immunopathogenesis of leprosy neuritis

proefschrift

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door
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geboren te Rosmalen in 1969

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βροτοισιν ουδεν εστ'απωμετον:
ψευδει γαρ η πινοια την γνωμην.

Sofokles, Antigone.

aan mijn ouders
voor Mariët

chapter 1
general introduction

For other illnesses found here
Wise doctors on the scene appear,
Who understand disease.
To hospital those sick are brought,
And for their plight a cure is sought.
Thus their ills are relieved,
And all their wounds are dressed.

We lepers can no doctors get:
Here must we stay and wait and fret,
Until our time is up.
Peter from prison did escape
Because on God's grace he did wait.
O God, break now the chains
Which bind our limbs with pains.

excerpt from “En Klagesang”, by Peder Olsen Feidie,
patient in St. George's hospital for lepers in Bergen from 1832 to 1849.

leprosy

a major health care problem

Leprosy is a chronic infectious disease caused by *Mycobacterium leprae*. Although leprosy was found almost worldwide in the past, the disease currently occurs mainly in tropical and subtropical countries. In endemic regions, the prevalence rates can exceed 5 per 10,000. The most prominent feature of leprosy is involvement of the skin and nerves, which can lead to severe physical deformities of hand, feet, face and eyes, and permanent disabilities. Overall, 2 to 3 million individuals are disabled as a result of the disease (Noordeen 1994). This is the main reason that leprosy represents a major social stigma. The introduction of multiple drug therapy (MDT) in 1982 has caused a major decline in the prevalence of leprosy (World Health Organization 1997). The estimated number of leprosy patients has declined from 10-12 million in 1985 to less than 2 million in 1995 as a result of this (World Health Organization 1995; Noordeen 1995). Despite the drop in prevalence, the number of newly detected cases of leprosy worldwide has remained stable at a level of approximately 600,000 new cases annually, thus creating a large population at risk of developing nerve damage (Smith 1997).

the bacillus

The causative agent of leprosy, *M. leprae*, is a virtually non-toxic, obligatory intracellular bacterium, which particularly infects macrophages and Schwann cells. G.A. Hansen discovered the bacterium as early as 1874 (Hansen 1874), but it is still impossible to culture the bacterium *in vitro*. The genus 'Mycobacterium' contains gram-positive bacteria, with a characteristic cell wall composition. The most characteristic feature is the presence of mycolic acids that reduce the permeability of the mycobacterial cell wall and are also responsible for the acid fast staining of the bacilli (Bishop and Neumann 1970; Jarlier and Nikaido 1994). Although mycobacteria share a number characteristics, *M. leprae* has some unique features, including the presence of phenolic glycolipid I (PGL-1) (Hunter and Brennan 1981; Hunter *et al.* 1982). These unique components may have the potential to detect *M. leprae* infected patients specifically (Lal *et al.* 1993; Luna-Herrera *et al.* 1996; Buhrer *et al.* 1998).

an immunological disease

Host immunity to *M. leprae* plays an important role in the clinical manifestations of the disease. More than 99% of the population is believed not to acquire the disease after having been in contact with the bacillus, due to protective immunity (Godal *et al.* 1972; Godal and Negassi 1973). Individuals that develop clinical disease can be classified into a 5 group spectrum (figure 1) (Ridley and Jopling 1966). On one side of the leprosy spectrum are lepromatous leprosy patients, which have a high humoral immune response and a low cellular response. These patients show an *M. leprae* specific cellular non-responsiveness and fail to clear the bacteria. On the other side of the spectrum are tuberculoid leprosy patients, which have a low humoral immune response but display both acquired cellular immunity and delayed type hypersensitivity against *M. leprae*. The bacterial load in these patients is low. The majority of the patients is positioned in between these two poles and is categorized as borderline lepromatous, borderline and borderline tuberculoid leprosy.

leprosy reactions

Superimposed on the leprosy spectrum, leprosy reactions can occur. Leprosy reactions can occur before treatment (Naafs and Wheate 1978), but are mainly observed

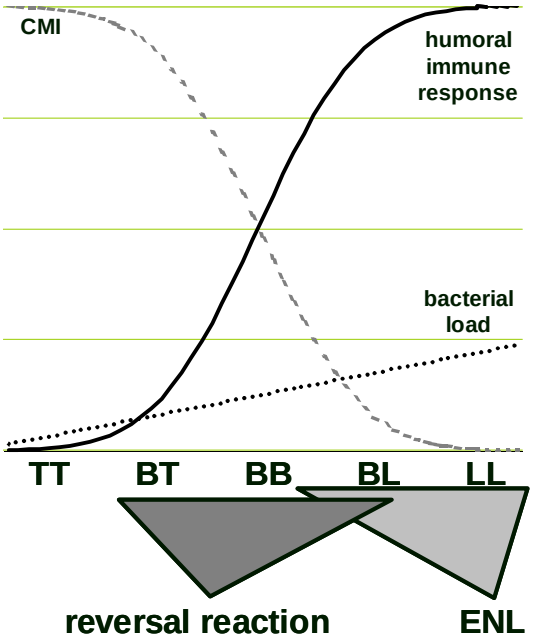

figure 1: the leprosy spectrum

during or after treatment and typically represent acute inflammatory episodes. Two commonly recognized types of leprosy reactions can be discriminated; 1) erythema nodosum leprosum (ENL) or type II leprosy reactions, which can occur in patients on the lepromatous side of the spectrum, and 2) reversal reactions (RR), also designated as type 1 leprosy reactions. The latter type occurs almost exclusively in patients of the borderline area of the spectrum, particularly during treatment (Lockwood *et al.* 1993). Reversal reactions are thought to represent episodes in which cell-mediated responses towards *M. leprae* are strongly increased, resulting in an inflammatory response in the areas of the skin and nerves affected by the disease (Modlin *et al.* 1983). Estimates of the overall prevalence of reversal reactions range from 8-30% in leprosy (Lienhardt and Fine 1994).

From the point of view of clearing bacteria, upgrading responses might be considered to be beneficial. However, the inflammation in nerve tissue often results in permanent damage and disability within a matter of days, if not treated adequately. Clinical neural involvement occurs in approximately 10% of pauci bacillary and 40% of multi bacillary leprosy patients, particularly in patients with reversal reactions (Richardus *et al.* 1996). It has, however, been suggested that sub-clinical damage takes place in virtually all leprosy patients and that 30% of the nerve fibers has to be destroyed before sensory impairment becomes detectable (Pearson and Ross 1975). Several pathogenic mechanisms may be responsible for the nerve damage in leprosy, like biochemical interference of *M. leprae* with host cell metabolism, mechanical damage due to the large influx of cells and fluid, or immunological damage. Since reversal reactions are accompanied by an increased cell mediated immunity (CMI), a role for the immune system in causing nerve damage during RR has long been suspected (Modlin *et al.* 1983; Naafs 1989). $CD4^+$ T cells are more abundant in skin lesions of patients with RR compared to lesions of leprosy patients without reactions. The T cells in the granulomas are predominantly $CD4^+$, whereas $CD8^+$ T cells are mostly present in the mantle area surrounding the granulomata (Modlin *et al.* 1983; Narayanan *et al.* 1984; Cooper *et al.* 1989). A strong increase of type-1 cytokines has been noted during reversal reactions (Cooper *et al.* 1989; Yamamura *et al.* 1991; Yamamura *et al.* 1992). T cell clones isolated from skin biopsies of patients with reversal

reactions are predominantly of Thelper (Th)1-type (Verhagen *et al.* 1997; Verhagen *et al.* 1998). The microanatomical location of serine esterase positive cells within tuberculoid granulomas and reversal reactions overlaps with the $CD4^+$ $CD45RO^+$ subpopulation (Cooper *et al.* 1989), indicating that these T cells contain cytotoxic granules. Analysis of *M. leprae* reactive $CD4^+$ cytotoxic T cell clones has confirmed that these cells are indeed highly cytotoxic (Mutis *et al.* 1993a). Thus, Th1 like cytotoxic T cells are believed to play a major role in the immunopathology of leprosy neuritis.

Demyelination and nerve damage could also be caused as a bystander effect of inflammation. Possible mediators are tumor necrosis factor (TNF)-α, proteases, and urokinase (Said and Hontebeyrie-Joskowicz 1992). Thus, besides the well documented protective effect of TNF-α during mycobacterial infections (Kindler *et al.* 1989; Appelberg 1994; Flynn *et al.* 1995; Kaneko *et al.* 1999), TNF-α may also be responsible for the observed pathology (Rook *et al.* 1989; Khanolkar-Young *et al.* 1995). In relation to leprosy, TNF-α mRNA and protein is more abundant in lesions of patients with reversal reactions (Yamamura *et al.* 1992; Khanolkar-Young *et al.* 1995). It is predominantly produced by *M. leprae* responsive type-1 T cells derived from patients undergoing reversal reactions (Verhagen *et al.* 1997), but also infected and activated macrophages can be responsible for the production.

early detection of leprosy reactions

Good markers for early detection of leprosy reactions are still lacking. Measurement of the acute-phase response and the ratio of serum amyloid A/C-Reactive Protein in particular have been suggested to be helpful in the clinical diagnosis of ENL (Hussain *et al.* 1995). Neopterin has recently been reported to be increased in patients with both ENL and RR (Hamerlinck *et al.* 1999). A marker more specific for RR is the presence of anti-PGL-1 IgM antibodies present in serum (Roche *et al.* 1991). Another risk factor correlated with the development of RR is post-pregnancy, since women are particularly susceptible 1 to 3 months after delivery (Rose and Waters 1991). Also the involvement of three or more body areas is related to an increased risk to develop reversal reactions (van Brakel *et al.* 1994). To prevent permanent nerve damage, prediction, early detection, and intervention are required. The treatment of choice for patients with RR is corticosteroids, suppressing detrimental T cell reactivity (Naafs 1996). ENL can also be treated with thalidomide, which strongly reduces TNF-α production in ENL (Barnes *et al.* 1992; Sampaio *et al.* 1993).

immunity against mycobacterial pathogens

T cells

T cells can be distinguished into two subpopulations based on their cytokine production profile. Polarized Th1 and Th2 cells were originally described among mouse $CD4^+$ cells (Mosmann *et al.* 1986). Th1 cells are defined by the production of interleukin (IL)-2, interferon (IFN)-γ and lymphotoxin, whereas Th2 cells are characterized by the production of IL-4 and IL-5. A third subset, designated Th0, secreted both Th1 and Th2 cytokines and is believed to differentiate into either Th1 or Th2 (Street *et al.* 1990). The same subsets have been identified in humans (Wierenga *et al.* 1990; Haanen *et al.* 1991; Romagnani 1991). The balance between Th1 and Th2 plays a crucial role during mycobacterial infections. In patients with tuberculoid leprosy, Th1 cells are dominant and correlate with bacterial clearance, while little or no Th2 cells can be found (Salgame *et al.* 1991; Haanen *et al.* 1991; Mutis *et al.* 1993b; Verhagen *et al.* 1997). Th2 cells have been documented in skin biopsies of patients on the lepromatous pole (Salgame *et al.* 1991; Verhagen *et al.* 1997), whereas they could not be found in peripheral blood (Mutis *et al.* 1993b). During RR, *M. leprae* reactive T cells derived from skin lesions almost exclusively produce IFN-γ and little

or no IL-4 (Verhagen *et al.* 1997).

Recently, a new $CD4^+$ T cell population was described, designated T-regulatory (Tr) (Groux *et al.* 1997). These Tr cells produce IL-10, but not IL-4 and exhibit immunosuppressive features. The presence of Tr cells in leprosy patients has not been analyzed in detail yet. Earlier studies, however, detected T cells in peripheral blood of leprosy patients which had suppressive effects on T cell proliferation and might thus explain non-responsiveness in lepromatous leprosy (Ottenhoff *et al.* 1986a; Mutis *et al.* 1994). These T cells do not suppress by IL-4 and IL-10 and thus may represent another, possibly unique subset of regulatory T cells.

Protective immunity against mycobacteria critically depends on the cell-mediated immune response. Animal studies using knockout models showed that both $CD4^+$ and $CD8^+$ T cells are required to eliminate mycobacteria from the host. $CD4^+$ T cells are essential for protection against tuberculosis, as illustrated by the increased incidence of tuberculosis in HIV patients (Barnes and Modlin 1996), and the increased susceptibility to experimental mycobacterial infections in CD4 -/- (Xing *et al.* 1998) and Major Histocompatibility Complex (MHC) class II -/- (Ladel *et al.* 1995) mouse models. Mycobacterium specific $CD4^+$ T cells expressing a Th1 profile and cytotoxic activity could be isolated from both patients and healthy contacts (Haanen *et al.* 1991; Mutis *et al.* 1993a). This population might therefore not only facilitate the elimination of bacilli by producing cytokines, but may also actively attack infected cells and kill them.

