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## INVITED REVIEW

## Invited Review Themed Issue

# In search of biomarkers for leprosy by unraveling the host immune response to *Mycobacterium leprae*

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**Abstract**

*Mycobacterium leprae*, the causative agent of leprosy, is still actively transmitted in endemic areas reflected by the fairly stable number of new cases detected each year. Recognizing the signs and symptoms of leprosy is challenging, especially at an early stage. Improved diagnostic tools, based on sensitive and specific biomarkers, that facilitate diagnosis of leprosy are therefore urgently needed. In this review, we address the challenges that leprosy biomarker research is facing by reviewing cell types reported to be involved in host immunity to *M leprae*. These cell types can be associated with different possible fates of *M leprae* infection being either protective immunity, or pathogenic immune responses inducing nerve damage. Unraveling these responses will facilitate the search for biomarkers. Implications for further studies to disentangle the complex interplay between host responses that lead to leprosy disease are discussed, providing leads for the identification of new biomarkers to improve leprosy diagnostics.

**KEYWORDS**

bacterial < infectious diseases, biomarkers, immune response, leprosy, *Mycobacterium leprae*

## 1 | INTRODUCTION

Leprosy is one of the oldest recorded human diseases<sup>1</sup> and ranks second as the most severe human mycobacterial disease after tuberculosis (TB). *Mycobacterium leprae*, the causative agent of leprosy, primarily affects the skin and peripheral nerves. Neuropathy caused by *M leprae* infection is known to result in severe, lifelong disabilities. Today, more than 4 million people still live with leprosy-associated handicaps. Besides the physical handicaps, leprosy not only heavily impacts the affected individuals and their family, with respect to their economic, but also impacts their social status due to the leprosy-related social stigma.<sup>2</sup>

In the mid-1980s, the introduction of multidrug therapy (MDT) significantly reduced the prevalence of the disease, from 5.4 million

cases at that time to 202 185 newly reported cases in 2019.<sup>3</sup> Since 2010, however, a fairly stable number of new leprosy cases of around 200 000 are reported each year indicating that only providing MDT upon diagnosis is not sufficient to eliminate leprosy.<sup>4</sup> A possible reason for the ongoing transmission is that the huge drop in prevalence on paper resulted in a reduction in leprosy control activities, especially since the World Health Organization (WHO) declared that leprosy was eliminated as a public health problem in 2000.<sup>5</sup> Reduced knowledge to recognize the signs and symptoms of leprosy frequently leads to delayed diagnosis, resulting in large numbers of undetected cases.<sup>6</sup> This is even more the case in endemic countries, especially in non-endemic countries the possibility of symptoms being signs of leprosy is often overlooked. For example, in the United Kingdom in more than 80% of the cases leprosy

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was not suspected at the first visit to a medical doctor with leprosy symptoms resulting in a median diagnostic delay of 1.8 years.<sup>7</sup> As migration rates are increasing, leprosy cases will again emerge in parts of the world where leprosy is not endemic and hence not expected. This development urges to raise awareness that leprosy is a disease that despite all the efforts is still prevalent today.

One factor that complicates the diagnosis of leprosy is the spectral presentation ranging from disseminated infection to a self-limited form of the disease. This spectral presentation is determined by the host immune response to *M leprae* infection. The complex interplay between innate and adaptive immune responses, influenced by host genetic background and environmental factors, influences the establishment of *M leprae* infection and subsequent progression to disease. Biomarkers based on the host immune response to *M leprae* are thus ideally suited to aid in the diagnosis of leprosy patients, especially since the detection of *M leprae* is difficult in self-limited and preclinical stages of the disease.<sup>8</sup>

## 2 | THE LEPROSY SPECTRUM

The spectral pathology of leprosy can be diagnosed using two co-existing classification systems. The WHO classification is based on the number of lesions and determines the treatment regimen.<sup>9</sup> Individuals with more than five lesions are classified as multibacillary (MB) patients, and individuals with less than five lesions are classified as paucibacillary (PB) patients. A more detailed five-part classification system based on histopathology was developed by Ridley and Jopling.<sup>10</sup> On one end of the spectrum, tuberculoid leprosy (TT) is defined as the presence of a single lesion with well-developed epithelioid granulomas and rare bacilli. At the other end, lepromatous leprosy (LL) is defined as multiple skin lesions in which foamy macrophages and large numbers of bacilli can be identified. The majority of patients, however, present with the borderline phenotypes: borderline tuberculoid (BT), mid-borderline (BB), and borderline lepromatous (BL). In these phenotypes, the bacterial load correlates with the histological features, BT being more closely related to TT patients and BL to LL patients.

The borderline states are immunologically unstable and susceptible to the occurrence of leprosy reactions. These aggressive, inflammatory episodes affect the peripheral nerves and are the main cause of leprosy-associated disabilities.<sup>11,12</sup> Two types of reactions can develop, type 1 reactions (T1R; reversal reaction) or type 2 reactions (T2R; erythema nodosum leprosum ENL). T1R are delayed hypersensitivity reactions associated with the development of *M leprae* antigenic determinants and an increase in cell-mediated immune responses in the lesions, thereby upgrading the response to *M leprae*.<sup>13,14</sup> T2R are antibody or immune complex responses to *M leprae* antigenic determinants associated with disposition of immune complexes in the circulation and in tissues, characterized by infiltration of neutrophils. T2R predominantly occur in BL/LL patients with high bacillary loads and have systemic effects including high fever and edema.<sup>15</sup> The nerve damage characteristic of leprosy

is thus a result of an over-active immune response against *M leprae*, similar to autoimmunity.

As indicated above, the appearance of macrophages in leprosy lesions can be used to define the type of leprosy<sup>10</sup> and these cells are important contributors to the innate immune response to *M leprae*. The different clinical manifestations of leprosy are also determined by the adaptive immune response, the classical paradigm being that the disseminated form of disease is associated with a Th2 immune response and the self-limited form with a Th1 immune response. Advanced knowledge on the immuno-pathological spectrum of leprosy has shown that newly identified T cell subsets (Th9, Th17, Th22, and regulatory T cells) also contribute considerably to the immunopathogenesis of leprosy.<sup>16</sup> Hence, this review will discuss the current knowledge on the different cell types that play a role in *M leprae* infection and host serum proteins such as cytokines/chemokines and growth factors (CCGF) produced by these cells in response to *M leprae*.

## 3 | CIRCULATORY BIOMARKERS: BEYOND THE ANTIBODY RESPONSE

The antibody response to phenolic glycolipid-I (PGL-I), a cell-wall component specific for *M leprae*, is the most widely evaluated biomarker for leprosy. It has been shown extensively that detection of  $\alpha$ -PGL-I antibodies only is not sufficient to identify all leprosy patients, as MB patients can be sensitively detected, whereas PB patients generally lack an antibody response.<sup>17-22</sup> Antibody responses to other *M leprae*-specific antigens such as lipoarabinomannan (LAM),<sup>23</sup> a recombinant fusion protein LID-1,<sup>24-26</sup> major membrane protein-II (MMP-II),<sup>27</sup> and several recombinant *M leprae* proteins<sup>23,28,29</sup> have also been examined. In general, these antibody responses showed a similar pattern as the anti-PGL-I response, with higher levels observed in MB patients. Hence, identification of PB patients based on the antibody response is very insensitive and requires additional host biomarkers.

The interferon-gamma release assay (IGRA) measures the hallmark cytokine of the Th1 response, IFN- $\gamma$ , and is used to identify TB patients. The production of IFN- $\gamma$  in response to the highly specific *M tuberculosis* (*Mtb*) antigens ESAT-6 and CFP-10, as applied in the Quantiferon-TB, enables the discrimination of (latently) *Mtb*-infected individuals from uninfected individuals.<sup>30,31</sup> The advantage of the IGRA test is the lack of cross-reactivity with the TB vaccine bacillus Calmette-Guerin (BCG) and the majority of non-tuberculous mycobacteria (NTM).<sup>32</sup> However, this assay does not discriminate active from latent TB nor indicates progression from infection to disease. Consequently, the IGRA is most useful as a diagnostic tool for TB in non-endemic countries.<sup>33</sup>

The identification of the ESAT-6<sup>34,35</sup> and CFP-10<sup>36,37</sup> homologues in *M leprae* enabled the assessment of the IFN- $\gamma$  response to these antigens, similar to the IGRA for TB. In the traditional paradigm, Th1 (IFN- $\gamma$ ) responses contribute to protective immunity in tuberculoid leprosy patients. Both *M leprae* antigens induced T-cell

dependent IFN- $\gamma$  production in leprosy patients, reflecting the leprosy spectrum with an absence of response at the lepromatous pole. Although responses were not observed in *M leprae*/*M tb*-non-exposed healthy individuals, both antigens were also recognized by TB patients and healthy individuals from areas where leprosy and TB are endemic.<sup>35,36</sup> Since TB is mostly endemic in areas where leprosy is found, IFN- $\gamma$  responses to *M leprae* ESAT-6 and CFP-10 cannot be utilized as a tool to diagnose leprosy.

