Immunopathology of community-acquired pneumonia

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Chapter 7

General discussion

GENERAL DISCUSSION

Lower respiratory tract infections are responsible for more than 2,49 million deaths annually, making this disease the 4th leading cause of death worldwide (1,2). Community-acquired pneumonia (CAP) - incurred in daily life - remains a major health care problem with *Streptococcus (S.) pneumoniae* as the most prevalent causative pathogen. Innate immune cells encompass several mechanisms to respond rapidly upon recognition of pathogens, with production of inflammatory mediators providing the first line of host innate immune defense. The studies described in this thesis, sought to obtain further insight into biomarkers and the regulation of innate host responses during CAP, particularly circulating ferritin levels and the macrophage activation-like syndrome (MALS) as indicators of hyperinflammation (**chapters 2 and 3**), the cytokine production capacity of blood leukocytes upon exposure to bacterial agonists as an indicator of immunosuppression (**chapters 4-6**), and leukocyte DNA methylation as a potential factor regulating the responsiveness of blood monocytes to stimulation (**chapter 6**). For this we used the ELDER-BIOME ("the effect of leukocyte DNA methylation and microbiome diversity on host defense mechanisms during community-acquired pneumonia") data collection and biobank study as backbone (**chapters 3, 4 and 6**), a prospective observational study in patients hospitalized for CAP. Two hospitals in the Netherlands participated in the inclusion of CAP patients admitted to the ward and intensive care unit (ICU), and extensive microbiological, clinical, interventional, outcome and 28-day follow up data was collected. Age- and gender matched volunteers were included at the outdoor patient clinic as control participants. Besides patients enrolled in the ELDER-BIOME study, we investigated critically ill patients admitted to the ICU with sepsis due to CAP, who were included in the MARS ("molecular diagnosis and risk stratification of sepsis") project, a prospective observational study in the mixed ICUs of two tertiary teaching hospitals in the Netherlands (**chapters 2 and 5**). Expanding our fundamental knowledge on the pathophysiology of CAP is needed in order to identify treatable components of the host response, and in the future assist in identifying patients who are more likely to benefit from a certain biological intervention, an approach known as "predictive enrichment" of the treatment population. As such, we need to identify therapeutic targets in CAP based on human translational biology, which can improve risk stratification and patient management.

In **chapters 2 and 3** we evaluated ferritin as a potential biomarker of hyperinflammation in patients with CAP admitted to the ICU or general ward respectively. Ferritin is an acute-phase protein of which the circulating levels are elevated during inflammation and infection (3). Extreme hyperferritinemia is seen in the macrophage activation syndrome (MAS),

a life-threatening condition characterized by systemic hyperinflammation, described in various diseases including hematologic malignancies, Still's disease and systemic lupus erythematosus (4). The Hellenic Sepsis Study Group recently introduced the term MALS to describe a hyperinflammatory state in patients with sepsis defined by a positive hemophagocytosis syndrome score and/or hepatobiliary dysfunction with disseminated intravascular coagulation (5). Selecting patients with characteristics of MALS might enrich the population for patients who are more likely to respond to anti-inflammatory therapy, such as interleukin-1 receptor antagonist (IL-1RA) (6). High ferritin concentrations (> 4420 ng/ml) were proposed as a surrogate biomarker for the presence MALS with high sensitivity (97%) and negative predictive value (98%) (5). More moderate hyperferritinemia (> 500 ng/mL) has been used to identify patients with hyperinflammation after infection with SARS-CoV-2, the cause of coronavirus disease 2019 (COVID-19) (clinicaltrials.gov identifiers NCT04530578, NCT04341675, NCT04443881). In COVID-19 hyperferritinemia is a predictor of a favorable response to treatment with recombinant IL-1RA (7), resembling previous findings in sepsis listed above (6). Knowledge of the incidence and pathophysiological implications of hyperferritinemia in patients with CAP is limited. In **chapter 2** and **chapter 3** we set out to determine the pathophysiological relevance of extreme hyperferritinemia (> 4420 ng/mL; indicating MALS) and more moderate hyperferritinemia (> 250 or 500 ng/mL) in CAP patients admitted to the ICU or the ward respectively. For this we measured a large set of plasma biomarkers providing insight into activation and deregulation of host response mechanisms implicated in the pathogenesis of sepsis. In **chapter 2** we report that 15 of 158 (9.8%) critically ill patients with sepsis due to CAP had an admission plasma ferritin level ≥4420 ng/ml (CAP-MALS). The presence of MALS was associated with an higher disease severity and enhanced lethality in this population. Other studies reported the incidence of MALS in sepsis patients to vary between 3 and 4% (8); however in these patients sepsis was defined according to the 1992 consensus definition (suspected infection plus at least two systemic inflammatory response criteria) (9) and the source of infection causing sepsis was mixed. More similar to our data, the incidence of MALS was 15.6% in patients with infection with organ failure (i.e., fulfilling the current sepsis definition) or septic shock (8). We showed that MALS in CAP ICU patients have a pro-inflammatory phenotype with aberrations in multiple host response pathways, characterized by exaggerated systemic inflammation, cytokine release, endothelial cell injury, reduced endothelial barrier dysfunction and coagulation activation. Hematologic malignancies are a predisposing condition for developing MAS (8,10–12) and five out of 15 CAP-MALS patients suffered from hematological malignancies. After exclusion of these patients differences in mortality between CAP-MALS and CAP-controls were not present anymore, but the exaggerated host response abnormalities in CAP-MALS patients remained,