First evidence for the involvement of $CD8^+$ lymphocytes in the protection against mycobacterial infections came from the β2m -/- model, in which MHC class I expression at the cell surface is impaired and $CD8^+$ T cells are almost absent (Flynn *et al.* 1992; Ladel *et al.* 1995). Mortality was higher in β2m disrupted mice after infection with virulent *M. tuberculosis*, compared to wild type mice. Furthermore, cytotoxic $CD8^+$ cells could be demonstrated in tuberculosis patients at low frequencies (Lalvani *et al.* 1998) and in bulk cultures from leprosy patients (Kaleab *et al.* 1990a). The fact that both the MHC class I and class II deficient mice are devoid of protective immunity against mycobacteria illustrate the close cooperation of $CD4^+$ and $CD8^+$ T cells in eliminating mycobacteria.

natural killer cells

Natural killer (NK) cells preferentially kill target cells that do not express MHC class I molecules (Ljunggren and Karre 1985; Piontek *et al.* 1985). This phenomenon can be explained by the presence of NK receptors that inhibit NK cell activation upon recognition of MHC class I (reviewed in (Lanier 1998)). Activated killer cells presumably originate from NK cells and are efficiently induced by *M. leprae* (Kaleab *et al.* 1990a) and *M. bovis* Bacille Calmette-Guerin (BCG) (Wolfe *et al.* 1977; Kaleab *et al.* 1990a; Mizutani and Yoshida 1994). BCG induced killer cells reduce mycobacterial growth in *in vitro* infected macrophages (Denis 1991). Furthermore, NK cells could be isolated from a number of TB patients, healthy *M. tuberculosis* responders as well as non-responders (Restrepo *et al.* 1990). Freshly isolated PBMC from all three groups were tested on the NK sensitive target cell K562. Patients and non-responders showed low target lysis, while healthy responders efficiently killed the target. Cytotoxic activity of PBMC from patients and responders could further be enhanced by pre-incubation with *M. tuberculosis*, whereas this effect was marginal in the non-responder group. It is still unclear which NK/target cell interactions are essential for effective target cell lysis. Expression of Neural Cell Adhesion Molecule (N-CAM), also called CD56, on both target and effector has been hypothesized to be of importance (Lanier *et al.* 1989).

N-CAM expression has also been observed on some $CD4^+$ T cells in relation to multiple sclerosis (Vergelli *et al.* 1996; Antel *et al.* 1998). The inducing agent in this case was myelin basic protein. These T cells were able to kill N-CAM positive oligodendrocytes,

the central nervous system equivalent of Schwann cells, in an antigen independent manner. These studies also revealed that co-adhesion via other molecules, such as CD54 and CD11a, was essential and that homotypic N-CAM interaction alone was not sufficient to establish target lysis. This mechanism may also be involved in leprosy neuritis, since T cells derived from inflamed neural tissue show increased N-CAM expression when compared to peripheral T cells (Kaleab 1992).

mechanisms of killing of mycobacterium infected targets

Effective immunity to mycobacterial pathogens most likely involves lysis of infected cells as well as killing of the invading pathogen. Different mechanisms may be used to reach this goal. After recognition of infected targets via MHC/peptide/T cell receptor (TCR) interactions by $CD4^+$ and $CD8^+$ T cells, or via yet unknown interactions between targets and NK cells, these cells can secrete lytic granules, containing granulysin and perforins. These two components were reported to act in concert in attacking the infected target cells (Stenger *et al.* 1998). Perforin permeabilizes the eukaryotic cell membrane, allowing the granulysin to enter the cells. Granulysin subsequently attacks the intracellular bacteria by altering their membrane integrity, resulting in bacterial death. Knocking out perforin, however, does not alter the capability of mice to control *M. tuberculosis* or BCG infections, indicating that alternative killing pathways are likely to exist (Laochumroonvorapong *et al.* 1997).

Aside to granule-mediated lysis, killing of cells via the FAS or FAS related 'death-receptors' appears to be an important pathway. Interaction between FAS and FAS-L on target and effector respectively, initiates an intracellular cascade that finally results in apoptosis of the target cell (Nagata and Golstein 1995). There has been some debate on the issue whether this pathway also results in elimination of intracellular bacteria. Reduced bacterial viability has been reported by some (Oddo *et al.* 1998), while others see no effect (Stenger *et al.* 1997; Mazzaccaro *et al.* 1998). These latter results are supported by the finding that the course of *M. bovis* BCG infection in FAS-receptor-defective mice was not altered (Laochumroonvorapong *et al.* 1997).

A third mechanism of apoptosis induction is via extracellular Adenosine Triphosphate (ATP) (Zanovello *et al.* 1990). ATP, which may also be produced by immune effector cells, can interact with P2Y, or P2Z receptors on target cells and induce apoptosis (Pizzo *et al.* 1992). Extracellular ATP has not only been shown to kill targets via P2 receptors (Molloy *et al.* 1994), but also to affect the viability of intracellular bacteria (Lammas *et al.* 1997). Interestingly, individuals can be classified into three groups, based upon their response to ATP (Kumararatne *et al.* 1996): some individuals show responses to ATP in the absence of IFN-γ, while others only respond intermediately. The response of the intermediate group can be enhanced by IFN-γ. A third group does respond neither to ATP alone, nor to ATP in combination with IFN-γ. These responses are correlated with the level of P2 receptors on their APC and may thus explain differences in susceptibility to ATP mediated apoptosis between individuals.

TNF-α plays an important role in protective immunity against virulent mycobacteria (Kindler *et al.* 1989; Appelberg 1994; Flynn *et al.* 1995; Kaneko *et al.* 1999). As discussed above, it can also be involved in pathogenicity (Rook *et al.* 1989; Khanolkar-Young *et al.* 1995). TNF-α hardly has a toxic effect on Schwann cells on its own, but in combination with Transforming Growth Factor (TGF)-β it has been reported to cause significant Schwann cell detachment and lysis (Skoff *et al.* 1998). Little is known about the effect of TNF-α mediated target killing on mycobacterial survival. TNF-α mediated lysis has been reported to have a similar effect on mycobacterial viability as FAS/FAS-L mediated lysis (Oddo *et al.* 1998), but for both FAS and TNF-α this topic is highly disputed.

Cytokines determine the outcome of inflammatory immune responses in various ways. Chemokines, chemoattractive cytokines, such as RANTES (Regulated upon Activation, Normally T cell Expressed and presumably Secreted), Monocyte Chemotactic Proteins (MCP)-1, IL-6, and IL-8, may attract specific cell populations. Some of these chemokine genes have been disrupted in mice. IL-6 was reported to play a crucial role in cellular defense against mycobacteria. It is produced in an early stage of mycobacterial infection and inhibits bacterial growth in macrophages (Flesch and Kaufmann 1990; Orme *et al.* 1993). Disruption of the IL-6 gene greatly reduced the capability to clear *M. avium* and *M. tuberculosis* and these mice subsequently died (Appelberg 1994; Ladel *et al.* 1997). Similar results were obtained using other parasites (Kopf *et al.* 1994; Dalrymple *et al.* 1995). As shown in IL-6 -/- mice, IL-6 also attracts macrophages and T cells to the nervous system (Eugster *et al.* 1998). Monocytes and $CD4^+$ $CD45RO^+$ T cells are central components of reversal reaction granulomata (Cooper *et al.* 1989). MCP-1, RANTES, and IL-8 play an important role in recruiting these cells and initiating local immune responses (Taub *et al.* 1995) (Schall *et al.* 1990; Wilkinson and Newman 1992). Thus, the increase in chemokine production after infection of tissue cells, including Schwann cells, with *M. leprae*, may be an important event in the initiation of inflammatory responses in neural tissue.

The final result of inflammation is the net balance of Th1 promoting cytokines such as IFN-γ and IL-12, versus deactivating cytokines, including IL-4, IL-10, and TGF-β. Mice with genes disrupted for the genes encoding for IFN-γ -/- (Cooper *et al.* 1993) or IFN-γR (Kamijo *et al.* 1993) were unable to generate protective immune responses against mycobacteria. Also disruption of the IL-12 (Magram *et al.* 1996) or IL-12R gene (Wu *et al.* 1997) lead to strongly impaired Th1 responses. IL-18 disruption had similar effects (Takeda *et al.* 1998; Sugawara *et al.* 1999). Individuals deficient in receptors for either IL-12 or IFN-γ were highly susceptible for infection with low-pathogenic mycobacterial species, such as *M. avium* or *M. bovis* BCG (Newport *et al.* 1996; Jouanguy *et al.* 1996; de Jong *et al.* 1998; Altare *et al.* 1998). So far, microsatellite segregation studies did not yield indications that certain microsatellite markers are indeed associated with leprosy or tuberculosis (Siddiqui *et al.* 1999). Since deficiencies in these patients were often the result of single nucleotide mutations, more precise and informative markers will be needed to resolve these issues.

IL-12 production can be inhibited by Th2 like cytokines TGF-β, IL-4, and prostaglandin E2 (PGE_2) (van der Pouw Kraan TC *et al.* 1995; Skeen *et al.* 1996) and thus suppress Th1 responses (Salgame *et al.* 1991; Sieling *et al.* 1993). TGF-β also attenuates the IL-12 responsiveness of Th1 cells via down-regulation of IL-12Rβ1 and IL-12Rβ2 expression (Gorham *et al.* 1998; Bright and Sriram 1998; Zhang *et al.* 1999). Furthermore, TGF-β reduces MHC expression (Geiser *et al.* 1993) and suppresses iNOS and NO production (Vodovotz *et al.* 1996). Thus, in general TGF-β drives immune responses away from Th1, toward Th2-like responses in which DTH is suppressed. IL-10 suppresses macrophage function at various levels, including MHC class II expression (de Waal Malefyt *et al.* 1991; Koppelman *et al.* 1997) and proinflammatory cytokine production (Gazzinelli *et al.* 1996; Neyer *et al.* 1997) and has been suggested to be responsible for the latent phase during mycobacterial infection (reviewed in (Murray 1999)).

Schwann cells may well be actively involved in modulating cell-mediated immune responses via the production of cytokines. A number of studies reported the production of TGF-β (Flanders *et al.* 1991; Unsicker *et al.* 1991), which even increases 3-fold after damage to neural tissue (Kiefer *et al.* 1995). TGF-β may be partly responsible for immune suppression in neural tissue, thus creating an immune privileged site, like has been suggested for the eye (D'Orazio and Niederkorn 1998). This effect may be amplified by the production of PGE_2 (Constable *et al.* 1994), which also has a Th1 suppressing effect (van der Pouw Kraan TC *et al.* 1995). Furthermore, two studies have reported the production of

IL-6 by Schwann cells (Bolin *et al.* 1995; Murwani *et al.* 1996).

peripheral nerve system

anatomy of peripheral nerves

The peripheral nervous system (PNS) connects the central nervous system (CNS) with the periphery. It includes the cranial nerves, the spinal nerves with their roots and rami, the peripheral nerves and the peripheral components of the autonomic nervous system. A nerve fiber consists of an axon that is almost completely enveloped in a sheath of Schwann cells. Axons can be divided in myelinated and unmyelinated ones. Myelinated peripheral axons have a myelin sheath interposed between the Schwann cells and the axon (figure 2a). This myelin sheath is derived from the Schwann cells. Unmyelinated axons lack a myelin sheath and lie in deep grooves in the surface of the Schwann cells, with multiple axons enveloped by the same cell (figure 2b).

Externally, Schwann cells are covered by a basal lamina, which, in turn, is surrounded by endoneurial tissue (figure 2c). Several Schwann cell/axon units, which are embedded in endoneurial tissue, are surrounded by the relatively impermeable perineurium, consisting of randomly orientated and highly concentrated collagen fibers. Tight junctions between endothelial cells of the capillaries and the basement membrane separate the endoneurium from the circulation. This isolation, also referred to as blood/nerve barrier, is believed to be important for maintaining the appropriate physicochemical environment for the axons and for protecting them from harmful agents. However, the junction can provide a route through which bacteria or leukocytes can ultimately enter the PNS.

function of myelin

Myelin formation results from the dense winding of cell membranes around axons. These membranes partly fuse and thus form lipoprotein complexes. This substance is a perfect insulator and prevents almost all ion flow, increasing the resistance to ion flow through the membrane approximately 5000-fold. However, at the juncture between each two successive Schwann cells along an axon, a small, non-isolated area remains, the node of Ranvier, where ions can flow easily between the extracellular fluid and the axon. Action potentials can therefore only occur at the nodes, which increases the velocity of signals five-fold, as well as reduces energy used by axons a hundred-fold, since depolarization has to be established at the nodes only.

Schwann cells and myelin formation

Schwann cells are the myelin forming cells of the peripheral nerve system and are generated from the neural crest cells in embryonic life (Le Douarin *et al.* 1991; Anderson 1993). This process involves the generation of an intermediate stage, the Schwann cell precursor, that differs from both crest cells and immature Schwann cells in several ways, including survival requirements, antigenic phenotype, and morphology (Jessen *et al.* 1994; Gavrilovic *et al.* 1995). During late embryogenesis, these cells develop into S100-positive, bipolar immature Schwann cells, which in turn differentiate into myelin- or non-myelin-forming Schwann cells, depending upon the diameter of the axon they ensheath (figure 2a-b) (Jessen and Mirsky 1991). Myelin-forming Schwann cells wrap concentrically around a single large diameter axon (>1 μm), while non-myelin-forming Schwann cells ensheath multiple small diameter axons.