As an alternative to ESAT-6 and CFP-10, several *M leprae* unique antigens, identified by comparative genomics of the *M leprae* genome to the genomes of other mycobacteria,<sup>38</sup> were tested for their ability to induce T-cell-dependent IFN- $\gamma$  production.<sup>39-45</sup> In one study, five antigens expressed as recombinant proteins induced significant T-cell responses in PB patients and household contacts exposed to *M leprae*, but not in TB patients or community controls.<sup>39</sup> Household contacts are an important study group, they are continuously exposed to *M leprae* but do not show any signs of leprosy, indicating that their immune system is able to effectively clear the bacterium. Although *M leprae* peptides are presented via hypervariable HLA-molecules, they are less likely to induce aspecific responses than recombinantly produced *M leprae* proteins.<sup>46</sup> T-cell responses to 50 synthetic peptides spanning the amino acid sequence of the earlier identified *M leprae* unique proteins were examined, showing a combination of four peptides that was recognized by both PB patients and household contacts of leprosy patients.<sup>39</sup>

To examine the applicability of the *M leprae* unique proteins and peptides in different populations, T-cell responses at five different sites (Brazil, Nepal, Bangladesh, Pakistan, and Ethiopia) were compared,<sup>47</sup> revealing that T cells of endemic controls could also respond to these proteins and to a lesser extent to peptides. Endemic controls live in an area where *M leprae* is prevalent but do not have known contacts with leprosy patient. To identify to what extent the level of leprosy endemicity influenced the cellular immunity to *M leprae* unique antigens, groups of endemic controls with different degrees of exposure to *M leprae* were evaluated in a subsequent study in which whole blood was stimulated with a panel of *M leprae* antigens followed by assessment of multiple cytokines. This analysis identified two *M leprae* unique proteins, ML0840 and ML2478, inducing high IFN- $\gamma$  responses in TT/BT patients and community controls from high-prevalence areas, but none in controls from areas where leprosy is not endemic anymore.<sup>48</sup> This was in contrast to *M leprae* WCS, which mounted comparable IFN- $\gamma$  responses in all control groups. Although useful for detection of *M leprae* exposure, the IFN- $\gamma$  response did not differ between patients and contacts from the same endemic area, rendering this IGRA not applicable in leprosy endemic areas. Of the additionally assessed cytokines, IP-10 showed a similar pattern as IFN- $\gamma$ , indicative of exposure, whereas IL-1 $\beta$ , CCL2, and CCL4 in response to *M leprae* WCS and ML2478 could discriminate patients from endemic controls, potentially identifying pathogenic immune responses to *M leprae*.<sup>48</sup> This study provided the first identification of cellular biomarkers other than IFN- $\gamma$  for detection of leprosy in endemic areas.

Evaluating host CCGF profiles in response to both WCS and ML0840/ML2478 in WBAs was explored further. In multiple independent cohorts from Bangladesh, ApoA1, CCL4, CRP, IL-1Ra, IL-10, IP-10,  $\alpha$ PGL-I IgM, and S100A12 were identified as biomarkers covering the entire leprosy spectrum.<sup>22</sup> For the detection of MB leprosy,  $\alpha$ PGL-I IgM, IP-10, and CRP were most useful, whereas IL-1Ra and CCL4 in these stimulated WBA allowed detection of PB patients. In addition, IL-10, S100A12, and ApoA1 were markers for both disease types, but seem more indicative of *M leprae* infection than disease as the levels of these markers also differentiated household contacts from endemic controls. These findings were corroborated in additional studies, also studying these CCGF in cohorts in China, Brazil, and Ethiopia with a different genetic background.<sup>19,49</sup> However, most PB leprosy patients and household contacts responded similarly to the *M leprae* antigens. Further research on the mechanisms that determine progression of *M leprae* infection to disease may allow the identification of biomarkers differentiating PB from healthy exposed individuals. In this review, we investigate potential candidates for biomarkers, especially for PB leprosy, by discussing the current knowledge on the different cell types involved in *M leprae* infection.

## 4 | FIRST ENCOUNTER: SKIN OR NASAL EPITHELIUM?

Understanding the pathogenesis of leprosy is obstructed by the inability to culture *M leprae* in vitro and the slow doubling time (14 days) in vivo.<sup>38</sup> A proposed route of *M leprae* entry is via nasal epithelial cells or poorly differentiated keratinocytes, fibroblasts, and endothelial cells of the skin through minor injuries. These four cell types are most likely the first line of defense against *M leprae*, and tropism for these cells is potentially mediated via mammalian cell entry protein 1A (Mce1A).<sup>50-52</sup> Mce1A-specific antibodies have been detected in leprosy patients, especially IgG antibodies were significantly higher in both MB and PB patients compared to controls.<sup>53</sup> This is in contrast to the general assumption that antibodies are predominant in the MB side of the leprosy spectrum only, and Mce1A-specific antibodies therefore show potential for the identification of PB patients as well. The four cell types that can internalize *M leprae* via Mce1A will be discussed next.

### 4.1 | Epithelial cells: director of IgA antibody responses?

The presence of *M leprae* DNA in nasal swabs of household contacts of leprosy patients is in line with the hypothesis that the airways are an important *M leprae* entry route. Human airway epithelial cells are able to sustain survival of *M leprae*<sup>54</sup> and contribute to the mucosal immune response. The functional expression of pattern recognition receptors by epithelial cells, including Toll-like receptors (TLRs), enables the activation of innate immune cells such as macrophages and dendritic cells (DCs).<sup>55</sup> Epithelial cells actively regulate the local

immune response, among others by chemokines, and can direct the mucosal DCs to stimulate IgA class switching of B lymphocytes.<sup>56</sup>

The presence of IgA antibodies to whole *M leprae* in saliva identified a possible role for mucosal immunity in protective responses to *M leprae* infection.<sup>57,58</sup> Contacts of untreated leprosy patients, which are continuously exposed to *M leprae*, showed higher salivary IgA levels to *M leprae* than endemic controls, indicative of an activated mucosal immune response.<sup>59</sup> High salivary IgA levels in response to LAM or PGL-I compared to controls were also observed in household contacts,<sup>60,61</sup> especially in frequent contacts of MB patients.<sup>62</sup> The level of exposure to *M leprae* therefore seems to correlate to the salivary IgA levels. The IgA antibodies observed in the mucosal airways might confer protection to *M leprae* infection by efficiently clearing the bacterium at the site of infection. In contrast to the almost mutually exclusive observation of humoral-mediated immunity at one extreme (LL) and systemic, cellular-mediated immunity at the other extreme of the leprosy spectrum (TT), the local mucosal immune response to *M leprae*, potentially regulated by epithelial cells, combines both humoral- and cell-mediated immunity required to induce the IgA class switching. Gaining more insight in the local immune response at the site of *M leprae* entry would provide information on the ability of *M leprae* colonized individuals to clear the bacterium before infection can be established.

## 4.2 | Keratinocyte activity reveals PB leprosy

Another possible route of entry for *M leprae* is through minor injuries in the skin. The most outer layer of the skin, the epidermis, consists of 90% of keratinocytes. Differential expression of genes associated with keratinocyte biology have been identified, especially in BT patients, confirming a role of these cells in leprosy pathology.<sup>63</sup> *M leprae* attachment to keratinocytes is probably mediated via laminin-5, the predominant form of laminin in the dermis.<sup>64</sup> Subsequent phagocytosis of *M leprae* by keratinocytes induced the production of antimicrobial cathelicidin, TNF- $\alpha$ , and IL-1 $\beta$  in vitro, suggesting a key role for keratinocytes in initiating the primary immune response in the skin.<sup>65</sup> Keratinocytes can also function as an antigen-presenting cell (APC) and have been shown to present *M leprae* antigens to CD4 T-cells via MHC class II.<sup>66</sup> In turn, production of IFN- $\gamma$  by CD4 T-cells induced the secretion of IP-10 and IL-7 by keratinocytes.<sup>67,68</sup> In line with the Th1/Th2 paradigm, IFN- $\gamma$ -induced keratinocytes were observed only in lesions of tuberculoid leprosy patients. Keratinocyte-derived biomarkers that reflect this effective immune response at the site of disease are potential candidates to identify PB patients.

## 4.3 | Fibroblasts: healing leads to damage

Fibroblasts play an important role in wound healing and maintain the structural integrity of connective tissue.<sup>69</sup> Excessive accumulation of collagen because of chronic activation of fibroblasts can lead to fibrosis, which is the severe end stage of leprosy neuropathy