indicating that MALS occurs more often in CAP patients with hematological malignancies but that the excessive activation of different host response pathways also occurs in the absence of this comorbidity. In chapter 3 we studied CAP patients hospitalized on a general ward and assessed the frequency of hyperferritinemia, defined by two different cut-off values (500 ng/ml, frequently used in SARS-CoV19 studies to identify patients with hyperinflammation; and 250 ng/ml, reference value used in most laboratories) and its association with aberrations in key host response mechanisms. Forty-six patients (26%) had ferritin levels ≥500 ng/ml; 90 patients (52%) had ferritin concentrations ≥250 ng/ml. Both cut-off values were linked with stronger abnormalities in key host response pathways including systemic inflammation, neutrophil activation, cytokine release, endothelial cell activation and dysfunction, and coagulation activation. The results from **chapter 2** and **chapter 3** taken together suggest that hyperferritinemia defined by different cut-off levels identifies CAP patients with wide-ranging aberrations of various key host response mechanisms implicated in the pathogenesis of sepsis. Whilst our data do not indicate a causative role of high ferritin levels in hyperinflammation, they do suggest that the presence of hyperferritinemia can assist in identifying patients who might benefit from interventions targeting distinct pathophysiological mechanisms.

During infection the innate immune response is necessary for efficient clearance of infectious agents; a balanced response entails a brisk pro-inflammatory reaction, characterized amongst other by the recruitment of leukocytes, the release of cytokines, and activation of the complement and coagulation systems, combined with anti-inflammatory responses that seek to limit potential collateral damage inflicted by inflammation and to initiate tissue repair mechanisms (13). Sepsis is associated with an unbalanced immune response consisting of both excessive inflammation and immune suppression (13). Immune suppression is a well-known phenomenon in which cells are reprogrammed to a state of transient refractoriness, including a reduced capacity to produce pro-inflammatory cytokines (e.g., tumor necrosis factor alpha, TNF-α) upon stimulation with various antigens, such as lipopolysaccharide (LPS) (13)(14,15). While in recent years it has become clear that hyperinflammation and immune suppression are concurrently present in patients with sepsis on hospital admission, the nature and timing of these seemingly opposite reactions are still in debate. Moreover, these responses have almost exclusively been studied in critically ill patients on the ICU; knowledge of the existence and extent of immune suppressive responses in CAP and whether these are associated with the presence of sepsis and concurrent pro-inflammatory responses is limited (16,17). The relationship between the degree of immune suppression and systemic inflammation in patients with severe infection is crucial information for (planned) clinical trials testing immune stimulatory agents as a novel therapeutic approach. Responsiveness of whole