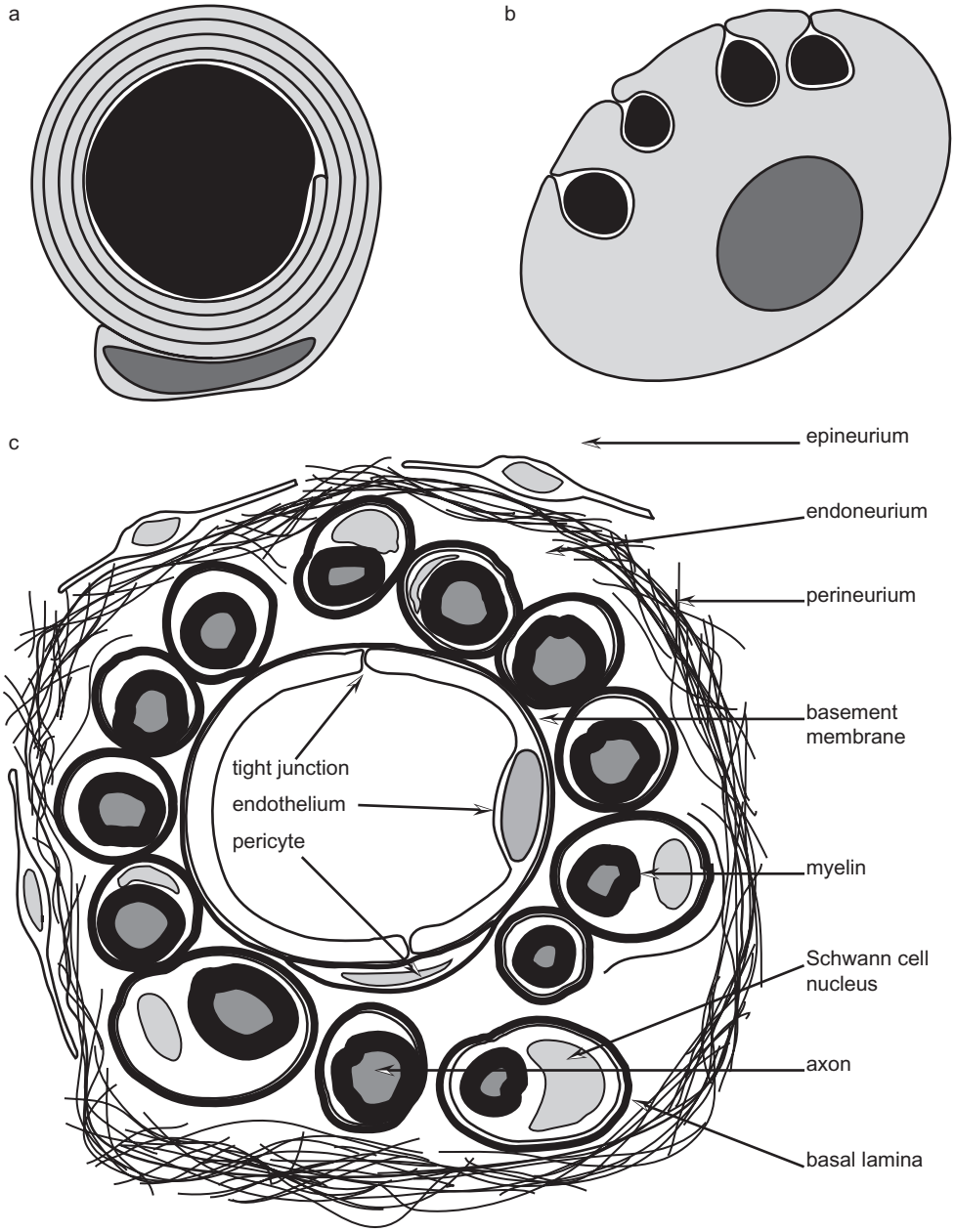

figure 2: Myelinating (a) and non-myelinating (b) Schwann cells and the structure of peripheral nerve tissue (c).

demyelinating neuropathies

Demyelinating neuropathies are characterized by disruption of the myelin sheath and segmental demyelination. A wide range of traumatic, hereditary, toxic, infectious and immune mediated processes may be associated with, or present as peripheral neuropathies. Examples of neuropathies in which the immune system may actively be involved in causing demyelination are chronic inflammatory demyelinating polyradiculoneuropathy and Guillain-Barré syndrome. Target tissues were shown to display increased expression of MHC class II (Pollard *et al.* 1986; Mancardi *et al.* 1988; Mitchell *et al.* 1991). A similar phenomenon was observed in leprosy, where Schwann cells were found to express MHC class II (Narayanan *et al.* 1990). Schwann cells may therefore well be actively involved in the immunopathology of leprosy neuritis by presenting *M. leprae* antigens to cytotoxic T cells. This hypothesis is supported by the finding that $CD8^+$ T cells were able to lyse murine Schwann cells in an MHC class I restricted, mycobacterial antigen dependent manner (Steinhoff and Kaufmann 1988). As a result of antigen recognition, Schwann cells were killed. Schwann cells were also able to stimulate $CD4^+$ T cells via MHC class II (Wekerle *et al.* 1986; Ford *et al.* 1993). So far, it is unknown to what extent these rodent studies can be extrapolated to leprosy neuritis, largely as a result of the inability to culture human Schwann cells, thus precluding such analyses in humans.

peripheral nerve system - *M. leprae* interactions

cellular receptors

Mycobacterium leprae, the causative agent of leprosy, resides intracellularly in macrophages and in the nerve surrounding Schwann cells (Boddingius 1974). Various receptor-mediated mechanisms, similar to those exploited for macrophage invasion, may play a role in invasion of human Schwann cells by mycobacteria. Candidates are Fc receptors (Vedeler *et al.* 1989), complement receptor 1 (Vedeler *et al.* 1989; Schorey *et al.* 1997), the fibronectin binding protein (Schorey *et al.* 1995), and mannose receptors (Schlesinger 1993). These mechanisms are, however, not restricted to Schwann cells and thus do not explain why *M. leprae* specifically homes to neural tissue. The neurotropism of *M. leprae* may be attributed to its affinity for laminin-α2, present in the basal lamina of Schwann cells (Rambukkana *et al.* 1997).

laminins

Laminins are extracellular matrix proteins that consist of alpha, beta and gamma chains (figure 3a) (Timpl and Brown 1994). In merosin, or laminin-2, the alpha chain is of the α2 isoform. The tissue distribution of laminin-2 is restricted to the basal lamina of Schwann cells, striated muscles, and trophoblasts of the placenta (Leivo and Engvall 1988; Engvall *et al.* 1990; Engvall 1993). The heavy α-chain can be subdivided into a number of domains (Engvall and Wewer 1996). The N terminal part or short arm of the α2 macromolecule spans the domains IV-VI. This short arm can interact with cellular receptors via various domains. Domain VI contains binding sites for integrin α1/β1 and α2/β1 (Colognato *et al.* 1997), and an integrin binding RGD site is present in domain IV (Aumailley *et al.* 1990; Aumailley *et al.* 1991; Schulze *et al.* 1996). The C terminus is characterized by the presence of five homologous G domains. These domains have been reported to be involved in binding to receptors as α/β dystroglycan (DG) (Yamada *et al.* 1996) and a number of integrins (Nomizu *et al.* 1996).

M. leprae exploits laminin-α2 and dystroglycan to bind to Schwann cells (figure 3b) (Rambukkana *et al.* 1998). It is likely that other receptors are also involved in *M. leprae*/

Schwann cell interaction, since blocking of the DG complex could not inhibit bacterial adhesion completely (Rambukkana *et al.* 1998). Candidates are integrins, which are also able to bind to laminin-α2. Furthermore *M. leprae* has been reported to interact with a yet uncharacterized 28-30 kDa phosphoprotein which is expressed by Schwann cells (Suneetha *et al.* 1997), thus providing other receptor/ligand interactions for *M. leprae* to bind to Schwann cells than via laminin-α2/dystroglycan alone.

mycobacterial receptors

Mycobacteria are well known to possess mechanisms to interact specifically with host cells. Recently, a laminin binding receptor of 21 kDa on *M. leprae* has been identified,

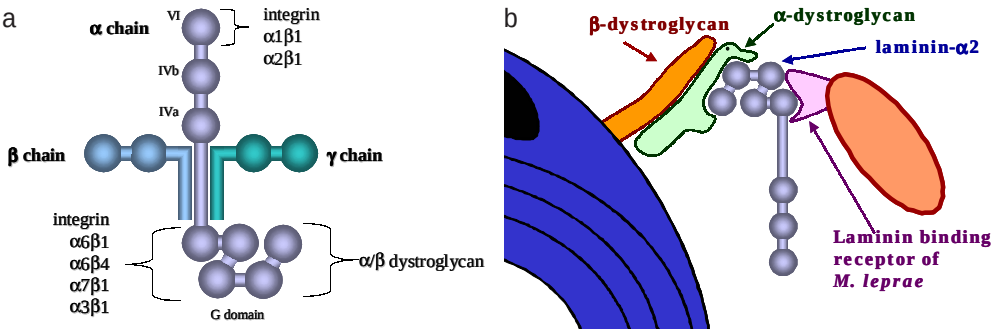

figure 3: a) Schematical structure of laminins. b) Molecules involved in *M.leprae*/Schwann cell interaction.

being a histone like protein (HLP) (Shimoji *et al.* 1999; Pessolani *et al.* 1999). This mycobacterial receptor may function as a critical surface adhesin that facilitates the entry of *M. leprae* into Schwann cells. Other mycobacterial receptors described to be involved in eukaryotic cell invasion include the mammalian cell entry (*mce*) proteins. Sequencing of the complete *M. tuberculosis* genome yielded four similar mce operons consisting of 8 open reading frames (Cole *et al.* 1998). Non invasive bacteria, as *Escherichia coli*, become invasive after introduction of *mce* genes (Arruda *et al.* 1993) and disruption of the mce gene of BCG reduced the ability to invade the non-phagocytic cells as compared to wild-type BCG (Flesselles *et al.* 1999).

aims and outlines of this thesis

The success of MDT in the control of leprosy is currently overshadowed by the increased frequency of leprosy reactions during treatment and the lack of decline of leprosy incidence. Particularly during the first 6 months of treatment, reversal reactions may occur in as much as 80% of the patients (Roche *et al.* 1991). Nerve damage is a prominent feature of leprosy reactions. Besides macrophages, Schwann cells are predilection sites for *M. leprae*. Nerve damage is at least partly immunologically mediated, since reversal reactions are clearly associated with an increased cell-mediated immune response. Many fundamental aspects of nerve damage in leprosy regarding the role of Schwann cells, T cells, and antibodies in the immunopathology of leprous neuritis have remained unresolved, partly as a result of the inability to culture human Schwann cells and *M. leprae*. This thesis aims to help resolving these issues by addressing the following questions:

- Is it possible to establish a human Schwann cell system for the study of immunological Schwann cell/T cell interactions?
- Are human Schwann cells able to present mycobacterial antigens to T cells?
- What are the consequences of T cell mediated antigen recognition for Schwann cell function, in particular Schwann cell killing as a cause of nerve damage?
- How do *Mycobacteria* interact with Schwann cells?
- To what extent can human Schwann cells modulate potentially harmful Th1 responses after exposure to *M. leprae*?
- In what respect do T cells from leprosy nerve lesions possibly differ from peripheral T cells regarding function and phenotype?
- Can T cell mediated, antigen independent cytolytic mechanisms be identified that are related to Schwann cell damage in leprosy?
- Can antibodies towards nerve related antigens predict nerve damage or leprosy reactions?

Chapter 2 deals with the establishment of a human *in vitro* cultured Schwann cell system. The antigen presenting capacity of these cells in relation to *M. leprae* (2a) and Schwann cell expressed alloantigens (2b) is described. The inability to establish long term Schwann cell cultures and to culture *M. leprae in vitro* have been important obstacles in studying these issues so far. Schwann cell cultures were phenotypically characterized and used to test the hypothesis that they function as non-professional antigen presenting cells for *M. leprae* specific $CD4^+$ Th1 cells, which are abundantly present in inflamed neural tissue during leprosy reactions. It is shown that Schwann cells are damaged by the cytolytic activity of these T cells during the process of antigen recognition. This may provide an important novel mechanism of immune mediated Schwann cell destruction.

In chapter 2b experiments are performed to test the capacity of human Schwann cells to prime antigen specific responses in an allogeneic model, using peripheral blood mononuclear cells of HLA-DR matched and mismatched individuals as responder cells. Artificial nerve grafts in combination with Schwann cells have already successfully been added to the lumen of such grafts in syngenic rat models to facilitate nerve regeneration. Rejection of allografted rat Schwann cells, however, has previously been reported. Since *in vitro* cultured human Schwann cells express MHC class II and can function as antigen presenting cells to activate specific T cells (chapter 2a), they may also induce immunological recognition and rejection of donor material by host cells. This study provides *in vitro* data confirming this hypothesis.