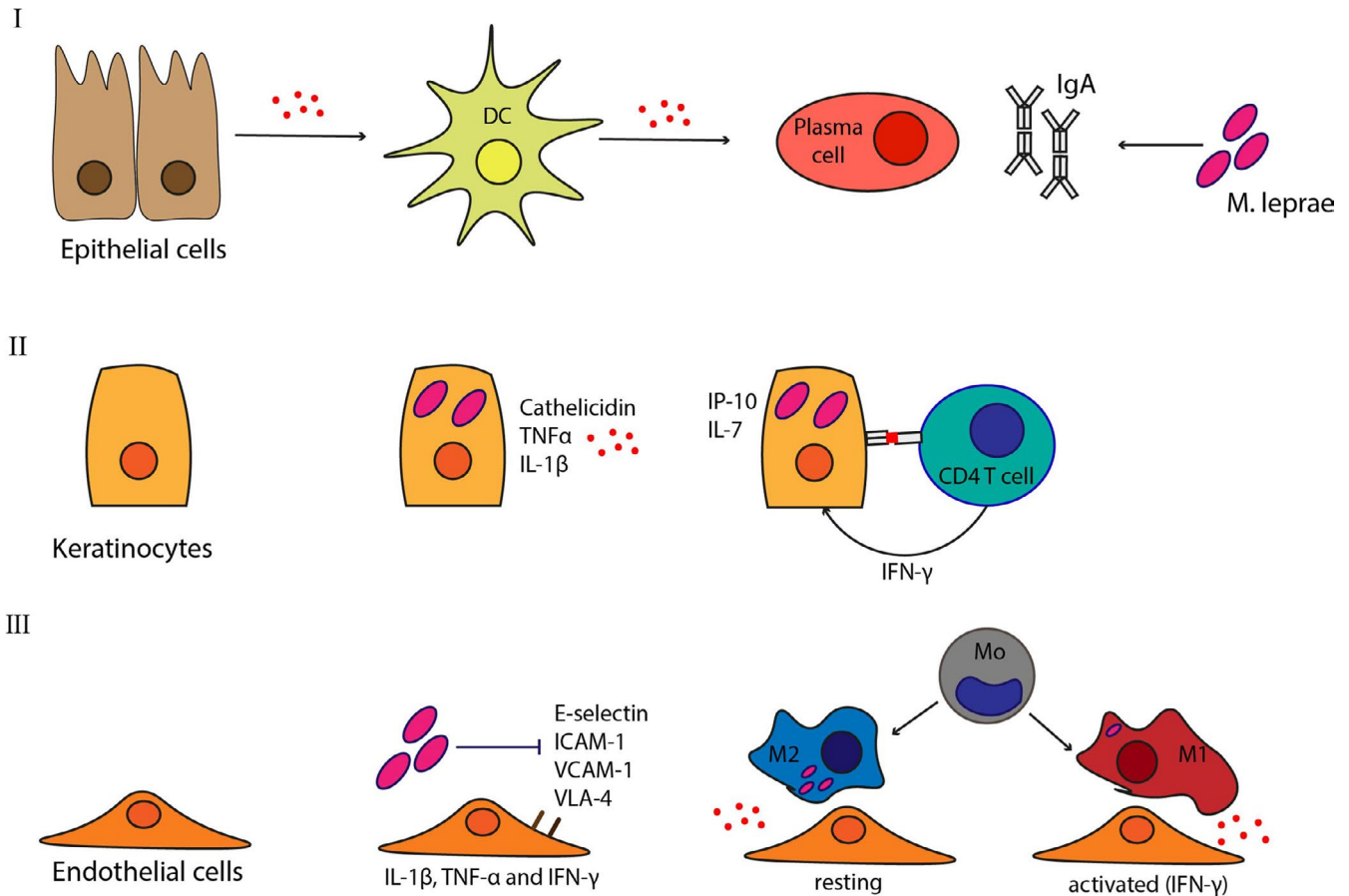
causing irreversible nerve impairments.<sup>70</sup> Fibroblasts express TLR and chemokine receptors and are able to produce cyto- and chemokines such as CCL2, CCL4, and IP-10.<sup>71</sup> First, immune cells are recruited by fibroblasts at the site of infection to clear the bacteria, before organizing tissue repair.<sup>72</sup> *M leprae* is able to invade fibroblasts, and thereby, these cells possibly contribute to the dissemination of infection throughout the body.<sup>73</sup> Whether immune responses are elicited in fibroblasts upon uptake of *M leprae* has not been studied so far.

## 4.4 | Nerve endothelial cells: activation state determines response to *M leprae*

Endothelial cells of the peripheral nerve vasculature are considered an important reservoir of *M leprae* before the invasion of Schwann cells, the primary target of *M leprae* and part of the peripheral nervous system.<sup>74</sup> Once activated, the endothelium allows transmigration of immune cells to the site of infection. Adhesion molecules are induced upon recognition of inflammatory stimuli by MHC class I and pattern recognition receptors expressed on endothelial cells in their steady state. In addition to the upregulation of adhesion molecules, MHC class II is expressed allowing antigen presentation followed by secretion of pro-inflammatory cytokines and chemokines.<sup>75</sup> In skin biopsies, inflammatory mediators IL-1 $\beta$ , TNF- $\alpha$ , and IFN- $\gamma$  and the adhesion molecules E-selectin, ICAM-1, VCAM-1, and VLA-4 were more frequently expressed in tuberculoid lesions compared to lepromatous lesions.<sup>76,77</sup> The reduced endothelial activation observed in lepromatous patients indicates that the endothelium is important in determining the type of immune response at the site of infection.

Resting endothelial cells instruct monocytes to differentiate to anti-inflammatory macrophages (M2), which are able to phagocytose *M leprae* but do not mount an antimicrobial response.<sup>78</sup> In contrast, pro-inflammatory macrophages (M1) have a strong antimicrobial response. Stimulation of endothelial cells with IFN- $\gamma$  instructed monocytes to differentiate to M1 macrophages via jagged1, which was indeed more frequently expressed in lesions of tuberculoid patients. This indicates that an effective response to *M leprae* at the site of infection can be achieved by proper instruction of monocytes by endothelial cells. By phagocytosing *M leprae* at the primary site of infection, macrophages may also play a role in dissemination of the bacteria from the epithelial tissue to the endothelial cells of the nerve vasculature. The role of macrophages in *M leprae* infection will be discussed in more detail later.

Endothelial cells, as well as epithelial cells, fibroblasts, and keratinocytes provide a directive role in the local immune environment at the primary site of infection (Figure 1). Rather than only providing a type of cell for *M leprae* to enter the host, these cells effectively contribute to the initial immune response which might be decisive in the establishment of infection. As the majority of exposed individuals (approximately 90%) is naturally immune to *M leprae* infection,<sup>79</sup> the possible role of the local immune response in this resistance deserves more attention from researchers. The local immune response



**FIGURE 1** Immune responses at the initial site of *M leprae* infection. (I) Epithelial cells can instruct mucosal dendritic cells (DCs) via cytokines and chemokines (red dots) to stimulate IgA class switching of B lymphocytes. *M leprae*-specific IgA in saliva of exposed individuals may then confer protection against infection. (II) Phagocytosis of *M leprae* by keratinocytes induces the production of antimicrobial cathelicidin, TNF- $\alpha$ , and IL-1 $\beta$  in vitro. Keratinocytes can also present *M leprae* antigens to CD4 T-cells via MHC class II. In turn, production of IFN- $\gamma$  by CD4 Th-cells induces the secretion of IP-10 and IL-7 by keratinocytes. These IFN- $\gamma$ -induced keratinocytes are observed only in lesions of tuberculoid leprosy patients. (III) Endothelial cells activated by inflammatory mediators such as IL-1 $\beta$ , TNF- $\alpha$ , and IFN- $\gamma$  induce the upregulation of adhesion molecules (E-selectin, ICAM-1, VCAM-1, and VLA-4) to allow the transmigration of immune cells to the site of infection. In lepromatous patients with high bacillary loads, this upregulation was not observed, indicating that *M leprae* affects this upregulation. Resting endothelial cells instruct monocytes to differentiate to anti-inflammatory M2 macrophages, which do not mount an antimicrobial response, whereas IFN- $\gamma$ -activated endothelial cells instruct monocytes to differentiate to pro-inflammatory M1 macrophages which can restrict bacterial growth. In tuberculoid leprosy patients, activated endothelial cells are observed at the site of infection inducing an effective antimicrobial response by M1 macrophages

of individuals susceptible to *M leprae* infection allows a permissive environment for *M leprae* replication. The ability of these cells to present antigens and direct the polarization of macrophages could be vital in the initial establishment of infection of the host.

## 5 | TARGET OF CHOICE: SCHWANN CELLS

The neurodegenerative effect of *M leprae* infection is unique among bacterial infections. Biomarkers related to the tropism of *M leprae* for Schwann cells therefore represent interesting targets for leprosy diagnosis. Schwann cells maintain the axonal energy metabolism and produce the myelin sheath around neuronal axons, vital for proper nerve function. The PGL-I trisaccharide is highly specific for *M leprae* and binds to laminin-2 on Schwann cells.<sup>80</sup> Besides the

PGL-I-specific antibody response observed in leprosy patients with high bacterial loads, PGL-I is thus also responsible for the unique tropism of *M leprae* for Schwann cells. PGL-I is essential for Schwann cell internalization and induces the expression of CD206 via peroxisome proliferator-activated receptor gamma (PPAR- $\gamma$ ). The cross-talk between CD206 and PPAR $\gamma$  modulates the Schwann cell phenotype to allow intracellular persistence of *M leprae*.<sup>81</sup>

### 5.1 | How to survive?

Several factors have been described that enable *M leprae* to survive in Schwann cells. *M leprae* modulates the metabolism of Schwann cells for its benefit, increasing the uptake of glucose and lipid synthesis and downregulating the mitochondrial activity leading to a

reduction in intracellular oxidative stress.<sup>82</sup> For intracellular survival, it is vital to avoid host cell apoptosis, and the induction of Schwann cell survival factor insulin-like growth factor-I (IGF-I) by *M leprae* in vitro showed the bacterium's ability to survive in infected cells.<sup>83</sup> Another factor upregulated by *M leprae* in infected Schwann cells is 2'-5' oligoadenylate synthetase-like (OASL), which is associated with a type I IFN signature, and impairs bacterial killing activation mechanisms such as autophagy and antimicrobial peptide expression.<sup>84</sup>

Apart from the mechanisms for immune evasion and avoiding apoptosis, nerve-impairing demyelination is also beneficial for *M leprae* survival and is actively enhanced upon infection as lipids provide a source of nutrition and are thereby essential for *M leprae* survival.<sup>85</sup>

