What does not kill you,

Makes you stronger.

(Metallica)

10 | General discussion

HOST-PATHOGEN INTERACTIONS

This thesis consists of studies on innate immune reponses in (myco) bacterial meningitis and focuses on pathogenesis and outcome of disease. Interactions between host and pathogen determine these phenomena. On the one hand, outcome of bacterial meningitis (BM) depends on the infecting organism and the timing of antimicrobial therapy. On the other hand, aggravating knowledge on host factors has proven to be unequivocally important [1]. In other words, the pathogen determines the host response but not every host responds identical. Differences in host immune responses are largely driven by genetic variation [2,3]. Immune responses are aimed at killing or silencing pathogens, but the armamentarium of the host consists of recognizing receptors, signaling proteins, phagocytosing cells, and ultimately, reactive oxygen radicals and proteolytic enzymes which also frequently result in collateral damage of healthy cells and tissues, thereby influencing outcome of disease [4, 5].

PATHOGENESIS OF (MYCO) BACTERIAL MENINGITIS

Tuberculous meningitis

The pathogenesis of tuberculosis (TB) and the development of tuberculous meningitis (TBM) is a complicated interplay between mycobacteria and the specific host immune response. Granuloma formation is a very characteristic hallmark of mycobacterial infection [6]. TBM results from hematogenous dissemination of *Mycobacterium tuberculosis* (*M. tub*) to the central nervous system (CNS) after primary pulmonary infection. However, TBM develops not directly by hematogenous spread of bacilli to the meninges but by release of bacilli into the cerebrospinal fluid (CSF) from focal granulomatous lesions, called *Rich foci*, which are typically located subpial or subependymal [7]. Recent studies show the dynamic interactions between mycobacteria and granuloma: infected macrophages attract uninfected macrophages and aggregate in an organized way to form granuloma, the hallmark of mycobacterial infection [8].

   Animal models designed to study the pathogenesis and immune response in TBM are reviewed in Chapter 3. Evaluating the available animal models to study TBM, the rabbit model seems to most closely mimic human TBM. The mouse model however, is superior in terms of studying the immunological response. We developed a new mouse model to study the pathogenesis of TBM (Chapter 4). We were able to develop a reproducible *in vivo* model to study the inflammatory response in TBM. However, this model has limitations in terms of translation to human disease: although the route of infection mimics the natural way, mice did not show clinical signs of disease, contrary to human disease, and also the cytokine profile was different from that of human

CHAPTER 10

disease. On the other hand, we did find bacterial growth of TB in the CNS that leads to a chronic inflammatory response in terms of chemokine production. We intend to use this model to further analyze the role played by the innate immunity in TBM, especially the role played by Toll-like receptors (TLRs) and other signaling pathways that orchestrate the immunological response. Other authors have used the mouse model since then for detailed studies of TBM pathogenesis. Be *et al.* focused on the crucial step in the pathogenesis of TBM, which is entering the CNS after primary pulmonary infection upon bacteremia. Using the mouse model after intravenous infection with *M. tub* they were able to identify genetic determinants for CNS invasion by pooled infection with genotypically defined *M. tub* mutants. They were able to identify five bacterial genes required for invasion or survival in the CNS [9]. Lee *et al.* described the specific immune response in the CNS after direct intracerebral infection of mice with *Mycobacterium bovis* BCG strain and found specific T-cells in the brain, similar as in peripheral blood but also a subset of activated T-cells unique to the CNS. These cells originate from microglial and dendritic sources indicating a specific local inflammatory response inside the CNS [10].

The ultimate goal of these kind of experimental studies is to translate the results into disease modifying strategies in humans and apply them to prevention, early recognition, targeted therapy and guided follow up. Against this background we retrospectively studied a large cohort of 554 children with TBM in South Africa (Chapter 2) and found that TBM starts with non-specific symptoms and is often only diagnosed when brain damage has already occurred. Recent poor weight gain, low-grade fever, vomiting, and recent contact with a TB patient are important clues for early diagnosis of TBM and outcome is directly associated with stage of disease. Ethnicity, progressed stage of disease, headache, convulsions, affected motor functions, brainstem dysfunction, and cerebral infarctions were independently associated with poor outcome in multivariate logistic regression analysis.

We are currently studying the inflammatory response in a prospective cohort of children with TBM in South Africa to further unravel the cells, proteins and genes involved in human TBM pathogenesis.