Chapter 3 describes the cytokine profile of *in vitro* cultured human Schwann cells and the effect of these cytokines on T cell activation. The outcome of local immune responses not only depends on a T cell/APC interaction, but also on the local balance between inflammatory and anti-inflammatory cytokines. In addition to functioning as non-professional antigen presenting cells, Schwann cells might also modulate local immune responses by releasing cytokines such as TGF-β, PGE_2, IL-4 and IL-10.

Lesional T cells are likely to be the most relevant T cell population to study in relation to nerve damage. Chapter 4 describes the characterization of *M. leprae* reactive T cells isolated from inflamed neural tissue of a leprosy patient. The study was focused on a special subset of T cells, expressing N-CAM. N-CAM is a neural cell adhesion molecule, which is expressed on neurons, Schwann cells, NK cells and some T cell populations. N-CAM expressing T cells have recently been reported to be involved in autoimmune mediated cell damage in multiple sclerosis. Homotypic N-CAM/N-CAM interactions may play a role in T cell mediated antigen independent Schwann cell killing. To address this issue, expression of N-CAM on T cells from nerve lesions and peripheral blood was compared.

Furthermore, N-CAM expression on peripheral T cells after exposure to *M. leprae* was analyzed in patients with and without type 1 or type 2 leprosy reactions. The relation between N-CAM expression and lysis of N-CAM positive targets was investigated.

In chapter 5 the presence of an *M. leprae* operon with high similarity to the mammalian cell entry protein of *M. tuberculosis* is described. Genes encoded by these operons have been reported to be directly involved in cell entrance. *M. leprae* may exploit these proteins for host cell invasion. The presence of HLP/Laminin-α2/Dystroglycan independent cell entrance mechanisms was also suggested by the finding that other mycobacteria, like *M. smegmatis*, are also able to enter human Schwann cells (chapter 2a).

Chapter 6 analyses the presence of antibodies in leprosy patients towards nerve related antigens. Neural antibodies are possible candidates for early detection of nerve damage. One such antigen is sulfatide, which is expressed as a surface determinant of myelin in the central and peripheral nervous system. Antibodies towards sulfatides have already been detected in several neuropathies, including insulin dependent diabetes mellitus, Guillain Barré syndrome, Miller Fisher syndrome and multiple sclerosis.

Finally, chapter 7 provides a synthesis of the described findings as well as their implications for the immunopathogenesis of leprosy Schwann cell and nerve damage.

chapter 2a
Mycobacterium leprae specific, HLA class II restricted killing of human Schwann cells by CD4$^+$ Thelper-1 cells: a novel immunopathogenic mechanism of nerve damage in leprosy

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adapted and submitted for publication

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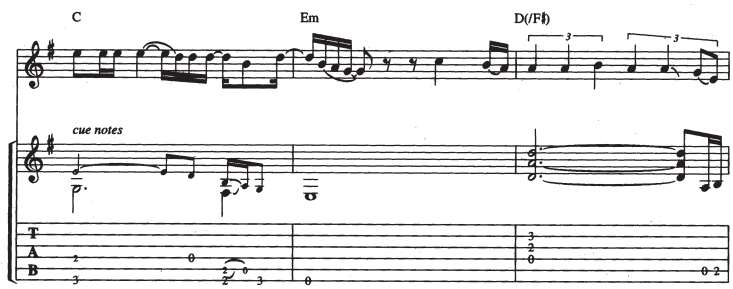

abstract

Peripheral nerve damage is a major complication of reversal reactions in leprosy. The pathogenesis of nerve damage remains largely unresolved, but detailed *in situ* analyses have suggested that type-1 T cells play an important key initiating role. *M. leprae* is known to have a remarkable tropism for peripheral nerve associated Schwann cells that surround, or deposit myelin sheets around axons. Reversal reactions in leprosy are accompanied by severe and irreversible nerve destruction and are associated with increased cellular immune reactivity against *Mycobacterium leprae*. Thus, a likely immunopathogenic mechanism of Schwann cell- and nerve damage in leprosy is that infected Schwann cells process and present antigens of *M. leprae* to antigen specific, inflammatory type-1 T cells and that these T cells subsequently damage and lyse infected Schwann cells. Thus far it has been impossible to study this possibility directly, due to the inability to culture human Schwann cells *in vitro*. We have now established long-term human Schwann cell cultures from sural nerves and show here that such Schwann cells express Schwann cell markers including S-100β, GFAP and CNPase, as well as MHC class I and II, ICAM-1 and CD80, surface molecules involved in antigen presentation. It is demonstrated that human Schwann cells efficiently process and present *M. leprae*, as well as recombinant proteins and peptides in an HLA class II restricted manner, and are efficiently killed by activated Th1 cells. These findings demonstrate that human Schwann cells are facultative antigen presenting cells for the presentation of *M. leprae* antigens to T cells and are killed in this process. This is likely to represent an important novel mechanism with regard to the immunopathogenesis of nerve damage in leprosy.

introduction

Acute reactional episodes are major complications in leprosy. Type-1 reversal reactions (RR) in particular can result in irreversible tissue damage and nerve destruction. Such reactions are characterized by strongly increased cellular immune responses in peripheral blood and lesions (Modlin *et al.* 1983), accompanied by the abundant presence of local $CD4^+$ T cells (Modlin *et al.* 1983; Narayanan *et al.* 1984; Cooper *et al.* 1989) and type-1 cytokines (Cooper *et al.* 1989; Yamamura *et al.* 1991; Yamamura *et al.* 1992; Verhagen *et al.* 1997). In tuberculoid and RR granulomas, cells that express serine esterase, a component of cytotoxic granules, colocalize with $CD4^+$ $CD45RO^+$ memory T cells (Cooper *et al.* 1989), and analysis of *Mycobacterium leprae* reactive T cells confirmed that these T cells indeed produce serine esterase *in vitro* (Mutis *et al.* 1993a). The induction of cytolytic $CD4^+$ Th1 like cells during mycobacterial infections has been documented extensively (Ottenhoff *et al.* 1988; Kaplan *et al.* 1989; Kaleab *et al.* 1990b; Mutis *et al.* 1993a), further suggesting that cytotoxic Th1 cells may play a major role in the protection against and the immunopathology of mycobacterial infections. However, direct evidence for a pathogenic role of T cells in Schwann cell damage is lacking. Better insight into the immunopathogenesis of Schwann cell damage in leprosy is urgently required, given the major impact of nerve damage in leprosy.

M. leprae, has a remarkable affinity for Schwann cells, the molecular basis of which has been elucidated recently: *M. leprae* binds specifically to the G-domain of the extracellular matrix protein laminin-α2 which ligates to α/β-dystroglycan receptor-complexes on myelinating Schwann cells (Rambukkana *et al.* 1997; Rambukkana *et al.* 1998). Thus, *M. leprae* exploits interactions between matrix- and cytoskeletal-linked glycoproteins to target and infect Schwann cells. The recent elucidation of this mechanism now provides novel opportunities to disrupt interactions between *M. leprae*, Schwann cells, and inflammatory T cells, and is of potential value in the prevention or treatment of nerve damage.

Previously, Steinhoff *et al.* showed in a mouse model that Schwann cells can be lysed by $CD8^+$ T cells in an antigen specific manner (Steinhoff and Kaufmann 1988), suggesting that murine Schwann cells are susceptible to killing by $CD8^+$ T cells. In this study, however, $CD4^+$ T cells, which form the major compound of granulomatous lesions in leprosy, were not examined. Furthermore, due to the inability to culture human Schwann cells, the immunological role of Schwann cells in leprous neuritis has not been addressed so far.

In the present study, the antigen presenting capacity of human Schwann cells and their susceptibility to killing by T cells is analyzed. Our data show that human Schwann cells can process and present *M. leprae* to antigen specific T cells, and are subsequently killed during this event. We propose that this may be an important mechanism in nerve damage during reversal reactions.

materials and methods

establishment of human Schwann cell cultures

Human Schwann cells were isolated from sural nerve biopsies from amputated material and propagated as described before (van den Berg *et al.* 1995). Briefly, sural nerve specimens were cut into small pieces and incubated in 85% IMDM, supplemented with 10% LAK cell supernatant, 5% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml PHA (Murex Diagnostics, Dartford, UK), in humidified 5% CO_2 at 37°C. After 10 days non-adherent cells were removed, and adherent cells propagated in 80% IMDM, supplemented with 5% LAK cell supernatant, 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, supplemented with 0.6% glucose.

RT-PCR

Total RNA was isolated from trypsinized Schwann cell cultures using the High Pure RNA isolation kit following the instructions of the manufacturer (Boehringer Mannheim GmbH, Mannheim, Germany). First strand cDNA was generated from oligo(dT) primed RNA by incubation at 42°C for 60 minutes in 20 μl RT mix containing 75 mM KCl, 50 mM Tris-HCl (pH 8.3), 3 mM $MgCl_2$, 0.4 mM of each dNTP, 10 units AMV reverse transcriptase, and 25 units RNasin (Promega, Madison, WI).

All PCR reactions contained 20 pmol of each primer in 50 μl of a reaction mixture containing 10mM Tris/HCl (pH 8.4) buffer, 50 mM KCl,4mM $MgCl_2$, 0.5 mM dNTPs and 1 unit Taq polymerase (Perkin Elmer Roche Molecular Systems Inc., Branchburg, NJ). Primers used were as follows: for 2',3'-cyclic nucleotide-3'-phosphohydrolase (CNPase), 5'-CAGGCGTGCTGCATTGCACAA-3' and 5'-CCTTGCGTGGGCACAGGTTTG-3'; for S-100β, 5'-CCCTCATCGACGTTTTCCACC-3' and 5'-GCCAGTCAGCTTACACACAGG-3'; for glial fibrillary acidic protein (GFAP), 5'-TATGAGGCAATGGCGTCCAGC-3' and 5'-TGGTGATCCGGTTCTCCTCGC-3'. Amplification started with a denaturation step of 5 min at 95°C. Cycling conditions were 95°C for 30 s, 60°C for 30 s, and 72°C for 90 s during 35 cycles, followed by an extension of the last step with 5 min. Following amplification, 10 μl of each PCR product was applied to a 2% agarose gel, visualized under UV after ethidium bromide staining and photographed.

immunostaining of cultured Schwann cells

Human Schwann cells were cultured on gelatin coated cover slides, fixed and stained with hematoxilin. The slides were incubated with anti-S100-β (Sigma Aldrich, St. Louis, MO), anti-GFAP (Sigma), or anti-α-prolyl 4-hydroxylase (DAKO, Glostrup, Den-

mark) in PBS, 0.1% BSA for 16 h. After washing 3 times the slides were incubated with a goat-anti-mouse antibody labeled with horse-radish peroxidase. After 2 hours, the slides were washed and immunostained with di-amino peroxidase. Cells were examined visually by light microscopy.

phagocytosis of mycobacteria

Schwann cells were seeded into 75cm^2 culture flasks (Becton Dickinson, Mountain View, CA) and allowed to adhere for 24 hours. *Mycobacterium smegmatis* was grown on 7H9 culturing medium for two days. Bacteria were washed twice and added to the Schwann cell cultures at a ratio of 100 to 1. After 72 hours, the cultures were rinsed three times with PBS and fixed in 0.1% glutaraldehyde in 0.14 M cacodylate buffer (pH 7.4) for 45 minutes at room temperature. After rinsing twice with PBS, the cells were postfixed in 1% osmium teroxide in Millonig phosphate buffer (pH 7.4) for 45 minutes at 4°C, washed in PBS and dehydrated in a graded series of ethanol. The cells were embedded flatly in culture dishes in epoxy resin LX-112 (Ladd Research Industries, Burlington, VT) and polymerized at 60°C for 60 hours. Ultra thin sections (60 nm) were cut on an ultratome (Reichert OM U3), collected on copper grids, stained with uranyl acetate and lead hydroxide and examined in a Philips EM 410 LS transmission electron microscope.

phenotypic characterization

Expression of HLA, adhesion and co-stimulatory molecules by human Schwann cells was examined by immunofluorescence. Antibodies recognizing HLA-DP (B7/21), HLA-DQ (SPV-L3), HLA-DR-FITC, CD80-FITC, CD86-PE, ICAM-1-FITC, LFA-1-FITC (Becton Dickinson) and FAS (CLB, Amsterdam, The Netherlands) were applied for FACScan® analysis. Cultured cells were incubated with mAbs for 60 minutes at 4°C. When non-conjugated antibodies were used, samples were subsequently incubated with goat anti-mouse-FITC (Becton Dickinson) for another 60 minutes if necessary. The cells were then washed extensively and fixed in 2% paraformaldehyde. Percentage of positive cells were scored by FACScan® (Becton Dickinson) while gating on the viable Schwann cell population, as defined by forward and side light scatter.