## 5.2 | Reprogramming

*Mycobacterium leprae* reprograms the Schwann cells to a non-myelinating phenotype by downregulation of myelin basic protein (MBP), myelin protein zero (MPZ), and Krox-20.<sup>86</sup> In skin biopsies, Schwann cells with less Krox-20 expression were associated with a higher degree of disability of the patient.<sup>87</sup> This indicates that the degree of demyelination impacts the grade of disability. Demyelination can also be mediated by matrix metalloproteinases (MMPs), which degrade protein components.<sup>88</sup> The production of MMP2 and MMP9 in response to *M leprae* in Schwann cells and the expression in nerve lesions,<sup>89</sup> as well as elevated MMP9 levels in serum of patients experiencing reactional episodes causing tissue damage,<sup>90</sup> indicated that these MMPs could serve as additional biomarkers for demyelination. However, these MMPs have also been described as biomarkers for active TB, rendering these biomarkers not specific for nerve demyelination only.<sup>91</sup>

The dedifferentiated Schwann cells are highly susceptible to bacterial invasion.<sup>92</sup> These progenitor/stem-like cells<sup>93</sup> could passively transmit infection to skeletal and smooth muscle cells, as they possess migratory capacity and are reported to spontaneously differentiate under inflammatory conditions to these cell types. The increased secretion of chemoattractants by these cells recruits macrophages, further enabling bacterial expansion and dissemination.<sup>93</sup> The bacterial transfer from reprogrammed Schwann cells to fibroblasts was also more efficient compared to non-reprogrammed cells, which retained the bacteria allowing a niche for replication.<sup>73</sup> The de-differentiation of Schwann cells induced by *M leprae* is therefore an important mechanism that permits *M leprae* to disseminate throughout the body.

## 5.3 | Immune cell-induced nerve damage

In the early phase of reprogramming, expression of innate immune and inflammatory genes was upregulated (including TLRs, IP-10, and CCL2), pointing toward an important role for the innate immune response in the initiation of neuropathogenesis.<sup>94</sup> This finding was corroborated in a zebrafish model for leprosy, showing that

the production of nitric oxide in response to *M leprae* PGL-I by macrophages that patrol the axons damaged the axonal mitochondria, initiating demyelination.<sup>95</sup> The recruitment of macrophages to the site of infection and the subsequent damage to mitochondria are important drivers of the neuropathy caused by *M leprae*. Besides the innate immune response, the adaptive immune response also contributes to the nerve damage; Schwann cells presenting antigens to MHC class II-restricted CD4 cytotoxic T cells were subsequently killed by these activated T cells.<sup>96</sup>

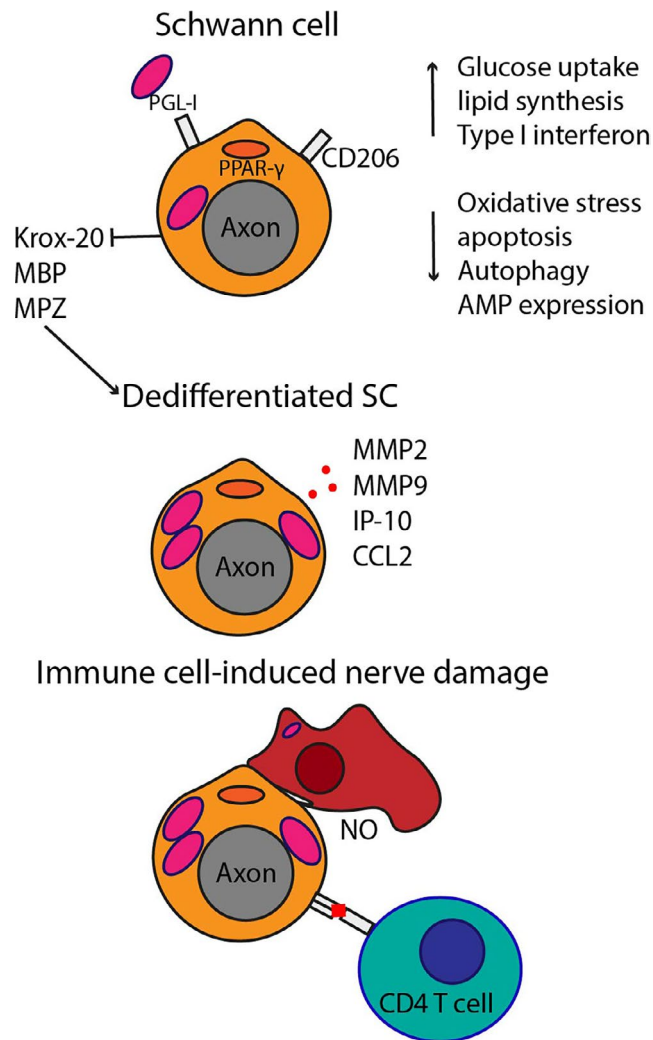
In summary, the production of CCGF in response to *M leprae* by Schwann cells permits on one hand survival of the bacterium and contributes to the nerve damage by recruitment of immune cells that initiate demyelination on the other hand (Figure 2). The demyelination process of Schwann cells induced by *M leprae* is not induced by other bacteria; biomarkers specific for this process are promising candidates to identify (early) nerve damage characteristic for leprosy patients and may reveal *M leprae*'s specific presence.

## 6 | MACROPHAGES: KEY DETERMINANTS OF THE INNATE RESPONSE TO *M leprae*

As mentioned above, macrophages are key players in the pathogenesis caused by this obligate intracellular pathogen. In conjunction with the Th1/Th2 paradigm, macrophages have also been classified as M1 (pro-inflammatory) and M2 (anti-inflammatory). In tuberculoid patients, IL-15 induces antimicrobial activity and the vitamin D-dependent antimicrobial program in M1 macrophages restricting bacterial dissemination.<sup>97</sup> In contrast, in lepromatous patients a scavenger receptor program is induced by IL-10, leading to foam cell formation by increased phagocytosis of mycobacteria and oxidized lipids, resulting in the persistence of *M leprae* in M2 macrophages.<sup>14,98</sup> Both M1 and M2 macrophages thus represent a significant source of CCGF that contribute to the clinical phenotype of leprosy.

### 6.1 | From monocyte to macrophage, impact of *M leprae*

The differentiation of macrophages from monocytes to M1 or M2 is determined by microenvironmental signals in the tissue; IFN- $\gamma$  is required for M1 polarization and IL-4/IL-13 for M2 polarization.<sup>99,100</sup> Exposure of monocytes in vitro to *M leprae* before M1 polarization reduced the levels of IL-6, IL-1 $\beta$ , TNF- $\alpha$ , and IL-12 in these macrophages compared to unexposed monocytes.<sup>101</sup> Interestingly, increased production of IP-10 and CCL2 was observed in M1 macrophages exposed to *M leprae* before polarization. *M leprae* exposed-monocytes polarized to M2 macrophages did not show significant differences in the production of IL-10 and IL-1Ra compared to unexposed monocytes; *M leprae* thus mainly affects the M1 phenotype. As indicated for Schwann cells, PGL-I also showed to play an important role in this immunomodulatory



**FIGURE 2** Response to *M leprae* in infected Schwann cells. *M leprae*-specific PGL-I binds to laminin-2 on Schwann cells and is essential for Schwann cell internalization, inducing the upregulation of CD206 via peroxisome proliferator-activated receptor gamma (PPAR- $\gamma$ ). To survive in Schwann cells, *M leprae* actively up- and downregulates several processes. Up: glucose uptake, lipid synthesis, and type I interferon-related genes. Down: oxidative stress, apoptosis, autophagy, and the expression of antimicrobial peptides (AMPs). Nerve demyelination is also induced by *M leprae* infection by downregulating Krox-20, myelin basic protein (MBP) and myelin protein zero (MPZ) resulting in dedifferentiated Schwann cells. These Schwann cells are highly susceptible to invasion and produce matrix metalloproteinases (MMPs), IP-10, and CCL2. IP-10 and CCL2 are chemoattractants of macrophages; the production of nitric oxide (NO) in response to *M leprae* PGL-I by macrophages and the subsequent mitochondrial damage in axons initiated nerve demyelination. Antigen presentation via MHC class II to CD4 cytotoxic T-cells lead to killing of infected Schwann cells, also contributing to the nerve damage

effect of *M leprae* on monocytes/macrophages in vitro.<sup>102,103</sup> The parallel continues with respect to the production of IGF-I which enabled the persistence of *M leprae* in macrophages as well via the induction of the suppressor of cytokine signaling-3 (SOCS3).<sup>104</sup>

The antimicrobial activity was restored in infected macrophages by blocking of IGF-I, again promoting bacterial killing. These studies showed that *M leprae* actively modulates the macrophage environment, allowing intracellular persistence.

## 6.2 | Vitamin D, not so healthy for *M leprae*

The vitamin D-dependent pathway is essential for the bacterial killing in M1 macrophages. This response is intrinsically activated in *M leprae*-infected macrophages but blocked through the induction of type I IFN by *M leprae*.<sup>105</sup> The production of IFN- $\beta$  and OAS1 in infected monocytes/macrophages in vitro inhibited CYP27B1, which converts inactive prohormone substrate 25-hydroxyvitamin D (25D) to active vitamin D hormone 1 $\alpha$ ,25-dihydroxyvitamin D required for the antimicrobial response. In conjunction with these in vitro studies, in lesions of lepromatous patients high expression of type I interferon genes and a low expression of CYP27B1 were observed.<sup>106</sup> On the other hand, in tuberculoid lesions the type II interferon IFN- $\gamma$  and vitamin D-dependent antimicrobial genes were higher expressed. The ability to trigger this antimicrobial response is inhibited by IL-27, which was also strongly expressed in lepromatous lesions, colocalizing with IL-10 and IFN- $\beta$  in macrophages.<sup>107</sup> These studies showed that the production of different CCGF in response to *M leprae* contributes to the polarization of monocytes to cells that either restrict or allow bacterial growth.