Bacterial meningitis

The sequential steps in the pathogenesis of non-mycobacterial BM from the pathogen's perspective are: (a) nasopharyngeal colonization with bacteria that have the potential to cause BM; (b) epithelial disruption by bacterial components, enabling these bacteria to enter the bloodstream where they replicate and cause bacteremia [11, 12]; (c) pathogen specific passage of the BBB and bacterial multiplication inside the subarachnoidal space; (d) bacterial recognition inside the CNS by microglia and

astrocytes and by non-neuronal structures in direct contact with the cerebrospinal fluid (CSF), such as dendritic cells and macrophages, all expressing pathogen recognition receptors (PRRs) including TLRs and nucleotide-binding oligomerization domain (NOD) proteins. Activation of PRRs triggers an intracellular signaling cascade resulting in (e) transcription of pro-inflammatory cytokines and chemokines inside the CNS. Cytokine induced increased permeability of the BBB and chemokine induced influx of inflammatory cells from the bloodstream into the CNS then results in enhancement of the local inflammatory response inside the brain. The clinical consequence of these events is brain edema, raised intracranial pressure, infarction and neuronal injury. The ability of a host to sense CNS invasion by microbes and to respond appropriately to control the local infection is essential for killing microbes but the inflammatory response also results in the production of several cytotoxic mediators responsible for damage to healthy neuronal cells and thus for adverse disease outcome [13].

GENETIC VARIATION IN IMMUNE RESPONSE GENES

Immune response genes have been found to be polymorphic. This genetic variation allows for a more sophisticated repertoire and enables the host to better withstand microbial challenges. While this is most probable advantageous on a population level and acts as evolutionary drive, there may be less favorable outcomes for individuals in terms of suppressed or aggravated immune responses affecting susceptibility and severity of infectious diseases.

In Chapter 5 we have discussed the stages of BM pathogenesis in detail and have reviewed studies on SNPs in genes involved in pathogen acquisition, epithelial interactions, and mechanisms that predispose for bloodstream infection. Next, we focused on the effect of genetic variation in pathogen recognition and the subsequent inflammatory response, both in the bloodstream and inside the CNS. Although we did not find studies that exclusively focused on BM, we identified several SNPs predisposing to invasive pneumococcal and meningococcal disease.

In order to specifically study the role of genetic variation in immune response genes on susceptibility to pneumococcal and meningococcal meningitis we designed a case-control study with 472 survivors of childhood BM (83 patients with pneumococcal meningitis [PM; 18%] and 389 patients with meningococcal meningitis [MM; 82%]). We found a significant effect of genetic variation in the gene encoding for Toll-like receptor 9 (TLR9) (Chapter 6). Genotype frequencies of two *TLR9* SNPs were compared with healthy ethnically matched adults and *TLR9* +2848-A alleles occured significantly more frequent in controls than in survivors of MM, implying a protective

effect. A functional biological explanation of this phenomenon, which we studied *in silico*, is that meningococci have a strong immune inhibitory potential upon triggering of TLR9, an intracellular endomosal receptor, and that the polymorphism affects this potential in favor of the immune response.

In a second experiment in the same cohort we studied eight SNPs in five immune response genes and compared the genotype frequencies with healthy ethnically matched controls that were genotyped for the same genes. In the single gene analysis we found that *TLR4* +896 and *NOD2* SNP8 were significantly associated with susceptibility to develop MM. In a genetic trait analysis we found that the combinations of *TLR2* with *TLR4* SNPs and *TLR4* with *NOD2* SNPs were strongly associated with susceptibility to MM (Chapter 8).

After genotyping our cohort of BM survivors we also analyzed the effect of the SNPs in immune response genes on the severity of BM. We clustered our patients according to thirteen clinical and laboratory severity parameters described in the literature and compared genotype frequencies in order to relate SNPs to severity parameters. In Chapter 7 we described that two *TLR9* SNPs were associated with protection against bacteremia. Carriers of the mutant alleles of these SNPs also showed enhanced local inflammatory responses inside the CNS reflected by higher leukocytes and lower glucose levels in CSF. In other words, carriage of mutant alleles for *TLR9* protect against bloodstream infection upon nasopharyngeal carriage; in the unanticipated case that meningococci eventually reach the bloodstream and cross the blood-brain barrier, carriers of the mutant alleles have a better immune response inside the CNS aimed at effective removal of the pathogen from the CNS. The immune response attempts to achievie bacterial clearance but neuronal and cochlear damage does also occur, as described in Chapter 9. In the latter study we used a multigene approach to study the relation between genetic variation of immune response genes and severity of BM. The single gene analysis showed that *TLR4* +896 was associated with post-meningitis hearing loss. In a trait analysis, we found that combined carriage of this SNP with *TLR9* -1237 mutant alleles or *TLR2* wild type enhanced this association.

The most relevant SNPs we found in our BM susceptibility and severity studies were in *TLR2*, *TLR4*, *TLR9*, and *NOD2*. Besides the identification of statistically significant differences in genotype frequencies in a case-control comparison, the challenge is to identify the functional relation between genetic variation in individuals and the effective response in case of pathogen acquisition. Animal models are very suitable in this perspective as are *in vitro* studies, and in an era of swiftly developing computational possibilities, *in silico* analyses are also of added value.