T cell clones

HLA restriction and antigen specificity of CD4$^+$ Thelper-1 like clones L10B4, L10C11, R1E4, R3F7, and Rp15 1-1 have been reported before (Mutis *et al.* 1993a; Thole *et al.* 1999). The epitope recognized by DR11 restricted, 15kDa protein reactive clone D1B2 (Janson *et al.* 1991) has not been described before and the antigen recognized by the DR11 restricted T cell clone D2C1 has not been determined yet.

Schwann cell antigen presentation assay

Human Schwann cells were seeded in 96 well flat-bottomed microtiter plates (Greiner GmbH, Solingen, Germany) at 2000 cells/well. After 24 hours, the cells were washed three times with IMDM, 10% pooled human serum and pulsed with antigens (10^5 bacilli/well *M. leprae*, 25 μg/ml of sonicated *M. leprae*, 10 μg/ml mycobacterial proteins or 10 μg/ml peptides) for 40 hours. The cells were washed three times and co-cultured with 10^4 T cells for 88 hours. During the last 16 hours 0.1 μCi ^{3}H-Thymidine per well was added. Incorporation of ^{3}H-Thymidine was measured by liquid scintillation counting. When indicated, monoclonal and polyclonal antibodies were added in the assay at 1:200 dilution.

In other experiments, antigen pulsed Schwann cells were labeled with 40 μCi/ml ^{51}Cr (Sodium Chromate, New England, Boston, USA) for 2 hours at 37°C in a total volume

of 100 µl and washed three times. Freshly cultured effector T cells were added into the wells at an E:T ratio of 40:1 in a final volume of 200 µl. Target cells were incubated with either medium alone or with 0.5% Triton-X to determine the spontaneous and maximum ^{51}Cr release respectively as described before (Ottenhoff *et al.* 1988). Cell free supernatants were collected from the wells after 6 hours and the ^{51}Cr release was measured by gamma counting. The percentage specific lysis was calculated as follows: percentage specific lysis = [(experimental ^{51}Cr release – spontaneous ^{51}Cr release)/(maximal ^{51}Cr release - spontaneous ^{51}Cr release)] x 100%. Spontaneous releases did not exceed 15% of the maximal release. Experiments were performed in quadruplicates.

results

characterization of Schwann cell cultures

Schwann cell cultures were analyzed by RT-PCR and by immunostaining with antibodies to determine expression of Schwann cell specific markers. All human Schwann cell cultures expressed the Schwann cell markers CNPase and S-100β by RT-PCR (figure 1a), whereas GFAP mRNA was weakly detectable in two out of three cell cultures. Control fibroblasts and PBMC were negative in RT-PCR for all Schwann cell markers. All Schwann cells, fibroblasts and PBMC expressed GAPDH mRNA, as expected.

Immunostaining with GFAP and S-100β mAbs confirmed expression of these Schwann cell markers by all cells in the cultures (figure 1b-c), while the expression of fibroblast marker 5-prolyl-hydroxylase could not be detected in Schwann cell cultures (data not shown). A typical feature of these cultures was the low division rate, with an average doubling time of 1-2 weeks.

One advantage of such *ex vivo* isolated human Schwann cells is circumventing the need of transformed cell lines. Such lines are often dedifferentiated, can display altered functional phenotypes, and mostly represent the progeny of low numbers of precursor cells that may not be fully representative of the original population.

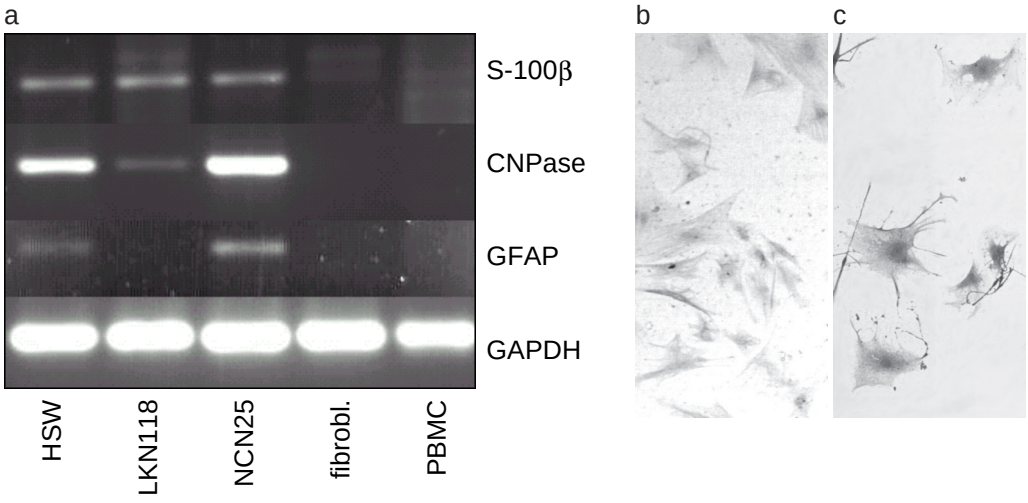

figure 1: Characterization of human Schwann cells by RT-PCR (a) and immunostaining for GFAP (b) and S-100β (c).

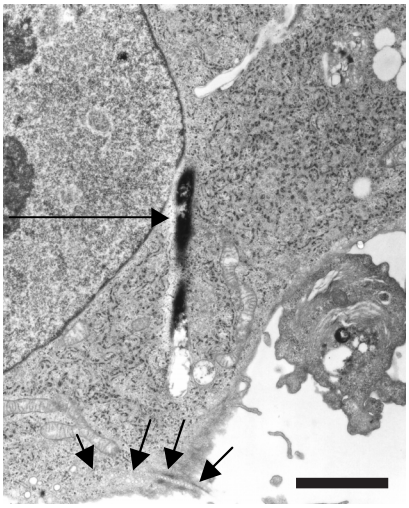

figure 2: Phagocytosis of mycobacteria by in vitro cultured human Schwann cells. Mycobacterium smegmatis (long arrow) was found intracellularly in a membrane bordered compartment. The bacilli were partly degraded. A large number of pinocytotic vesicles (small arrows) were observed (bar = 1μm).

table 1: expression of membrane molecules involved in antigen presentation and T cell stimulation.

	HSW	NCN25	LKN118	LKN109	LKN116
HLA-DP	28%	32%	83%	83%	48%
HLA-DQ	10%	70%	7%	6%	19%
HLA-DR	98%	93%	99%	94%	98%
CD1a	20%	3%	3%	2%	6%
ICAM-1 (CD54)	98%	93%	99%	94%	98%
LFA-1 (CD11a)	5%	6%	4%	7%	7%
LFA-3 (CD58)	21%	23%	82%	21%	31%
CD70	23%	3%	3%	4%	5%

Schwann cells phagocytose mycobacteria

After incubating human Schwann cells with *M. smegmatis* for 72 hours, bacteria could be detected inside Schwann cells by electron microscopical analysis. Bacilli were present in membrane bordered vesicles and were in some cases already degraded (figure 2). Beside phagocytosis, also pinocytotic vesicles, coated pits and coated vesicles were seen. Thus, human Schwann cells are capable of taking up mycobacteria directly.

phenotypic characterization of Schwann cells

In table 1, it is shown that human Schwann cells express a variety of relevant membrane molecules characteristic for antigen presenting cells. All five Schwann cell cultures expressed high levels of HLA-DR and ICAM-1, whereas HLA-DP and LFA-3 were expressed to a lesser extent, varying from 21% to 83%. HLA-DQ could be detected on 2 out of 5 Schwann cell cultures. Also CD80 expression was observed (figure 3a). Expression of neither LFA-1, nor CD1a/b/c, molecules involved in presentation of nonpeptide antigens (Sieling *et al.* 1995; Mazzaccaro *et al.* 1998), could be detected.

Since the expression of HLA-DR and ICAM-1 might have been induced by cytokines present in the culture medium, Schwann cells were also maintained in absence of LAK supernatant for 2 weeks. Subsequently, the cells were exposed to IFN-γ to examine the induction of surface expression. Although expression of HLA-DR and ICAM-1 was lower upon 2 weeks culturing on IFN-γ free medium (figure 3b), especially the expression of HLA-

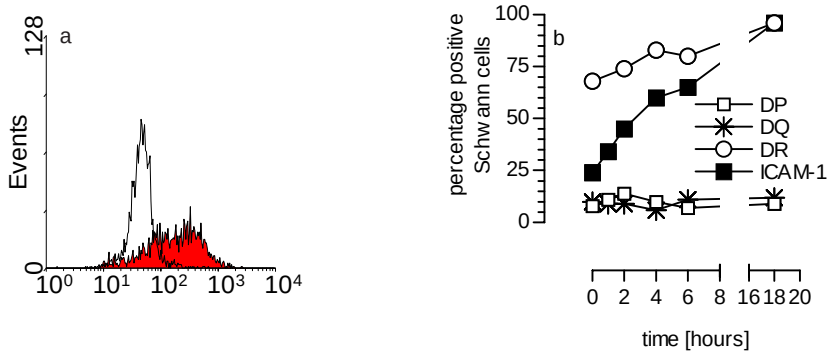

figure 3: Surface expression of molecules involved in antigen presentation. Significant expression of co-stimulatory molecule CD80 (a: solid profile) was observed when compared to an isotype matched control antibody (open profile). Induction of MHC class II and ICAM-1 by IFN-γ (b). In the absence of IFN-γ, HLA-DR (open circles) and ICAM-1 (solid squares) expression could be observed. Addition of rIFN-γ drastically increased the expression of these membrane molecules, but had no effect on HLA-DP (open squares) and HLA-DQ (asterices).

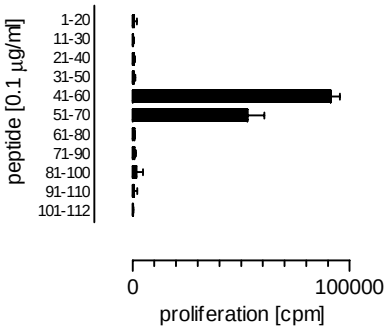

figure 4: Mapping of a HLA-DR11 restricted, 15 kDa specific peptide epitope recognized by T cell clone D1B2. Overlapping 20-mer peptides of the 15 kDa protein of M. leprae were synthesized and tested for T cell recognition using HLA-DR matched monocytes. The epitope recognized was situated between amino acids 51 and 60.

DR remained strongly positive. Moreover, addition of recombinant IFN-γ restored the initial level of expression within 18 hours. Thus, *in vitro* cultured human Schwann cells express constitutive and IFN-γ inducible cell surface molecules involved in antigen presentation.

mapping of a novel peptide epitope on an M. leprae 15 kDa protein

One of the Schwann cell cultures obtained expressed HLA-DRB1*1101. T cell clone D1B2 has been reported to recognize an *M. leprae* protein with a molecular weight of 15 kDa in the context of DR11 (Janson *et al.* 1991). In order to be able to compare recognition of peptides versus protein or *M. leprae*, overlapping 20-mer peptides of the 15 kDa protein of *M. leprae* were synthesized and tested for recognition by D1B2. The epitope recognized was situated between amino acids 51 and 60, as depicted in figure 4.

human Schwann cells efficiently present antigen to M. leprae specific type-1 inflammatory T cells

Since Schwann cells are non-professional phagocytes which can be infected with *M. leprae* (Steinhoff and Kaufmann 1988; Ford *et al.* 1993), we investigated whether *ex vivo* isolated human Schwann cells are capable of processing and presenting exogenous *M. leprae* antigens to inflammatory type-1 T cells. Varying numbers of Schwann cells ranging from 20 to 2500 cells per well were tested as antigen presenting cells in cultures to which a constant number of mycobacterium specific $CD4^+$ T cells was added. As depicted in

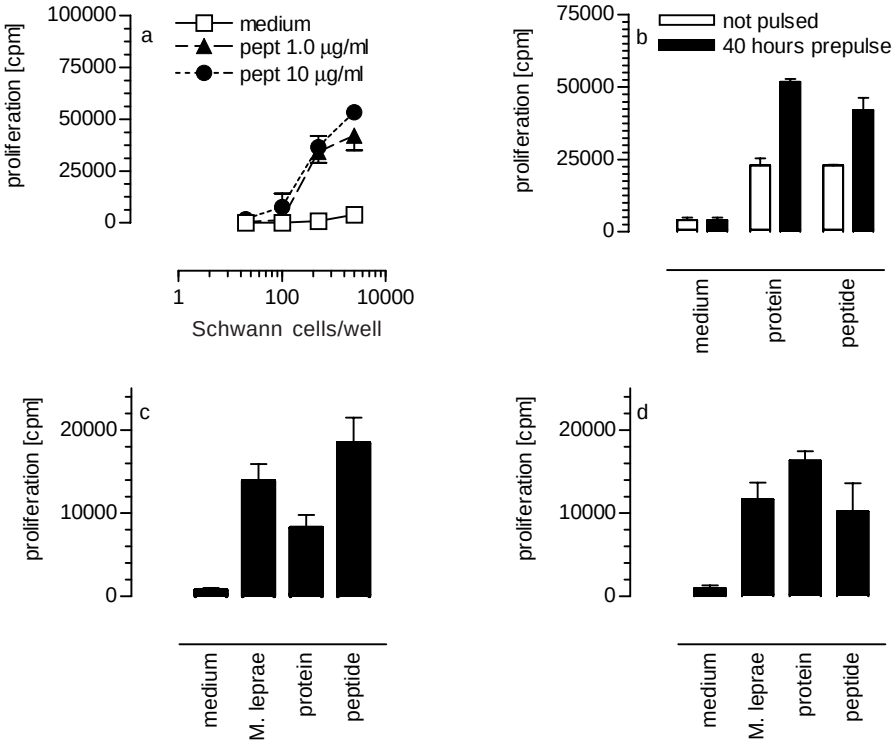

figure 5: Presentation of mycobacterial antigens by human Schwann cells. a) Human Schwann cells (HSW) are able to present peptides to CD4⁺ positive T cells (D1B2). Optimal presentation is achieved at cell densities between 1000 and 2000 Schwann cells per well. b) Pulsing for 40 hours enhances antigen presentation of both peptides and proteins. c-d) In addition to peptide, protein and M. leprae sonicate are also presented efficiently. Schwann cell/T cell couples used in the latter two experiments are HLA-DR11 restricted combination HSW/D1B2 (c) and the HLA-DR3 restricted combination NCN25/Rp15 1-1 (d).

figure 5a, 1000 Schwann cells/well already induced strong antigen specific T cell proliferation when HLA-DR matched T cells were added. Although addition of protein antigens directly to the assay induced significant T cell proliferation, pre-incubation of Schwann cells with antigens for 48 hours yielded a 2 fold stronger response (figure 5b).