## 6.3 | Avoiding apoptosis and autophagy

To persist in macrophages, the M2 phenotype is favorable for *M leprae*. This is in line with the described association between skewing of *M leprae*-infected macrophages in vitro to the M2 phenotype and the inhibition of autophagy and apoptosis.<sup>108</sup> This inhibition might prevent effective antigen presentation to T cells. Uptake of apoptotic cells by uninfected macrophages enabled antigen presentation, but infected macrophages were not efficiently engulfed.<sup>108</sup> The inhibition of autophagy was created by a negative feedback loop: Initially, live *M leprae* elevated the autophagy level of macrophages, which concomitantly expressed lower levels of pro-inflammatory cytokines. These macrophages preferentially primed anti-inflammatory T cells producing high levels of IL-10, suppressing further induction of autophagy.<sup>109,110</sup> Inhibition of apoptosis and autophagy is vital to enable the dissemination of infection. In lesions of tuberculoid patients, a higher density of apoptotic cells has been observed, which increased the uptake of *M leprae* specifically in M1 macrophages.<sup>111</sup> Removal of these apoptotic cells by phagocytosis in the presence of *M leprae* skewed the M1 macrophages to a M2 phenotype in vitro, reducing the levels of IL-6 and IL-15 and increasing the level of IL-10, TGF- $\beta$ , and arginase. Sustaining a sufficient level of apoptosis could be an important mechanism for the effective clearance of *M leprae* in PB leprosy patients.



## 6.4 | Genetic predisposition influences the macrophage response to *M leprae*

The innate macrophage response influences both the initial killing of *M leprae* and the subsequent adaptive response by T-cells to *M leprae*, resulting in either the MB or PB phenotype. What determines the differential macrophage response to *M leprae* in MB and PB patients is, however, largely unknown. GWAS and segregation studies in different populations have identified several genetic risk variants related to the macrophage response: Variants in IFN- $\gamma$ , the vitamin D receptor (M1), IL-10, and TGF- $\beta$  (M2) were associated with leprosy subtypes.<sup>112</sup> From in vitro studies, we learned that downregulation of the leprosy-susceptibility gene Parkin in macrophages decreased the level of IL-6 and CCL2 in response to mycobacteria.<sup>113</sup> Mutations in the well-studied leucine-rich repeat kinase 2 (LRRK2) also increased the susceptibility to mycobacterial infection; the knockout of LRRK2 increased the basal levels of type I interferon-related genes detrimental for effective bacterial killing.<sup>114</sup> These examples of risk variants indicate that the host genetic background influences the macrophage response to *M leprae*, predisposing individuals to less effective antimicrobial responses. The induced CCGF response therefore provides information on the effectivity of the macrophage to contain the infection, which might be vital to progress from *M leprae* infection to disease (Figure 3).

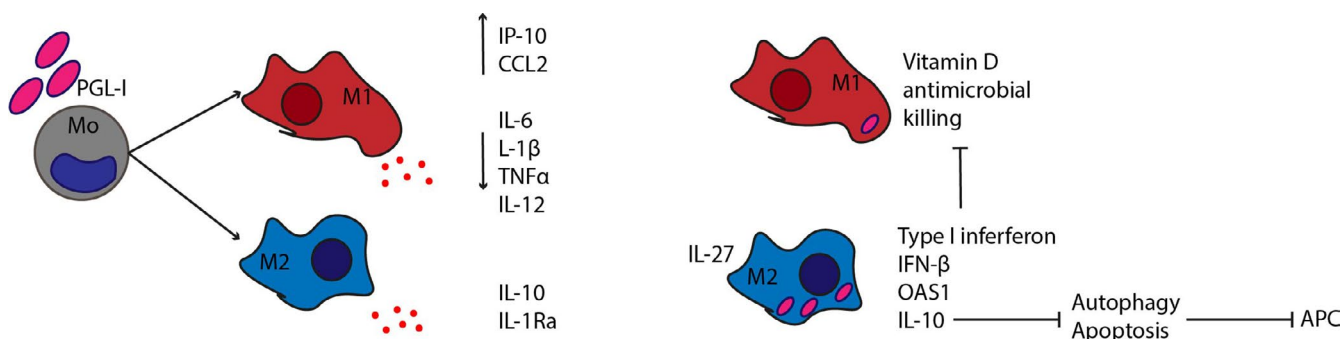
## 7 | DENDRITIC CELLS: PRESENTING *M leprae* TO T-CELLS

Besides macrophages, monocytes can also differentiate to dendritic cells (DCs) which represent the link between innate and adaptive immunity. For the in vitro production of DCs, these antigen-presenting cells require the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-4. In vitro DCs exposed to *M leprae* did not induce DC maturation or activation, which is required for

efficient antigen presentation, resulting in reduced T cell priming.<sup>115</sup> DCs can express the PGL-I antigen, masking this antigen enhanced the DC-mediated T cell response showing that PGL-I presentation dampens the T-cell response.<sup>116</sup> The expression of MHC class I and II antigens on DCs was downregulated in a bacterial dose-dependent manner, suggesting that in the early stages of infection, when bacterial loads are still low, the T cell response is already not sufficiently activated. Stimulation of DCs with purified MMP-II of *M leprae* could increase the expression of MHC class I and II, CD86 and CD83 molecules via TLR2 signaling, which is indicative of DC activation.<sup>117</sup> These MMP-II-pulsed DCs activated both CD4 and CD8 T-cells, inducing the production of IFN- $\gamma$  in these T cells.<sup>118</sup> It is well described that via the production of IL-12 by DCs, T cells start to produce IFN- $\gamma$  indicative of an effective Th1 response. Although in vitro differentiation of monocytes to DCs was similar for lepromatous and tuberculoid patients, the production of IL-12 in response to *M leprae* was weak in lepromatous patients, in conjunction with the lack of cellular-mediated immunity at this site of the leprosy spectrum.<sup>119</sup>

Recently, it has been shown that monocytes can also differentiate to DCs in an IL-32-dependent manner; these DCs are more efficient in antigen presentation to MHC class I-restricted CD8 T-cells compared to the GM-CSF-induced DCs.<sup>120</sup> The NOD2 receptor on monocytes recognizes the muramyl dipeptides (MDPs) from *M leprae*, inducing the production of IL-32 and the subsequent differentiation to DCs.<sup>121</sup> In monocytes of lepromatous patients, high IL-10 levels blocked the differentiation to DCs via NOD2/IL-32 and the in vitro addition of recombinant IL-32 restored the DC differentiation in these patients.<sup>120</sup> IL-32-induced DCs thus seem to play an important role in the host defense to *M leprae*.

A special type of DCs is Langerhans cells that reside in the skin or mucosa and uniquely express langerin (CD207). In lesions of tuberculoid patients, higher levels of IFN- $\gamma$  co-localized with Langerhans cells compared to lepromatous lesions.<sup>122</sup> IFN- $\gamma$  treatment of *M leprae*-infected Langerhans cells induced autophagy and increased the production of cathelicidin, which are both required to



**FIGURE 3** Influence of *M leprae* on macrophage polarization. Monocytes exposed to *M leprae*/PGL-I before the differentiation to macrophages showed an increased production of IP-10 and CCL2 in M1 macrophages and a decreased production of IL-6, IL-1 $\beta$ , TNF- $\alpha$ , and IL12, whereas the IL-10 and IL-1Ra levels in M2 macrophages were not altered by pre-exposure of monocytes to *M leprae*. The vitamin D-dependent pathway which is essential for bacterial killing in M1 macrophages is inhibited by type I interferon genes, including IFN- $\beta$  and OAS1, and IL-10, which are upregulated via IL-27. These genes are all strongly expressed in lepromatous leprosy lesions, coinciding with the M2 phenotype of macrophages. High levels of IL-10 suppress apoptosis and autophagy, resulting in less availability of antigens for antigen presentation to T-cells (APC)

activate the antimicrobial activity in these cells. The increased autophagy in Langerhans cells enabled the degradation of *M leprae*, releasing antigens for the presentation to resident T cells. As described for macrophages, a sufficient level of autophagy is an important mechanism to restrict bacterial growth. The ability of *M leprae* to affect maturation and differentiation of DCs and the presentation of antigens on the cell surface of DCs provides an important mechanism in establishing disseminated infection (Figure 4). Biomarkers of a sufficiently activated DC response, such as IL-32, are indicative of the response in PB leprosy patients, limiting the bacterial outgrowth.

