathway leading to oxidase priming is a subject of tight regulation. Such a strict control may prevent the cells from getting too easily into a hyper-activated state, before reaching the site of infection.

Stimulation of TLRs also leads to the activation of transcription factors such as NF-κB or IRFs, and subsequent transcription of genes encoding inflammatory mediators, e.g. cytokines, chemokines and interferons. Human neutrophils are able to produce only a few of those, including TNF-α, and various chemokines, such as IL-8, MIP-1α and -1β, and RANTES. Production of those mediators is important for further development of inflammatory responses, such as auto- and paracrine activation of neutrophils by IL-8, or attraction of additional types of immune cells by other chemokines. According to the gene transcription profile obtained by microarray analysis, the expressing of major chemokines is strongly downregulated in GTX neutrophils, with IL-8 being the most affected. IL-8 can be produced by granulocytes in in vitro cultures after stimulation of the cells with either TLR ligands, TNF-α or GM-CSF. Stimulation of GTX neutrophils with TLR ligands increases the expression of IL-8 mRNA, with similar kinetics as observed in control cells. However, the absolute amounts of mRNA remain dramatically lower, suggesting an impaired protein production. To our surprise, GTX neutrophils were able to produce massive amounts of IL-8 in response to TLR stimulation, in some cases even exceeding those delivered by control neutrophil. This suggests that TLR signalling not only activates transcription of the IL-8 gene, but also mRNA translation, and that the translation rate was highly increased in GTX neutrophils. However, the possible mechanism of this phenomenon remains to be elucidated.

As shown in Chapter 8, also the levels of mRNA for MIP-1α and -1β are severely reduced. In contrast to IL-8 MIPs cannot be rescued by cell stimulation of GTX neutro-
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Phils. Following mRNA, also the release of MIP-1 protein by GTX neutrophils in response to TLR ligands, was strongly diminished, which is in contrast with the IL-8 data. Whether this could potentially impair the effectiveness of GTX granulocytes in clearing infections remains elusive. Obviously, it would be of particular interest to identify the differences in the signalling pathways involved in the expression of those particular chemokines. This would potentially lead to the explanation of high IL-8 protein production despite low mRNA levels. Moreover, it would be interesting to investigate whether such an increase in the translation rate also applies to other proteins whose expression is regulated by similar pathways, or whether it is strictly IL-8 related.

In sum, granulocytes mobilized for transfusion purposes represent cells that - at first glance - are not different from those observed in the circulation during steady-state conditions. Nonetheless, the current standard mobilization procedure modifies specific functions of the mobilized neutrophils (e.g. life span and cytokine production), resulting in a granulocytic cell pool that theoretically may be more appropriate to fight the infections.

GTX have been shown in multiple recent studies to help controlling severe infections that progress despite the use of appropriate antibiotics. The clinical response rates vary between 40 and 80%. The variation in clinical efficacy may result – among many other reasons – from patient characteristics, clinical indications or inclusion criteria, and local antimicrobial policies. Because GTX are not commonly used in patients, only very few centers are currently able to provide transfusions in a timely fashion which may bias efficacy rates as well. Further studies are needed to clarify the optimal starting time, dosage and frequency of transfusions of granulocytes, as well as optimization of the preparation of granulocyte concentrates, both for direct use and potential storage conditions.

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