blood leukocytes to LPS is often used as a readout for immune suppression and in this thesis we stratified patients with CAP (mostly without sepsis; **chapter 4**) or critical illness (mainly sepsis; **chapter 5**) in groups of increasing immune suppression based on LPS-induced TNF-α production by blood leukocytes. This selection of patients allowed us to compare differences between patients with and without sepsis, and by measuring plasma biomarkers providing insight in activation and/or disturbances in key pro-inflammatory pathways involved in the pathogenesis of severe infection we sought to determine the association between immune suppression and (concurrent) hyperinflammation. We demonstrate that CAP (in the absence of sepsis) as well as critical illness/sepsis are accompanied with a reduced cytokine production capacity of blood leukocytes in response to stimulation with LPS, indicating that immune suppression can be detected at the time of hospital admission in both sepsis and non-sepsis patients. The finding of impaired cytokine production by blood leukocytes in sepsis (chapter 5) is in line with earlier research (13,18–20). Patients with the most impaired capacity of their blood leukocytes to produce TNF-α concurrently showed enhanced inflammatory, endothelial and procoagulant responses, irrespective of the presence of sepsis. The results of **chapter 4** were confirmed and expanded in **chapter 6**, in which we showed that (besides blood leukocytes) also monocytes purified from blood of CAP patients display a reduced capacity to produce TNF-α upon exposure to LPS. Taken together these data suggest that immune suppression in CAP, as measured by ex vivo cytokine production capacity of blood leukocytes or monocytes, can already be detected in CAP patients who are not septic and that immune suppression and hyperinflammation are two co-existing immune response aberrations that already are present in those without sepsis. Beside TNF-α production by blood leukocytes another frequently used readout of immunosuppression is reduced monocyte HLA-DR expression; several studies reported an association of this response with a reduced capability of leukocytes to produce TNF-α after LPS exposure (18,21–25), providing further validity to the use of TNF-α production capacity of blood leukocytes to stratify patients in groups with different severities of immune suppression. The results of **chapters 4** and **5** suggest that if patients are selected for immune stimulatory therapy based on TNF-α production capacity of blood leukocytes, these subjects likely also have the strongest systemic inflammatory and endothelial cell responses. This knowledge is relevant for the development of precision medicine in critical care and selection of patients for treatment with immune stimulatory agents.

Innate immune cells constitute “first responders” to an infection, with high capacities for pathogen recognition, and elimination. Functionality of innate immune cells, as with other cell-types, is tightly intertwined with transcriptional programs that are regulated at multiple

levels, including via epigenetic modulators. Such mechanisms can impart both short and long-term effects on immune cells, including myeloid cells (26–29). The long-term functional consequences of epigenetic shifts in immune cells are exemplified by reports in monocytes of healthy volunteers who were vaccinated with BCG (Bacillus Calmette Guérin). After BCG vaccination, monocytes exhibited heightened cytokine responses after re-stimulation, relative to controls. The increase in cytokine production was associated with modest shifts in epigenetic patterns (30). This phenomenon has been termed trained immunity, wherein cells of the innate immune system are hypothesized to develop a long-term memory via epigenetic alterations to chromatin (31,32). Epigenetics represents a mechanism by which cells regulate gene activity, without changes to the DNA sequence, by chemical modifications to chromatin or DNA, for example DNA methylation. DNA methylation is an essential component of the epigenetic landscape where cytosines are converted to 5-methyl-cytosine by DNA methylases, with the methyl group donated by S-adenosyl methionine (SAM) (33). Whether DNA methylation influences the functional state of circulating monocytes in the context of CAP is unclear. Reports in sepsis patients provided preliminary evidence of shifts to DNA methylation patterns, which may also be correlated with the immune tolerance phenotype of monocytes(34). Evidence on *in vitro* stimulated monocytes or macrophages also points to epigenetic changes, in particular histone acetylation and methylation, which are understood to partially convey the effects of immune tolerance (35,36). In **chapter 6** we sought to identify epigenetic and transcriptomic features of immune tolerance in circulating monocytes obtained from blood of CAP patients on hospitalization (acute stage), after on-month follow-up (recovery stage), as well as age and sex-matched control participants. We approached this study via systems-based multi-omics modelling of cytokine production capacities, transcriptomics and DNA methylation profiling. Circulating monocytes of CAP patients during the acute stage showed diminished production of cytokines after ex vivo stimulation with LPS, indicating that during the acute stage of CAP circulating monocytes were reprogrammed to a state of immune tolerance (14,15,37). These findings are in line with previous studies in sepsis patients in which blood monocytes showed a diminished capacity to activate nuclear factor-κB and production of TNF-α upon stimulation (38–40). Furthermore, we observed that IL-6 production was still impaired in recovery monocytes, unlike TNF-α, IL-1β and IL-10, which were relatively resolved. Circulating monocytes have a lifespan of approximately one day, which cannot explain the ongoing impaired production of IL-6, suggesting that cytokine-specific footprints of immune tolerance in circulating monocytes are more far-reaching than currently appreciated. We speculate that our IL-6 observations may relate to long-term reprogramming of immune cells via alterations to hematopoietic progenitor cells. We also showed that IL-10 production of circulating monocytes contrasted those reported