TLR2, an extracellular receptor on immune cells, recognizes lipoteichoic acid (LTA), present in the cell wall of pneumococci, and in meningococcal porin [14,15].

TLR2 activation triggers intracellular signaling via myeloid differentiation protein 88 (MyD88), resulting in pro-inflammatory cytokine production. *TLR2* -/- mice, intracerebrally infected with SP, showed higher mortality, aggravated brain bacterial loads, higher tumor necrosis factor alpha (TNF-a) concentrations in brain homogenates, and more damage to the blood-brain barrier (BBB) [16]. Variation in *TLR2* has been reported to alter the susceptibility to various inflammatory and infectious diseases and has been suggested to cause impaired function of the intracellular domain of the TLR2 protein [17]. The impaired intracellular domain fails to interact with MyD88, which subsequently lowers the production of inflammatory cytokines especially IL-2. The polymorphism in *TLR2* +2477 has been reported to increase the risk of Gram-negative sepsis in a Caucasian population and is associated with susceptibility to mycobacterial infection [17,18]. In genetic trait analysis we linked this SNP to susceptibility and severity of MM in our studies.

TLR4 recognizes lipopolysacharide (LPS) in the outer membrane of Gram-negative bacteria. TLR4 triggering activates intracellular signaling via MyD88, resulting in NFkB transcription and subsequent cytokine production. *TLR4* SNPs are present in 10% of Caucasian populations and are reported to have a positive correlation with susceptibility to several infectious diseases, including Gram-negative sepsis. The SNPs disrupt the normal structure of the extracellular region of TLR4 and are therefore hypothesized to decrease responsiveness to LPS through alterations in binding. Furthermore, mutant *TLR4*-transfected cell lines have been shown to elicit a decreased LPS-induced immune response, resulting in lower levels of cytokine production [3]. *TLR4* +896 mutants cause hypo-responsiveness to LPS in mice and humans and are associated with human invasive meningococcal and pneumococcal disease [19-22]. We found that carriage of *TLR4* +896 was associated with susceptibility to MM, even stronger in a trait with *TLR2* +2477 or *NOD2*. Furthermore, we found that carriage of *TLR4* +896 was significantly associated with post-meningitis hearing loss. Traits with *TLR2* +2477 or with *TLR9* -1237 made the associations even stronger.

TLR9 is an intracellular PRR, which recognizes unmethylated Cytosine-phosphate-Guanine (CpG) motifs in bacterial DNA and TLR9 activation triggers the MyD88 dependant pro-inflammatory pathway [23]. Polymorphisms in *TLR9* have been described in association with asthma in a European-American population and with Crohn's disease [24, 25]. Lange *et al.* used an *in vitro* luciferase assay on a human cell-line to show that the CC genotype of the TLR9 -1237 SNP resulted in a higher TLR9 promoter activity [26]. Another study showed *in silico* that carriage of the variant C-allele creates a putative NFkB binding site. This extra binding site was postulated to enhance the transcriptional activation of *TLR9* and potentially affects activation upon triggering by bacterial DNA motifs [27]. We also used this method in our study on

CHAPTER 10

genetically determined MM severity that was described in detail by Macintyre *et al.* as a new technique for *in silico* regulatory SNP detection (is-rSNP) [28].

Clinical application of genetic susceptibility and severity associations we found is the next step. The rapidly evolving field of Public Health Genomics (PHG) translates immunogenetic knowledge into health care policies in order to enhance population health. PGH activities are, amongst others, represented by large EU funded consortia such as PHGEN-II and the international GRaPH-Int consortium. The European Center of PHG (ECPHG) promotes translational research on the integration of genome-based knowledge and technologies into policies and practices, applying methods such as health needs assessment (HNA) and policy impact assement (PIA). Together, these efforts should improve health care on national, European and international levels, establishing a knowledge database for evidence-based policy-making. ECPHG organizes meetings, which are open to the scientific community, policy-makers, relevant stakeholders and the general public.