Optimal numbers of Schwann cells were subsequently pulsed with intact *M. leprae* bacilli, recombinant *M. leprae* proteins or corresponding specific *M. leprae* peptides thereof, and co-cultured with HLA-DR matched or mismatched *M. leprae* reactive CD4⁺, Th1-like clones. As shown in figure 5c-d, T cells recognize processed protein and *M. leprae* presented by Schwann cells. Presentation of *M. leprae* leads to equally strong T cell responses as presentation of exogenously added, preprocessed peptides. No recognition of irrelevant peptides or proteins was observed (data not shown). Antigen presentation was HLA-DR restricted, as judged by blocking experiments using HLA specific antibodies (figure 6a) and by using HLA-DR matched and mismatched T cell/APC combinations (figure 6b-d). Finally, antigen presentation was also processing dependent, since fixation experiments excluded the presence of directly presentable peptides in protein and *M. leprae* preparations (data not shown).

antigen specific lysis of Schwann cells by CD4⁺ Th1 like clones

CD4⁺ Th1 like cells often have potent cytolytic activity towards a range of host cells, including non-professional APC (Ottenhoff *et al.* 1988; Kaplan *et al.* 1989; Hancock

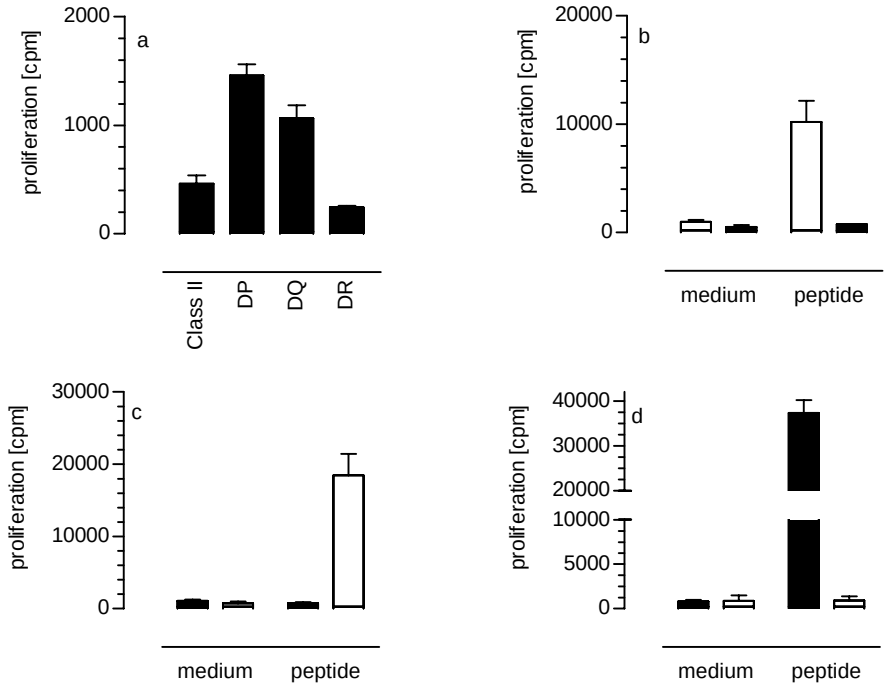

figure 6: Presentation of mycobacterial antigens by human Schwann cells. a) Human Schwann cells (HSW) are able to present peptides to CD4⁺ positive T cells (D1B2). Optimal presentation is achieved at cell densities between 1000 and 2000 Schwann cells per well. b) Pulsing for 40 hours enhances antigen presentation of both peptides and proteins. c-d) In addition to peptide, protein and M. leprae sonicate are also presented efficiently. Schwann cell/T cell couples used in the latter two experiments are HLA-DR11 restricted combination HSW/D1B2 (c) and the HLA-DR3 restricted combination NCN25/Rp15 1-1 (d).

et al. 1989; Kaleab *et al.* 1990b; Mutis *et al.* 1993a). We therefore investigated whether Schwann cells are susceptible to killing by CD4⁺ Th1 cells during Ag presentation. As shown in figure 7, *M. leprae* pulsed Schwann cells are highly susceptible to killing by type-1 CD4⁺ T cell clones from leprosy patients. Killing was antigen dependent and HLA-DR restricted.

Although human Schwann cells express FAS (figure 8a) and are susceptible to ATP mediated lysis (figure 8b), apoptosis-inducing FAS antibodies were not able to induce Schwann cell killing. The ATP inhibitor hexokinase strongly reduced ATP-, but not T-cell-mediated lysis of Schwann cells in response to specific peptides or to the mitogenic lectin concanavalin-A (figure 8b-c). Addition of $MgCl_2$-EGTA, however, reduced antigen specific, relevant for other inflammatory peripheral neuropathies. Furthermore, our data suggest that antagonism of molecular interactions between *M. leprae*, Schwann cells and inflammatory T cells provides a rational strategy to prevent Schwann cell and nerve damage in leprosy. T-cell-mediated lysis by approximately 35-40%, indicating that granule-mediated, but not FAS- or ATP-dependent, lysis is likely an important pathway for T-cell-mediated Schwann cell killing (figure 8d).

discussion

Using *ex vivo* isolated, cultured human Schwann cells that express appropriate and specific Schwann cell markers, we show here that human Schwann cells can take up,

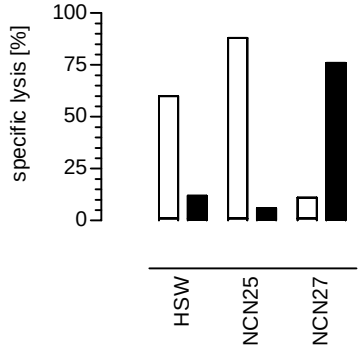

figure 7: M. leprae Ag presentation by human Schwann cells to CD4+ cytotoxic T cells results in HLA-DR dependent Schwann cell killing. Schwann cell cultures HSW (HLA-DR4, HLA-DR11), NCN25 (HLA-DR3, HLA-DR11), and NCN27 (HLA?DR2) were pulsed with peptides and incubated with T cell clone D1B2 (white bars, HLA?DR11 restricted) or T cell clone R2F10 (solid bars, HLA?DR2 restricted). Experiments were performed in quadruplicate using E:T ratios of 40:1.

process, and present mycobacterial antigens to MHC class II restricted $CD4^+$ cytotoxic T cells. Subsequently, Schwann cells are killed in an antigen specific fashion by *M. leprae* specific, inflammatory $CD4^+$ T cells. Molecules involved in co-stimulation and adhesion of T cells were strongly expressed by human Schwann cells and could be up-regulated by IFN-γ. Our findings indicate that human Schwann cells can act as non-professional antigen presenting cells and can therefore be a direct target for cytotoxic T cells in leprosy. This mechanism may play an important role in the immunopathogenesis of Schwann cell- and nerve damage in leprosy, particularly during inflammatory, $CD4^+$ T cell mediated reversal reactions. Analysis of leprosy lesions strongly suggests that $CD4^+$ T cells with cytotoxic capacity are abundantly present in lesions of patients with type 1 leprosy reactions (Cooper *et al.* 1989).

Presentation of *M. leprae* antigens resulted in both T cell proliferation and T cell-mediated Schwann cell lysis. Secretion of cytotoxic granules, a mechanism that not only leads to host cell lysis but also may reduce the viability of intracellular bacteria (Stenger *et al.* 1997), most likely is responsible for Schwann cell lysis, based on the observation that lysis could be inhibited by EGTA-$MgCl_2$, but not by the ATP inhibitor hexokinase. Moreover, although FAS was expressed by human Schwann cells, we were unable to demonstrate that this receptor was functional.

HLA class II expression by Schwann cells has been reported in lesions of patients with various neuropathies such as hereditary motor and sensory neuropathy type 1 and chronic inflammatory demyelinating polyradiculoneuropathy, (Cadoni *et al.* 1986; Pollard *et al.* 1986; Mancardi *et al.* 1988; Mitchell *et al.* 1991). This is also likely the case in inflamed neural tissue (Narayanan *et al.* 1990). Under inflammatory conditions infiltration of IFN-γ producing $CD4^+$ Thelper-1 cells, HLA class II expression was reported to be induced on rat Schwann cells *in vivo* (Bergsteinsdottir *et al.* 1992). Similarly, exposure to *M. leprae* can increase MHC class II expression by rat Schwann cells (Kingston *et al.* 1989). However, an electron microscopical study failed to demonstrate MHC class II expression on Schwann cells in leprosy lesions (Cowley *et al.* 1990).

Recent studies have indicated that *M. leprae* targets Schwann cells by binding uniquely to the extracellular matrix protein laminin-α2 (Rambukkana *et al.* 1997), which ligates to α/β dystroglycan receptors on the Schwann cell surface (Rambukkana *et al.* 1998). Our observation that human Schwann cells are also able to phagocytose *M. smegmatis*, however, may imply that mycobacteria, including possibly also *M. leprae*, could be internalized by Schwann cells through alternative receptor mediated uptake mechanisms. Since laminin-α2 is only present in the basal lamina of Schwann cells and *M. leprae* has a high affinity receptor for laminin-α2 (Shimoji *et al.* 1999), being a 21 kDa histone like protein (HLP), this mechanism has been proposed to explain the neural tropism of *M. leprae* (Rambukkana *et al.* 1997). However, soluble α-dystroglycan was able to block the interaction between laminin-α2 coated *M. leprae* and Schwann cells only partly.

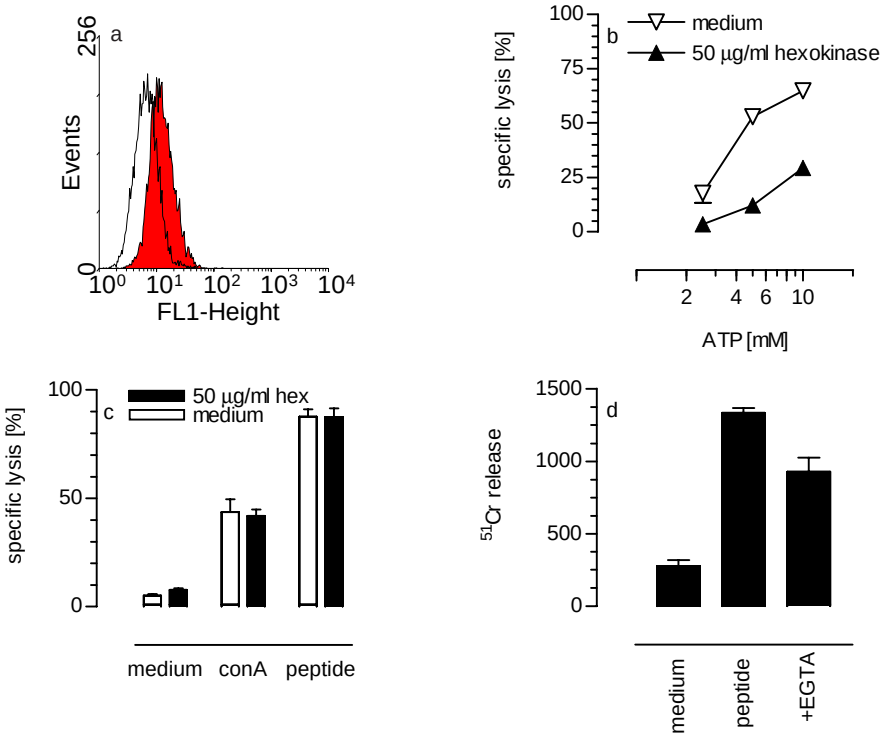

figure 8: Susceptibility of human Schwann cells to FAS-, ATP-, and granule-mediated cytotoxicity. a) Human Schwann cells express FAS molecules on their surface. Cytotoxicity inducing FAS antibodies, however, were unable to kill Schwann cells (not shown). Schwann cells are susceptible to extracellular ATP induced cell lysis (b, open triangles). Addition of ATP-inhibitor hexokinase (solid triangles) reduced lysis by exogenously added ATP (b), but not by T cells, either in response to specific peptides or to the mitogen con A (c). d) MgCl2 + EGTA blocked antigen dependent T cell mediated Schwann cell lysis with ± 35%, indicating that human Schwann cells are susceptible to granule mediated lysis.