## 8 | COMPLEMENTING LEPROSY

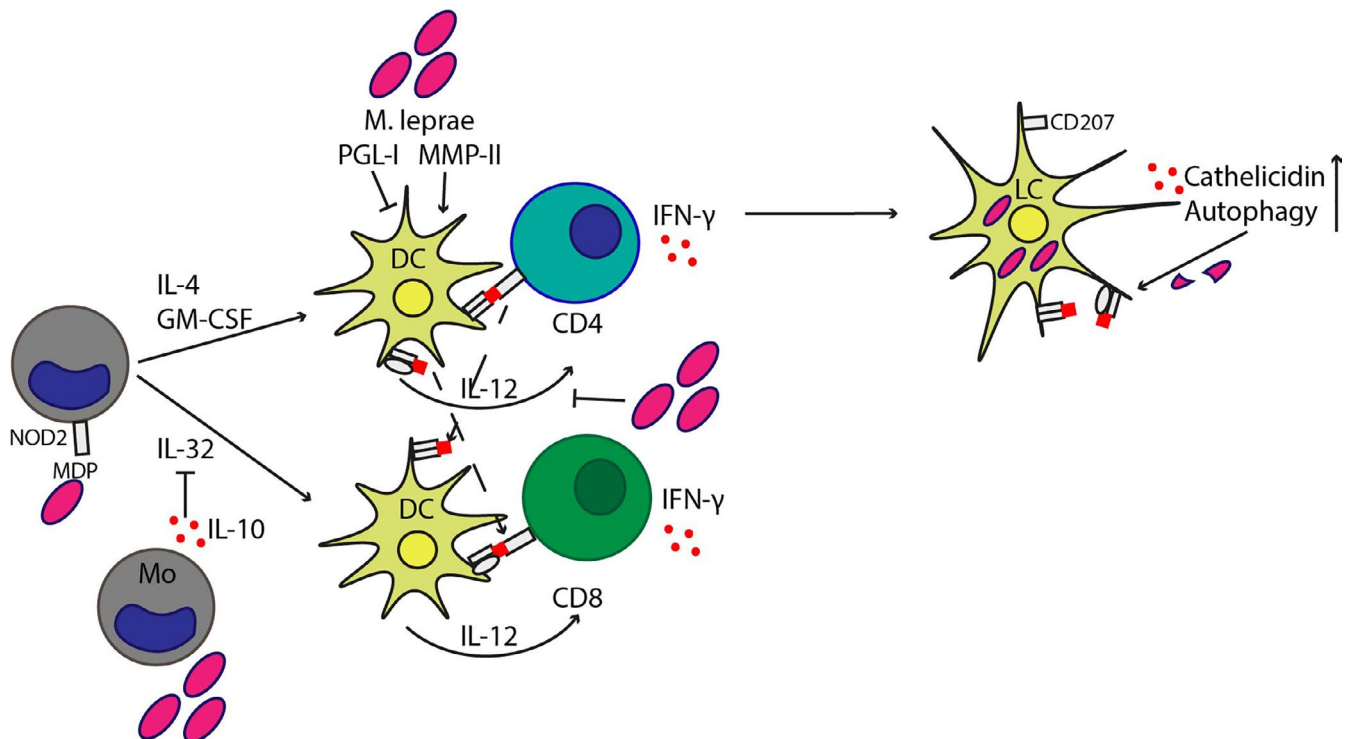
An important part of the innate immune system is the complement cascade, for which most soluble proteins are produced by the liver. The main functions of the complement cascade are opsonization, chemotaxis, and lysis. Genetic polymorphisms in genes of the complement cascade have been found to be associated with increased leprosy susceptibility.<sup>123,124</sup> Polymorphisms in complement receptor 1 (CR1) and mannan-binding lectin-associated serine protease 3

(MASP-3) regulated the serum levels of sCR1 and MASP-3 in leprosy patients. CR1, MASP1, and MASP2 all compete for binding to collectins and ficolins, which initiate the lectin pathway, while MASP3 activates the alternative pathway. However, both pathways result in internalization of C3b-opsonized *M leprae* via CR1. Genetic variants in the lectin and alternative pathway thus affect the phagocytic capacity of the host immune cells and the subsequent immune responses.

The membrane attack complex (MAC), which is formed as a result of complement activation, can also contribute to nerve damage in leprosy patients and is predominantly activated by LAM.<sup>125,126</sup> In skin biopsies, MAC deposition was mostly observed in lepromatous patients and was associated with C3d or LAM presence. As complement factors are readily detectable in serum, these factors are interesting biomarker candidates for both nerve damage and leprosy susceptibility.

## 9 | LYMPHOCYTE POPULATIONS IN LEPROSY PATIENTS

To gain more insight in potential host proteins as immune biomarkers for leprosy, the production of CCGF in the different lymphocyte



**FIGURE 4** Modulation of dendritic cells by *M leprae*. Monocytes can differentiate to dendritic cells via an IL-4/GM-CSF and IL-32-independent pathway. DCs present antigens via MHC class II to CD4 T-cells and via MHC class I to CD8 T-cells. *M leprae*/PGL-I downregulates the expression of MHC molecules, resulting in less T-cell priming. MMP-II, another cell-wall antigen of *M leprae*, induces the production of IFN- $\gamma$  by CD4 and CD8 T-cells via DCs. The production of IL-12 by DCs stimulates the production of IFN- $\gamma$  by T-cells; in lepromatous patients, the IL-12 production in response to *M leprae* is weak. IL-10 produced by monocytes in response to *M leprae* inhibits IL-32, blocking the differentiation to DCs, resulting in less IL-12. In the absence of IL-10, IL-32 is upregulated in monocytes upon the recognition of muramyl dipeptides (MDP) from *M leprae* via NOD2. Langerhans cells (LCs) reside in the skin or mucosa and uniquely express langerin (CD207). IFN- $\gamma$  treatment of *M leprae*-infected Langerhans cells induces autophagy and increases the production of cathelicidin, required to activate the antimicrobial activity in these cells. The increased autophagy in LCs enables the degradation of *M leprae*, releasing antigens for the presentation to resident T cells

subsets of T-cells, B-cells, and natural killer (NK) cells is essential, particularly subsets that confer protection to the establishment of *M leprae* infection.

## 9.1 | T-helper cells

T-cells are the most widely studied lymphocytes in the context of leprosy due to the crucial role in protection of disseminated infection. The classical paradigm is that the lepromatous pole is associated with a Th2 immune response and the tuberculoid pole with a Th1 immune response. More recently, additional subsets have been added to this paradigm showing that the T-cell response is less black and white as suggested. The response of T-helper (CD4) cell subsets Th1, Th2, Th9, Th17, Th22, and regulatory T cells (Tregs) in leprosy patients have been reviewed previously.<sup>16</sup> It is postulated that in tuberculoid patients mainly a Th1, Th9, and Th17 response is induced. Naïve CD4 T-cells differentiate to Th1 cells under the influence of IL-2 and IL-12, producing pro-inflammatory IFN- $\gamma$ , TNF- $\alpha$ , IL-6, and IL-1 $\beta$ . Another distinct T cell subset, Th9, is induced by IL-4 and TGF- $\beta$  and mainly produces IL-9 in these patients, which has a synergistic effect on IFN- $\gamma$ . Th17 cells produce IL-17 and require IL-6 and TGF- $\beta$  to differentiate. In lepromatous patients, Th2 and Treg responses predominantly produce IL-4, IL-10, and TGF- $\beta$ , whereas Th9 cells produce IL-10 and positively regulate TGF- $\beta$ . A combination of TNF- $\alpha$  and IL-6 differentiates naïve CD4 T cells to Th22 cells, which produce among others IL-13, IL-22, IL-26, and basic fibroblast growth factor (FGF-b), important for tissue repair. As the borderline forms of leprosy disease display characteristics of both the lepromatous and tuberculoid pole of the disease, they likely present with a mixture of these Th cell subsets. Combining biomarkers of different Th-cell subsets is essential to cover all clinical forms of leprosy.

## 9.2 | T follicular helper cells and natural killer T cells

In addition to Th cells, CD4 T-cells can also differentiate into T follicular helper (Tfh) cells or natural killer T cells (NKT cells). In the context of leprosy, Tfh cells have not been described. Tfh cells are essential for germinal center formation and provide help to B cells including affinity maturation of antibodies.<sup>127</sup> These cells are primarily found in the secondary lymphoid organs, which are difficult to sample, providing a possible explanation for the absence of literature on Tfh cells in leprosy.

NKT cells, sharing surface markers with NK cells, are innate-like lymphocytes that can rapidly secrete various CCGF once activated by recognition of antigens.<sup>128</sup> A specific subset of pro-inflammatory double-negative invariant NKT cells was reported to be reduced in leprosy patients,<sup>129</sup> and ex vivo unstimulated PBMCs showed lower percentages of NKT cells in leprosy patients

compared to control individuals as well.<sup>130</sup> However upon stimulation with PGL-I or mannose-capped LAM, the number of NKT cells increased especially in tuberculoid patients.<sup>130</sup> Moreover, the NKT cells of tuberculoid patients produced more IFN- $\gamma$  compared to controls and lepromatous patients in response to these antigens. These studies show that NKT cells contribute to the cell-mediated immune response to *M leprae*. Further studies are required to study the CCGF secreted by these cells in the context of *M leprae* infection.

## 9.3 | CD8 T-cells

Cytotoxic CD8 T-cells can directly kill infected cells, among others via the secretion of IFN- $\gamma$  and TNF- $\alpha$  or the release of cytotoxic granules. The frequency of CD8 T-cells expressing the cytotoxic proteins granulysin, perforin, and granzyme B was higher in peripheral blood of tuberculoid patients.<sup>131</sup> These specific CD8 T-cells could be expanded by IL-15 and mediated antimicrobial activity, corroborating that the presence of (a specific subset of) CD8 T cells is related to the protective response. The three cytotoxic proteins represent useful biomarkers for an effective antimicrobial response associated with the tuberculoid part of the leprosy spectrum, as well as for reactional episodes.<sup>132</sup> The innate counterpart of cytotoxic T-cells, natural killer cells, also contributed to the effective immune response in tuberculoid leprosy patients by upregulating the induction of cytotoxic T-cells in an IL-12- and IL-18-dependent manner.<sup>133</sup>

Leprosy was the first disease for which the presence of CD8 suppressor T cells was identified cloned from a borderline lepromatous patient that specifically suppressed the Th-response induced by mycobacterial antigens.<sup>134</sup> IL-10-producing CD8 Tregs were more abundantly present in these patients compared to household contacts, contributing to the T-cell anergy in lepromatous patients.<sup>135</sup> In conclusion, depending on the function of the CD8 T-cells they are either associated with PB leprosy (cytotoxic) or MB leprosy (Tregs).