by others (15,41). These seemingly opposing results can be explained mainly by differences in handling and culturing protocols of monocytes. The vast majority of studies involving monocyte functions have been performed using adherent plates, which is known to lead to differentiation of monocytes to macrophages conveyed by substantial changes in epigenetics and transcriptional activity (42–45). By means of next generation sequencing we revealed that transcriptomic profiles of circulating monocytes captured during acute CAP showed dramatic transcriptional changes of genes involved in various canonical signaling pathways, including up-regulation of cholesterol biosynthesis, IL-10 signaling, complement system, cyclins/cell cycle regulation genes. Down-regulated genes were associated with antigen presentation, IL-4 signaling and immunotherapeutic pathways among others. After one month, circulating monocyte transcriptomes from CAP patients were almost resolved, as compared to control subjects. To further dissect the molecular features underlying immune tolerance in circulatory monocytes, we performed DNA methylation analysis via reduced-representation bisulfite sequencing (RRBS), including validation by methylated DNA immunoprecipitation (MeDIP). Overall, the levels of DNA methylation in monocytes obtained from CAP patients were similar those from control subjects; however, two DNAse-hypersensitive sites (DNAse HSs) on chromosome 22 (hypermethylated) and chromosome 8 (hypomethylated) were partially altered in CAP acute stage versus controls, consistent with the blueprint epigenome signatures of monocytes. DNAse-hypersensitive sites represent regions within the chromatin that are sensitive to cleavage by DNAse-1 enzyme, and are known to harbor transcriptional enhancer or repressor elements. We applied an integrative bioinformatics approach to combine cytokine responses with RNA expression and DNA methylation. In doing so, we unmasked a potentially important mechanism of DNA methylation at specific DNAse HSs and diminished cytokine production via a transcriptomic signature attuned to the cholesterol biosynthesis pathway. Cholesterol partakes in many roles in cellular biology and innate immunity , particularly as an integral part of cell membranes, antibacterial activity, a substrate for production of corticosteroids, mineralcorticoids, sex hormones, vitamine D and biles acids (46,47). Changes in cellular metabolism are known to be important drivers of cell phenotypes. The cellular response to infection involves a shift in energy metabolism from oxidative phosphorylation towards aerobic glycolysis (48–52). Glycolysis leads to production of acetyl coenzyme A, which is an important metabolite for mevalonate, a metabolite from the cholesterol synthesis pathway (53). Several experiments revealed that initiation of the cholesterol synthesis pathway, without the synthesis of the product cholesterol, is essential for training of myeloid cells (54). Cholesterol plasma levels falls early in critically illness septic patients, with studies showing decreased cholesterol levels were associated with poor outcome (55). Our findings may be of interest in developing novel treatment to potentially

reverse the paralyzing effects of immunosuppression, for example via manipulation of the cholesterol synthesis pathway in inflammatory conditions. Notably, the patients studied in **chapters 4** and **6** overlap; as such, the results presented in these chapters demonstrate that the diminished responsiveness of blood leukocytes of CAP patients can also be found when studying purified monocytes.

CONCLUSION

The studies described in this thesis investigated two key aspects of the host response to severe infection: hyperinflammation and immune suppression. We utilized two observational studies in patients with CAP, one conducted as part of this PhD project (ELDER-BIOME; general ward) and one using stored samples and data of a recently completed investigation by our group (MARS; ICU). Thereby we obtained detailed information about the immune aberrations in patients with CAP of various disease severities and were able to show that hyperinflammation and immune suppression likely are connected phenomena, each entailing different cell types and organ systems. More specifically, whilst the proinflammatory responses studied in this thesis encompassed release of cytokines and activation of the vascular endothelium and the coagulation system, the immune suppressive features related a reduced cytokine production capacity of blood leukocytes (and monocytes). Observational studies in patients such as contained within this thesis are needed to unravel the immune response to infection and its underlying mechanisms. These cannot be replaced by in vitro system or animal models since these mimic human disease only to a limited extent. Integration of data from such model systems with measurements done in patients – while a challenge – is the way to move forward in the development of specific therapeutics that target immune dysregulation in CAP and other infections.

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