Moreover, other mycobacteria, including *M. tuberculosis* and *M. smegmatis*, have HLP homologues. Therefore other molecules present on Schwann cells may be involved in interaction with *M. leprae*. Candidates are Fc receptors (Vedeler *et al.* 1989), CR1 (Vedeler *et al.* 1989; Schorey *et al.* 1997), the fibronectin binding protein (Schorey *et al.* 1995), and mannose receptors (Schlesinger 1993). The role of these interactions in Schwann cell binding and phagocytosis of *M. leprae in vivo* and *in vitro* remains to be clarified.

Taken together, this study reveals a novel and potentially important mechanism of cytotoxic T cell mediated Schwann cell damage in leprosy. This mechanism may also be relevant for other inflammatory peripheral neuropathies. Furthermore, our data suggest that antagonism of molecular interactions between *M. leprae*, Schwann cells and inflammatory T cells provides a rational strategy to prevent Schwann cell and nerve damage in leprosy.

chapter 2b
allorecognition of artificial nerve guides filled with human Schwann cells – an *in vitro* pilot

Eric Spierings, Carmen L. A. M. Vleggeert-Lankamp, Enrico Marani,
Ralph T. W. M. Thomeer, and Tom H. M. Ottenhoff

Transplantation 2000. **69:** 455-456

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In the surgical repair of peripheral nerve injury, a gap between the proximal and the distal nerve stump is frequently encountered. The standard treatment involves bridging the gap with nerve autografts. These grafts, like the Wallerian degenerated distal nerve part, consist of empty tubes and Schwann cells (Francel *et al.* 1997). Regenerating axons are able to grow through these tubes and reconnect with their target organs. However, disadvantages of harvesting nerve autografts are donor site morbidity, limited supply, and prolonged surgical procedures (Keeley *et al.* 1993). Therefore, research has been focused on the production of artificial nerve grafts to bridge these gaps. In optimizing these grafts, adding Schwann cells to the lumen has been proven successful in rats (Keeley *et al.* 1993). Schwann cells secrete growth factors and produce extracellular matrix proteins, which are both stimulatory agents in nerve regeneration (Woolley *et al.* 1990). Artificial tubes can easily be filled with *in vitro* cultured Schwann cells from inbred rat strains. Rejection of allografted rat Schwann cells, however, has previously been reported (Gulati 1995). Similarly, when artificial nerve guides filled with allogenic Schwann cells have to be implanted in humans, the problem of immunological recognition is raised. We performed a pilot experiment to test allorecognition of human Schwann cells of two different hosts using peripheral blood mononuclear cells (PBMC) of HLA-DR matched and mismatched individuals.

Primary human Schwann cells were isolated and propagated as described before (van den Berg *et al.* 1995). Schwann cells were seeded into 96-wells, roundbottom plates (Greiner GmbH, Solingen, Germany) at a density of 500 cells per well. PBMC of 6 individuals were isolated using a Ficoll gradient and added at a density of 150.000 PBMC per well in triplicate. After 5 days, proliferation was measured by incorporation of ^{3}H-Thymidine, which was added 16 hours the assay was terminated. Significant differences were observed between proliferation of PBMC of matched and mismatched individuals (figure 1). Allorecognition was absent in HLA-DR compatible combinations. However, when PBMC were mismatched in one or both alleles, an identical increase in response was observed.

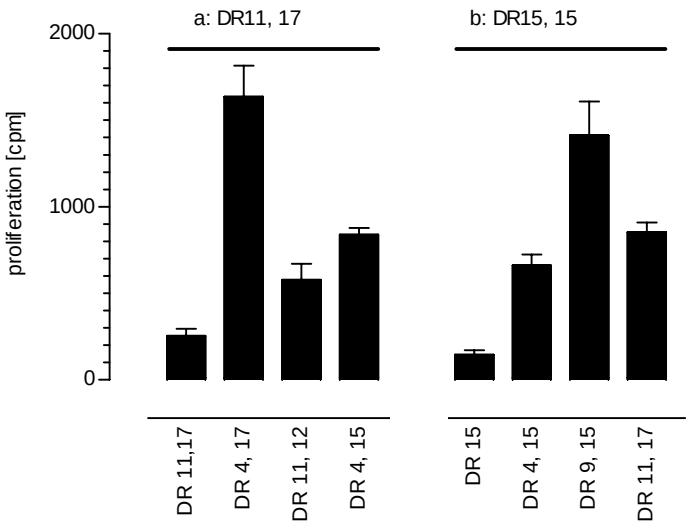

figure 1: Allorecognition of primary human Schwann cell cultures a and b by matched and mismatched PBMC. Results are depicted as mean counts/minute ± SEM. HLA-DR type of PBMC and Schwann cells are given in the graph. Allorecognition is equally present when PBMC are mismatched for one or two alleles and absent when matched.

Our results strongly suggest that implanted artificial nerve guides filled with allogenic human Schwann cells are likely to be rejected. Even mismatching of one of the DR-alleles results in a huge immunological response, showing its forceful character. To avoid lifelong administration of immunosuppressive drugs, the problem of allorecognition and subsequent rejection of the graft in humans could, theoretically, be overcome in different ways. First, Schwann cells could be collected from HLA-DR typed donors (postmortem). Otherwise, a piece of a small cutaneous nerve with neglectible sensory deficit may be taken from the patient to generate an artificial graft filled with cultured autologous Schwann cells. This method, however, not only requires two surgical interventions, but also the certainty at the time of presentation of the case that immediate repair is not likely to give better results. In elaborating this option, it would be interesting to analyze whether Schwann cell cultures can also be generated from the distal nerve stump, which could be tested in animal models first.

chapter 3
human Schwann cells produce immunomodulatory cytokines that regulate mycobacterium specific T helper-1 type responses

Eric Spierings, Tjitske de Boer, Enrico Marani,
and Tom H. M. Ottenhoff

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abstract

Peripheral nerve damage is a major complication of leprosy and often occurs during leprosy reactions, leading to permanent loss of nerve function. Peripheral nerve fibers are surrounded by Schwann cells, which are known to be predilection sites of *M. leprae*. Immune mediated destruction of infected Schwann cells may thus play a key role in the pathogenesis of leprosy nerve damage. We recently established a novel model to study human Schwann cell/T cell interactions *in vitro* and showed that type-1 T cells can efficiently kill Schwann cells in an *M. leprae* dependent fashion. Besides being facultative antigen presenting cells (APC), Schwann cells may also produce a variety of immunomodulatory cytokines that may influence local immune reactivity. Here we have investigated the production of pro- and anti-inflammatory cytokines by human Schwann cells and examined their influence on *Mycobacterium leprae* induced T cell activation. An unexpected observation was that human Schwann cells are able to produce IL-4 and IL-10, as determined by ELISPOT. To the best of our knowledge, this is the first demonstration that human IL-4 can be produced by non-hematopoietic cells. Both IL-4 and IL-10 were inducible by *M. leprae* only, but not by *Escherichia coli* derived LPS. Importantly, IL-4 neutralizing antibodies augmented Th1 cell proliferation when added to Schwann cells, but not when macrophages were applied as APC, thus demonstrating that production of endogenous IL-4 by *M. leprae* exposed Schwann cells can inhibit *M. leprae* specific T cell activation.

M. leprae as well as LPS were found to induce the production of IL-6, IL-8, and PGE_2 by Schwann cells, whereas TGF-β was produced constitutively. Production of RANTES was only induced by LPS but not *M. leprae*. No expression of IL-1β, IL-12, IL-18, TNF-α or IFN-γ could be detected in human Schwann cells. In line with these observations, neutralizing antibodies to IL-12 or IL-18 did not inhibit T cell proliferation induced by *M. leprae* pulsed Schwann cells, whereas these antibodies strongly inhibited T cell responsiveness to *M. leprae* presented by macrophages, indicating that Schwann cells fail to produce type-1 stimulatory cytokines. Our results demonstrate that human Schwann cells predominantly produce anti-inflammatory type-2 like factors, and fail to produce detectable levels of major type-1 cytokines. Thus, Schwann cells may act as type-2 antigen presenting cells. In this fashion Schwann cells likely create a peripheral nerve-tissue specific, immune privileged environment that skews cellular immune responses towards a Th2 type, rather than a potentially noxious, inflammatory Th1 dependent reaction.

introduction

Nerve damage is one of the most prominent and feared features of leprosy. Neuritis occurs in patients across the entire leprosy spectrum, but particularly during reversal reactions. Reversal reactions are observed in approximately 25% of all leprosy patients and represent a temporal upgrade of cellular immunity (Modlin *et al.* 1983). T cells in the granulomas are predominantly $CD4^+$, whereas $CD8^+$ T cells are mostly present in the surrounding mantle area (Modlin *et al.* 1983; Narayanan *et al.* 1984; Cooper *et al.* 1989). Strong increases of type-1 cytokines have been reported during reversal reactions (Cooper *et al.* 1989; Yamamura *et al.* 1991; Yamamura *et al.* 1992). T cell clones isolated from skin and nerve biopsies of patients with reversal reactions are predominantly of Th1-type (Verhagen *et al.* 1998; Verhagen *et al.* 1997; Spierings *et al.* Unpublished). Moreover, the microanatomical location of serine esterase positive cells within tuberculoid granulomas and reversal reactions overlaps with that of the $CD4^+$ $CD45RO^+$ subpopulation (Cooper *et al.* 1989). In line with these observations, *Mycobacterium leprae* reactive $CD4^+$ cytotoxic T cell clones have been shown to produce serine esterase and to be highly cytotoxic (Mutis *et al.* 1993a). Thus, Th1 like cytotoxic T cells are believed to

play a major role in the immunopathology of leprous neuritis.

Human Schwann cells are predilection sites for *M. leprae* (Boddingius 1974). The neurotropism of *M. leprae* has been attributed to specific binding of the bacillus to the α2-chain of laminin-2, a molecule that is almost exclusively expressed in the lamina basalis of Schwann cells (Rambukkana *et al.* 1997; Rambukkana *et al.* 1998). *M. leprae* infected Schwann cells can be targets for recognition by cytotoxic $CD8^+$ and $CD4^+$ T cells in mice (Steinhoff and Kaufmann 1988) and humans (Spierings *et al.* chapter 2a). Nothing is known, however, about the potential immunomodulatory role of human Schwann cells during infection and antigen presentation to T cells. Schwann cells may play an active role in modulating local T cell mediated inflammatory responses via the production of inhibitory or enhancing cytokines. Studies in mice and rats have revealed that rodent Schwann cells are able to produce interleukin(IL)-1β (Bergsteinsdottir *et al.* 1991), IL-6 (Bolin *et al.* 1995), Transforming Growth Factor-β (TGF-β) (Skoff *et al.* 1998), and Prostaglandin E_2 (PGE_2) (Constable *et al.* 1994). Furthermore, studies on human biopsy material suggested the production of IL-12 (Turka *et al.* 1995). These cytokines may modulate immune responses under pathological and non-pathological conditions.

Here we have studied the role of human Schwann cells in an *in vitro* model, developed to define immunopathogenic mechanisms of leprosy nerve damage. The capacity of Schwann cells to produce chemokines and pro- and anti-inflammatory cytokines was investigated, as well as the role of these cytokines in Schwann cell/T cell interaction.

materials and methods

reagents

M. leprae sonicate was provided through the WHO/IMMLEP programme by Dr. P. J. Brennan (Colorado State University, Fort Collins, CO). LPS of *E. coli* was purchased from Sigma (Sigma Aldrich, St. Louis, MO). Anti IL-12 and anti IL-18 antibodies were gifts of Dr. G. Trinchieri (Wistar Institute, Philadelphia, PA) and Dr. M. Kurimoto (Fujisaki Institute, Okayama, Japan) respectively. Recombinant IL-12 and IL-18 were purchased from R&D (R&D Systems, Minneapolis, MN). IL-4 antibody MP4-25D2 and anti IL-10 antibody JES3-9D7 were purchased from Pharmingen (Pharmingen, San Diego, CA) and Genzyme (Genzyme Diagnostics, Cambridge, MA) respectively.