FUTURE PERSPECTIVES

With the experience from studying genetic variation in BM susceptibility and severity, we plan to apply this to TBM as well. Many investigations have confirmed that genetic factors are involved in developing disease upon infection with TB and these include adoption studies, twin studies, genome-wide linkage, and population-based case–control association studies [29]. Additional evidence confirming that TB susceptibility is genetically determined was the discovery that individuals with mutations in genes of the interleukin (IL)-12/IL-23/interferon-gamma (IFN-γ) axis have an increased susceptibility to even non-pathogenic mycobacteria [30]. An extensive review of TB susceptibility genes summarizes polymorhisms in genes involved in cellular receptors such as TLRs, cytokines and chemokines, vitamin D and its receptor (VDR), intracellular transporters, antigen presentation, opsonization, bacterial killing by nitric oxide (NO), T-cell regulation, and apoptosis. The authors concluded that SNPs in IFNG, NRAMP1, and NOS2A have been validated in several ethnically different cohorts. SNPs with equivocal associations with TB susceptibility that have been described are those in TLR2, TLR9, IL10, CCL2, DC-SIGN, P2RX7, VDR, and SP110 [29]. The recently described TNFRSF1B SNP might also be associated with TB susceptibility but remains to be validated in replication cohorts [31]. Recently, a SNP on chromosome 18 (rs4331426) was strongly associated with susceptibility to TB in a genome wide association study in three cohorts from the Gambia, Ghana, and Malawi. Although it seems a susceptibility locus not associated with major histocompatibility complex genes, which is rare in infectious diseases, the functional consequence has not been
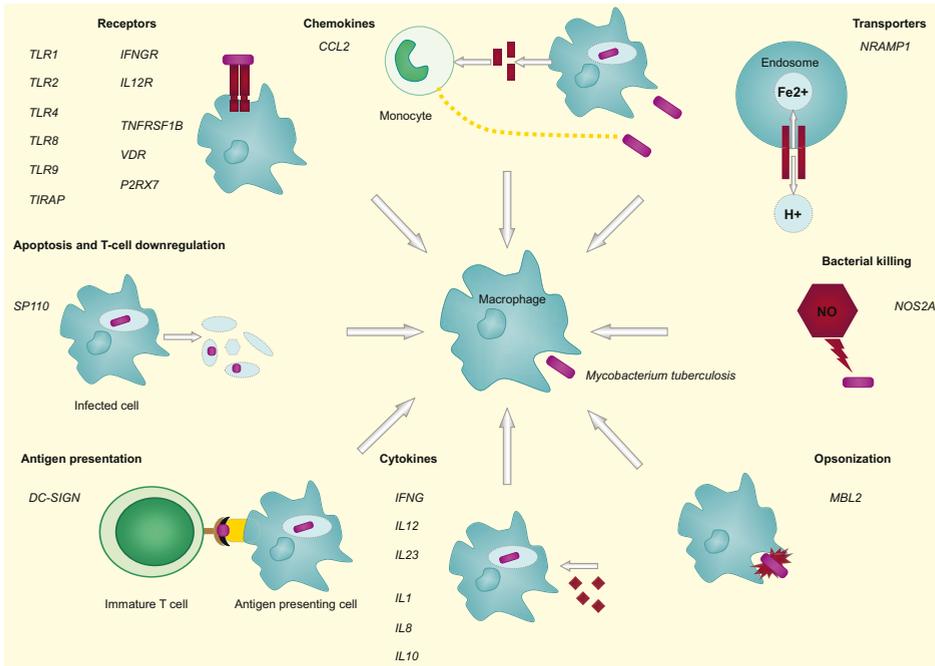
Figure 1. Human genes and immune response pathways potentially involved in susceptibility and severity of TBM

clarified yet [32]. In a hypothesis generating study, genetic variation of IL1B, VDR, and TLR2 was associated with an increased risk of extrapulmonary disease but the authors state that additional studies of the underlying mechanism of these genetic variants are warranted [33]. In a large Vietnamese cohort of TBM patients Thuong *et al.* found that a polymorphism in human *TLR2* is associated with increased susceptibility to TBM [34]. In two ethnically different cohorts in South Africa, Levin *et al.* found that genetic variation of TIRAP, a gene encoding for a signaling receptor downstream of TLR2 and TLR4, both associated with mycobacterial recognition, does not appear to be involved in childhood TB susceptibility, but might play a role in determining occurrence of TBM in specific ethnic populations [35]. All these studies show enough leads to perform a prospective genetic search in a cohort of TBM patients. *Figure 1* illustrates human genes and immune response pathways involved in susceptibility and severity of TB in general, which we intend to study in TBM specifically.

CONCLUDING REMARKS

Studying the pathogenesis of BM consists of several strategies. Disease may be studied in human cases or case series. A more structured approach is studying well

CHAPTER 10

defined cohorts and compare characteristics between groups. This may be done using a retrospective but preferably a prospective approach. Another method is to develop a disease model in which specific elements of disease pathogenesis might be studied in detail. Animal models are widely used but also *in vitro* techniques such as cell cultures, or *in silico* techniques, are valuable tools. These kind of studies are very suitable for testing hypotheses and therapeutic interventions.

SNPs in immune response genes contribute to differences in susceptibility and clinical severity as well as outcome of BM. Innate immune responses plays an important role in host defense to BM and subsequent neuronal and cochlear damage. Genetic markers may be used for identification of high-risk patients by creating prediction rules for post-meningitis hearing loss and other sequelae, and provide more insight in the complex immune response inside the CNS, possibly resulting in new therapeutic interventions.

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CHAPTER 10

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