## 9.4 | Memory

Immunological memory enables the host to respond more quickly and vigorously to a previously encountered antigen. *M leprae*-specific central CD4 and CD8 memory T-cells, determined by flow cytometry, were significantly increased in lepromatous patients that experienced a relapse compared to leprosy patients that were effectively cured by MDT.<sup>136</sup> Moreover, the frequencies of these memory cells correlated with the bacterial load and the number of skin lesions observed in these individuals. Central and effector memory T-cells have also been identified as possible inducers of the immune alteration in T2R.<sup>137,138</sup> These studies thus indicate that the presence of memory T-cells is not beneficial for leprosy patients, as they are associated with poor treatment outcome (relapse) or nerve-damaging episodes (T2R).

## 9.5 | Co-stimulation

T-cells require co-stimulation in combination with antigen presentation to become fully activated. The co-stimulatory molecule CD28 on T-cells interacts with CD80/CD86 on antigen-presenting cells, which were more strongly expressed in tuberculoid lesions.<sup>139</sup> In lepromatous lesions on the other hand, CTLA4 was more strongly expressed, providing a negative immunoregulatory signal upon interaction with CD80/CD86. Moreover, T-cell anergy observed in lepromatous patients could be reversed by affecting CD28 co-stimulation,<sup>140,141</sup> indicating that the absence of co-stimulatory signals is associated with the lepromatous phenotype. Another co-stimulatory molecule expressed on T-cells, CD40 ligand, showed a pattern comparable to CD28 and was also more strongly expressed in tuberculoid lesions.<sup>142</sup> The higher expression of CD40 ligand stimulated CD40-dependent production of IL-12 in monocytes, contributing to the Th1 cytokine profile associated with effective cellular-mediated immunity to *M leprae*. The CD40-CD40L interaction is also required for providing T-cell help to B-cells, necessary for class switching of antibodies. Whether the lower expression of CD40L observed in lepromatous patients affects the humoral immune response to *M leprae* is yet unexplored, and the correlation of antibodies to PGL-I and the bacterial load<sup>21</sup> indicates that although a sufficient quantity of antibodies is generated, these do not confer protection. The lack of co-stimulatory signals observed in lepromatous patients indicate that a protective T-cell response cannot be sufficiently induced in these patients.

## 9.6 | B-cells

Once the *M leprae* infection is established, the T-cell response seems to largely dictate the polarity of the leprosy phenotype. B-cell subsets have been less widely studied in the context of leprosy, probably because patients presenting with a predominant humoral immune response are not able to control the infection. As described by Ochoa et al,<sup>143</sup> in lesions of lepromatous patients B-cell pathways were indeed one of the most important differently regulated gene sets compared to tuberculoid patients. In the same study, IL-5 produced by T-cells was identified as a potential initiator of the high levels of IgM observed in plasma cells (CD138) at the site of disease. Immunohistochemical staining of leprosy lesions indeed showed more CD138+ cells in lepromatous patients, while at the other part of the spectrum CD20+, a B-cell marker which is absent on the terminally differentiated plasma cells, was more frequently observed.<sup>144</sup> The CD138- and CD20-positive cells showed an inverse gradient from the tuberculoid to the lepromatous pole, indicating that B-cells might not only contribute to disease pathogenesis in lepromatous patients. Dual-RNAseq of patients' lesions showed that bacterial viability was strongly correlated with the abundance of plasma cells.<sup>145</sup> The decreased viability of *M leprae* resulted in the expression of heat-shock proteins that triggered the maturation and survival of plasma cells producing class-switched antibodies (IgG,

IgA). Class switching might not be sufficiently induced in lepromatous patients due to the earlier described lack of co-stimulation, impairing effective antibody-mediated killing of *M leprae*.

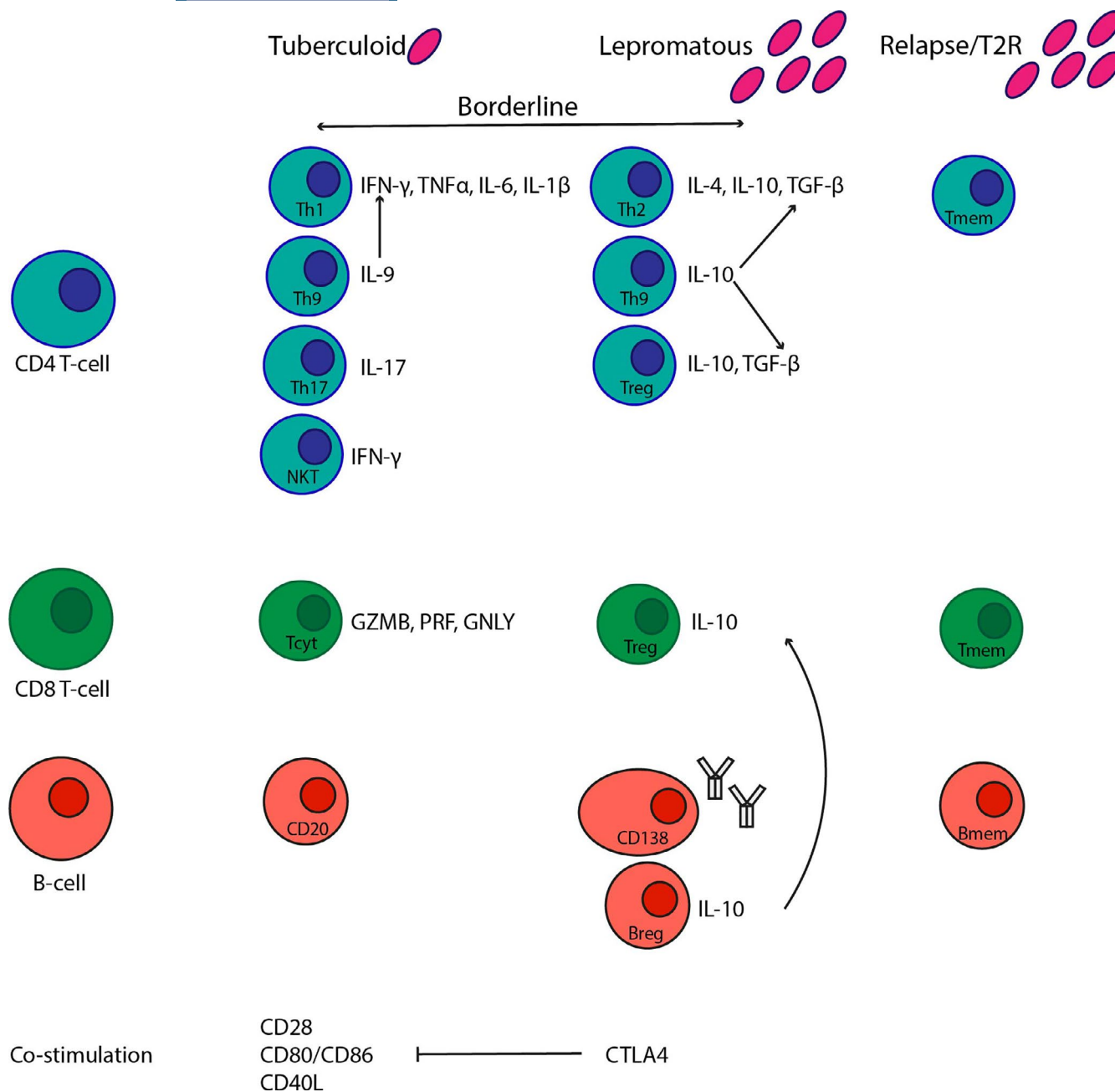
In the circulation, regulatory B-cells (Bregs) producing IL-10 have been identified in lepromatous patients and to a lesser extent in tuberculoid patients. Bregs induced a conversion from T effector cells to regulatory T cells coinciding with increased expression of FoxP3 and PD-1 on these cells.<sup>146</sup> This B-cell subset contributed to the production of the immunosuppressive IL-10 and might be important in the persistence of *M leprae*. Memory B-cells have only been described in the context of T2R,<sup>147</sup> just like the memory T-cells, indicating that immune memory can contribute to this pathological condition.

Few reports describe the direct correlation between circulating lymphocyte subsets and the serum CCGF profile.<sup>148,149</sup> To gain insight in the source of the CCGF that indicates a protective or susceptible immune response in serum, direct comparison of systemic CCGF to circulating immune cells is necessary. For diagnostic purposes, measurement of serum biomarkers requires less complicated procedures compared to immune cell subset characterization, which is especially important in low-income countries. In Figure 5, the different CCGF-producing lymphocyte subsets identified in both lepromatous and tuberculoid patients are summarized. The abundance of these biomarkers and the difference in concentration between different types of leprosy will determine the feasibility to incorporate them in point-of-care tests, which are generally less sensitive in measuring low concentrations than laboratory-based techniques.

## 10 | IMMUNOMETABOLISM

### 10.1 | Lipids

Apart from the more obvious immune biomarkers in response to *M leprae*, the identification of ApoA1 as a biomarker for leprosy<sup>22</sup> indicated that the lipid metabolism could also be affected in leprosy patients. ApoA1 is the predominant protein of high-density lipoprotein (HDL), which transports cholesterol back to the liver and can reverse foam cell formation through hydrolysis of oxidized lipids. The oxidation of ApoA1 induces dysfunctional HDL, resulting in impaired cholesterol removal from macrophages.<sup>150</sup> Foamy macrophages, characteristic for lepromatous leprosy, actively contribute to the increased survival of *M leprae* in the host, upregulation of genes related to lipid metabolism are not surprisingly predominantly found in lesions of these patients.<sup>151</sup> The decreased removal of oxidized lipids due to dysfunctional HDL in these patients resulted also in an inhibition of antigen presentation to T-cells by DCs, whereas HDL from healthy individuals preserved DC function. Tuberculoid patients also presented with dysfunctional HDL, but showed a less inhibitory effect of DC differentiation.<sup>151</sup> In line with this study, the lipidomic profile of HDL in lepromatous patients indeed showed an altered composition compared to tuberculoid patients and healthy controls.<sup>152</sup> These studies indicate that biomarkers of HDL functionality can be used to identify leprosy patients.



**FIGURE 5** Lymphocyte subsets in tuberculoid and lepromatous leprosy patients. Different lymphocyte subsets and cytokines and chemokines produced by these subsets in lepromatous and tuberculoid leprosy patients. CD4 T-cell subsets: T-helper (Th) cells, natural killer T-cells (NKT), regulatory T-cells (Treg), and memory T-cells (Tmem). CD8 T-cell subsets: cytotoxic T-cells (Tcyt), Treg, and Tmem. B-cells: antibody-producing plasma cells (CD138), regulatory B-cells (Breg), and memory B-cells (Bmem). Memory subsets were predominantly identified in patients that experience a relapse or type 2 leprosy reactions (T2R). Arrows indicate a stimulatory effect. Co-stimulation in combination with antigen presentation is required for T-cells to become fully activated, and CTLA4 inhibits the co-stimulatory molecules CD28, CD80/CD86, and CD40L

An interesting yet unexplored role for ApoA1 in leprosy is the description of the increased secretion of ApoA1 as a delayed response to neuronal injury. In the cerebrospinal fluid, ApoA1 levels increased after mechanical injury of the central nervous system and remained increased as a self-protecting mechanism to dampen the inflammatory response after injury contributing to the healing process.<sup>153</sup> Whether the ApoA1 response we observed in leprosy patients, particularly PB, can be related to mechanisms trying to reduce nerve

damage induced by *M leprae* or to the altered lipid metabolism, remains to be elucidated.

## 10.2 | Mitochondria

Apart from the lipid metabolism, comparative gene expression of nerve lesions from patients with peripheral neuropathy indicated

that several mitochondrial genes were downregulated in leprosy neuropathy compared to non-leprosy neuropathy.<sup>154</sup> This is in line with the earlier discussed study by Madigan et al,<sup>95</sup> where damaged mitochondria induced the nerve-damaging demyelination. Mitochondria generate adenosine triphosphate (ATP), the main energy source of the cell, via oxidative phosphorylation (OXPHOS). Dysfunctional mitochondria have been described in other neurodegenerative disease. In Alzheimer's disease (AD), OXPHOS dysfunction leads to the production of reactive oxygen species (ROS) and oxidative stress, inducing neuronal cell death.<sup>155</sup> Even before clinical diagnosis was determined, mitochondrial-encoded OXPHOS genes were upregulated in the blood of AD patients.<sup>156</sup> Impaired mitochondrial function has also been described as a possible cause for neurodegeneration in Parkinson's disease (PD), which shares genetic risk factors with leprosy.<sup>157</sup> One of these genes, PARK2, is a regulator of autophagy of damaged mitochondria, so-called mitophagy.<sup>158</sup> Defective mitophagy is described to contribute to the pathogenesis of neurodegenerative diseases as the accumulation of damaged mitochondria causes cellular dysfunction.<sup>159</sup> In addition, defective mitophagy can lead to chronic systemic inflammation as hyperactivation of inflammatory signals is induced via ROS and NLRP3. NLRP3 in turn, directly inhibits mitophagy via caspase-1-mediated proteolytic cleavage of PARK2, amplifying the mitochondrial defects. The balance between energy metabolism and the innate immune response is important for cell survival during bacterial infection as reviewed in detail elsewhere.<sup>160</sup> Mitophagy also plays a role in the above-described differentiation of macrophages to M1 or M2. Effective clearance of mitochondria stimulates glycolysis required for M1 polarization, whereas M2 predominantly uses OXPHOS as their energy source.<sup>160</sup> Mitophagy might provide the link between the immune response and metabolic phenotype observed in the spectral presentation of leprosy.

Mitochondria not only impact the immune response, but also play a role in nerve damage observed in leprosy patients. As nerve damage is observed in all clinical phenotypes of leprosy, biomarkers of mitochondrial dysfunction, which have also been observed in other neurodegenerative diseases, can be used to identify both MB and PB patients.

## 11 | FROM INFECTION TO DISEASE: HOUSEHOLD CONTACTS, IN VIVO DISEASE MODEL?

The inability to culture *M leprae* in vitro complicates research on the establishment of *M leprae* infection and subsequent development of disease. As *M leprae* strains are highly uniform, the host response to *M leprae* is the main determinant of the outcome upon *M leprae* infection. As discussed above, most studies have focused on the difference between immune cell subsets of lepromatous and tuberculoid patients, particularly in lesions, but the continuously exposed household contacts are less frequently studied. To unravel the mechanisms that are important for the development of leprosy in *M leprae*-infected

individuals, longitudinal follow-up and sampling of household contacts are vital, as these individuals have an increased risk of developing leprosy. This longitudinal follow-up enables to discern household contacts developing disease from those that are exposed but do not develop disease. Since 2012, extensive proteomic and transcriptomic profiling is performed on samples of household contacts in Bangladesh, providing clues on the involvement of the above-described host responses in the early stages of progression to disease.

In household contacts of lepromatous patients, the frequency of CD4 T-cells recognizing *M leprae*-specific antigens increased 6 months after the index case received MDT. The alteration in T-cell responses after MDT treatment of the index case<sup>161</sup> points toward immunomodulatory effects of *M leprae* to the continuously exposed household contacts, which can be reverted after adequate treatment of the index case. Exposure to *M leprae* also affects the innate immune response of contacts of lepromatous patients; levels of ApoA1 and S100A12 similar to those of PB patients have been observed in contacts of leprosy patients.<sup>49</sup> We hypothesize that in these individuals there is a constant battle between the host and the bacterium: In household contacts, the balance is in favor of the host, whereas in those who develop disease, the pathogen is the winner. Identification of the immune response that allows the pathogen to win is vital to diagnose leprosy at an early stage when it can still be treated efficiently.

## 12 | STAGE-SPECIFIC BIOMARKERS: FROM *M leprae* COLONIZATION TO MB LEPROSY

*Mycobacterium leprae*-infected individuals without clinical symptoms are difficult to identify, and their response to *M leprae* is highly similar to the response of PB leprosy patients. To discern infected individuals from those developing disease, we propose three different phases in the progression to disease that will enable the identification of stage-specific biomarkers:

1. *Colonization to M leprae infection.* The local immune response, instructed by keratinocytes, epithelial cells, and endothelial cells, allows a permissive environment for *M leprae* replication. The ability of these cells to present antigens and direct the polarization of macrophages is vital in the initial establishment of infection of the host.
2. *M leprae infection to early disease.* The response of macrophages recruited to the site of infection might be biphasic, low bacterial load of the macrophages still enables the M1 macrophages to contain the infection but as the amount of infected macrophages starts to increase the subversion to the M2 phenotype will be induced, allowing replication and further dissemination. In this phase, Schwann cells become infected, together with the infected macrophages patrolling axons, initiating pathological nerve damage.
3. *Early disease to leprosy subtype.* The activation of the adaptive immune system by innate immune cells aims to control the bacterial load. In case of low bacterial loads, antigen presentation by

DCs is still sufficient to induce a proper cellular immune response (PB). As the bacterial load increases, *M leprae* actively downregulates the ability of antigen presentation, reducing the activation of T cell-mediated immunity (MB). The reduction in the bacterial load and increased presence of antigens due to effective treatment can revert this downmodulation of antigen presentation, which can result in the hyperactivation of the adaptive immune response further aggravating the nerve damage.

The inability of the host to combat *M leprae* at any of these stages can result in either *M leprae* infection or progression to disease. Stage-specific biomarkers reflecting this host response will allow improved discrimination between *M leprae* infection, and PB and MB leprosy.

### 13 | CONCLUSION

Combining the current knowledge on the different cell types involved in the host response to *M leprae* leads to the hypothesis that the innate immune system is relatively more important in the initiation of nerve damage, indicative of the first manifestation of disease, whereas the adaptive immune system further aggravates the nerve damage and determines the type of leprosy. More research on the host response to *M leprae*, particularly in household contacts, is required to corroborate this hypothesis. Therefore, extensive proteomic and transcriptomic profiling of household contacts developing leprosy is currently ongoing. Further, unraveling the leprosy-causing mechanisms will provide insight in the yet unknown factors that are essential to allow progression from infection to disease. The identification of biomarkers based on these mechanisms will facilitate the diagnosis of leprosy patients, especially at an early stage when symptoms are less apparent.

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### CONFLICT OF INTEREST

All authors declare no competing or conflicting interests.

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