Schwann cell cultures

Human Schwann cells were isolated from sural nerve biopsies and propagated as described before (van den Berg *et al.* 1995). Briefly, the sural nerve specimens were cut into small pieces and incubated in 85% Iscove's modified DMEM (IMDM) (GIBCO BRL, Grand Island, NY), 10% lymphokine activated killer (LAK) cell supernatant (Lamers *et al.* 1992), 5% fetal calf serum (FCS), 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml phytohemagglutinin (PHA) (Murex Diagnostics, Dartford, UK). The nerve fragments were incubated in humidified 5% CO_2 at 37°C. After 10 days the non-adherent cells were removed and the adherent cells were propagated in medium consisting of 85% IMDM with 0.6% glucose (Sigma), 10% FCS, and 5% LAK cell supernatant.

cytokine induction

Human Schwann cells were harvested using trypsin-EDTA, and seeded at 2000 cells per well into 96 wells flat bottom plates (Greiner GmbH, Solingen, Germany). Cells were allowed to adhere for 24 hours at 37°C, 5% CO_2. After replacement of medium with IMDM, 10% FCS, cells were stimulated with LPS (100 ng/ml) or *M. leprae* sonicate (25 μg/

ml) and incubated for 48 hours. Supernatants were collected and stored at -20°C until analysis.

ELISA

Production of cytokines by cultured human Schwann cells was measured in the culture supernatant by ELISA. The detection level of each cytokine detection assay is given in brackets. For IL-4 (1 pg/ml), IL-6 (2 pg/ml), IL-8 (10 pg/ml), and IL-10 (5 pg/ml) measurement a sandwich ELISA was used (Pelikine, CLB, Amsterdam, The Netherlands). IFN-γ (100 pg/ml) (Boonstra *et al.* 1997), TGF-β (1 pg/ml), TNF-α, (100 pg/ml) MCP-1 (400 pg/ml) (van den Berg *et al.* 1998) and RANTES (50 pg/ml) (Deckers *et al.* 1998) sandwich ELISA were performed as described before. IL-12 (20 pg/ml), IL-1β (5 pg/ml), and PGE_2 (30 pg/ml) production was measured using Quantikine ELISA kits (R&D Systems). IL-18 ELISA (30 pg) was kindly provided by Dr. M. Kurimoto (Fujisaki Institute, Okayama, Japan).

ELISPOT assay

IL-4 and IL-10 secreting cells were detected and quantified by ELISPOT assay using human IL-4 and IL-10 ELISPOT systems (U-CyTech BV, Utrecht, The Netherlands), as described before (van der Meide *et al.* 1995) with minor modifications. After coating overnight with IL-4 or IL-10 specific monoclonal antibodies at 4°C, Schwann cells were added at a density of 500 cells/well, together with either medium, LPS (100ng/ml), or *M. leprae* sonicate (25 μg/ml) in triplicate. Schwann cells were removed 48 hours later by addition of ice water for 30 minutes. Subsequently, the wells were washed five times with PBS/0.01% tween. Visualization of IL-4 or IL-10 producing cells was performed as recommended by the manufacturer. The spots were examined under low magnification (7x). The number of cytokine producing cells was expressed per 1000 Schwann cells.

RT-PCR

Total RNA was isolated from trypsinized Schwann cell cultures stimulated with LPS or *M. leprae* using the High Pure RNA isolation kit following the instructions of the manufacturer (Boehringer Mannheim GmbH, Mannheim, Germany). First strand cDNA was generated from oligo(dT) primed RNA by incubation at 42°C for 60 minutes in 20 μl RT mix containing 75 mM KCl, 50 mM Tris-HCl (pH 8.3), 3 mM $MgCl_2$, 0.4 mM of each dNTP, 10 units AMV reverse transcriptase, and 25 units RNasin (Promega, Madison, WI).

All PCR reactions contained 20 pmol of each primer in 50 μl of a reaction mixture containing 10mM Tris/HCl (pH 8.4) buffer, 50 mM KCl,4mM $MgCl_2$, 0.5 mM dNTPs and 1 unit Taq polymerase (Perkin Elmer Roche Molecular Systems Inc., Branchburg, NJ). Primers used are listed in table 1.

Amplification started with a denaturation step of 5 min at 95°C. Cycling conditions were 95°C for 30 s, 50°C for 30 s, and 72°C for 90 s for 35 cycles, followed by an extension of the last step with 5 min. Following amplification, 10 μl of each PCR product was applied to a 2% agarose gel, visualized under UV after ethidium bromide staining and photographed.

antigen presentation assay

Schwann cells were collected and seeded 2000/well as described above. After 24 hours, 25 μg/ml *M. leprae* sonicate was added to the cells in IMDM, 10% human serum. After pulsing with antigens for 40 hours, cells were washed three times with IMDM and subsequently co-cultured for 88 hours with 10^4 T cells. T cell clone R1E4 recognizing HSP70 of *M. leprae* has been described before (Janson *et al.* 1991). Sixteen hours before

termination of the assay, 50 µl supernatant was collected for IFN-γ measurement. Subsequently, 10 µCi ^{3}H-Thymidine per well was added. The incorporation of ^{3}H-Thymidine was assayed by liquid scintillation counting. Anti-IL-12 (1 µg/ml), anti-IL-18 (1 µg/ml), anti-IL-4 (20 µg/ml, 5.0 µg/ml, and 1.25 µg/ml), rIL-12 (2.5 µg/ml) and rIL-18 (100 ng/ml) were added to the cultures for determination of the production of these cytokines.

results

cytokine ELISA

Cytokine production by human Schwann cells was determined after stimulation with LPS or *M. leprae*. No IFN-γ, TNF-α, IL-12, IL-18 or IL-1β production could be detected by ELISA (figure 1). PGE_2 (figure 2c), TGF-β (figure 2d), IL-6 (figure 3a) and MCP-1 (figure 3d) were produced constitutively in the absence of stimuli, while low levels of RANTES (figure 3c) and IL-8 (figure 3b) were detectable prior to stimulation. Stimulation with *M. leprae* enhanced IL-6, IL-8, and PGE_2 production but had no effect on the production of RANTES, MCP-1, and TGF-β. Like *M. leprae*, stimulation with LPS raised production of IL-6, IL-8, and PGE_2. In contrast to *M. leprae*, LPS induced the production of RANTES and MCP-1 (figure 3c-d).

detection of IL-4 and IL-10 production by ELISPOT assay

To determine the production of IL-4 and IL-10 a more sensitive technique was used: different human Schwann cell cultures were tested for IL-4 and IL-10 production after stimulation with LPS or *M. leprae* in ELISPOT. As shown in figure 2a-b and figure 4, *M. leprae* was able to induce IL-4 and IL-10 production in a significant number of Schwann cells. In contrast, LPS failed to induce significant production of IL-4 or IL-10 by Schwann cells. All Schwann cell cultures showed similar numbers of IL-4 producing cells. IL-4 staining was very intense, suggesting that positive cells produced relatively high amounts of IL-4. The intensity of IL-10 spots was weaker than in the case of IL-4 (figure 4), but the number

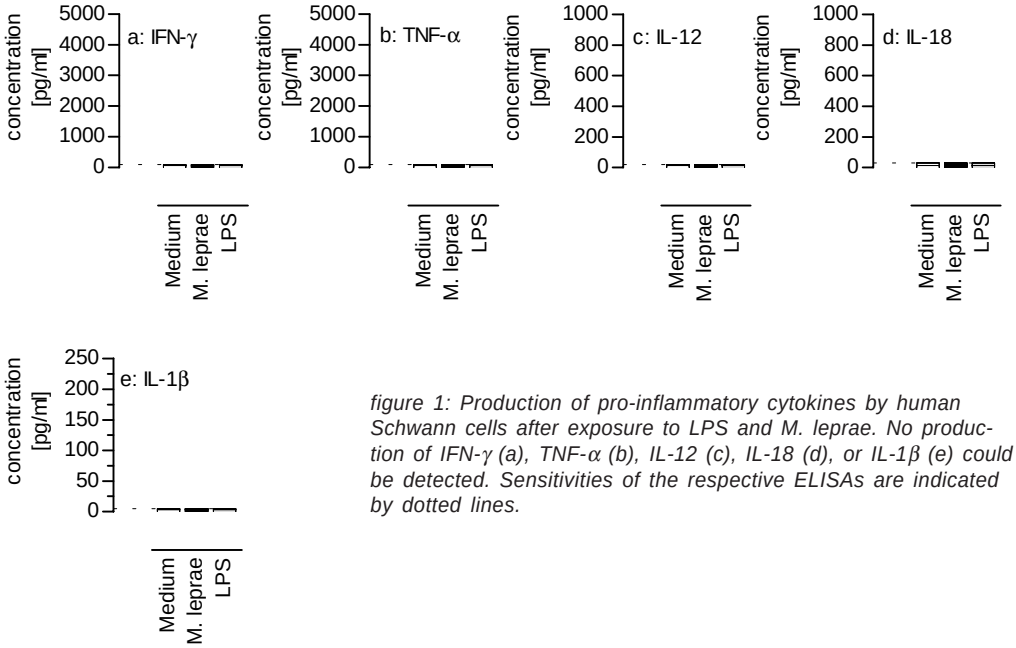

figure 1: Production of pro-inflammatory cytokines by human Schwann cells after exposure to LPS and M. leprae. No production of IFN-γ (a), TNF-α (b), IL-12 (c), IL-18 (d), or IL-1β (e) could be detected. Sensitivities of the respective ELISAs are indicated by dotted lines.

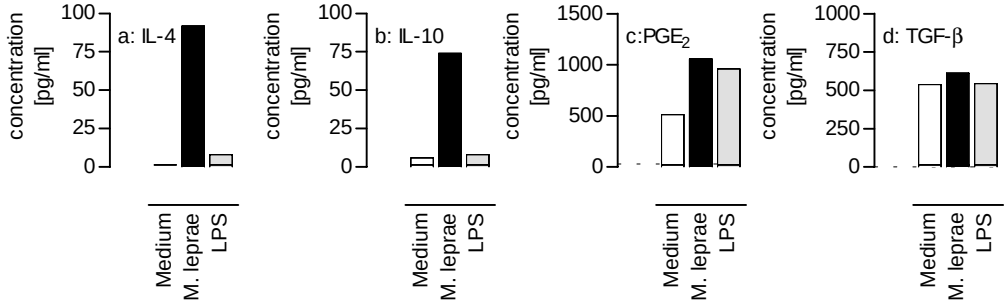

figure 2: Production of type 2 factors by human Schwann cells after exposure to LPS and M. leprae as tested in ELISPOT (IL-4 and IL-10) and ELISA (PGE_2 and TGF-β). M. leprae but not LPS was found to induce IL-4 (a) and IL-10 (b) production by human Schwann cells. Increased levels of PGE_2 can be found after stimulation with both M. leprae and LPS (c). TGF-β is constitutively produced and the production remains unchanged after addition of M. leprae or LPS (d). Sensitivities of the PGE_2 and TGF-β ELISAs are indicated by dotted lines.

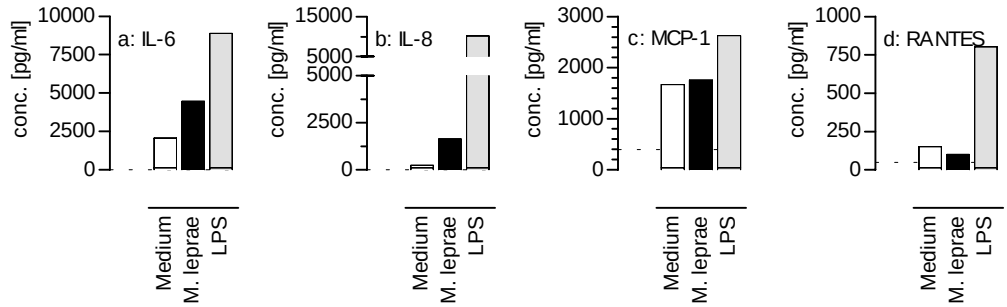

figure 3: Production of chemoattractants by human Schwann cells after exposure to LPS and M. leprae. Both M. leprae and LPS induce IL-6 (a) and IL-8 (b) production by human Schwann cells. Increased levels of RANTES were found after stimulation with LPS, but not M. leprae (c). MCP-1 is constitutively produced and can only be enhanced by LPS (d). Sensitivities of the respective ELISAs are indicated by dotted lines.

of positive spots was of the same magnitude (figure 2). Induction IL-10 by *M. leprae*, but not by LPS was confirmed by RT-PCR (figure 5).

significance of IL-4 in repressing T cell proliferation

IL-4 is a type-2 cytokines that inhibits Th1 development and function. In order to investigate whether IL-4 produced by Schwann cells is able to modulate Th1 responses, IL-4 neutralizing antibodies and control antibodies were used. Proliferation of a $CD4^+$ Th1 type T cell clone which does not produce IL-4 itself, increased threefold in the presence of anti IL-4 but not in case of control antibodies (figure 6a). Enhancement of T cell proliferation was dependent on the dose of IL-4 antibodies added. In contrast, addition of IL-4 antibodies did not enhance T cell proliferation when HLA-DR matched PBMC derived monocytes were used as APC, ruling out that IL-4 antibodies are mitogenic and confirming that macrophages do not produce IL-4 in the presence of *M. leprae*. These results strongly suggest that IL-4 production is specifically induced by *M. leprae* in human Schwann cells and has a direct inhibitory effect on Th1 activation.

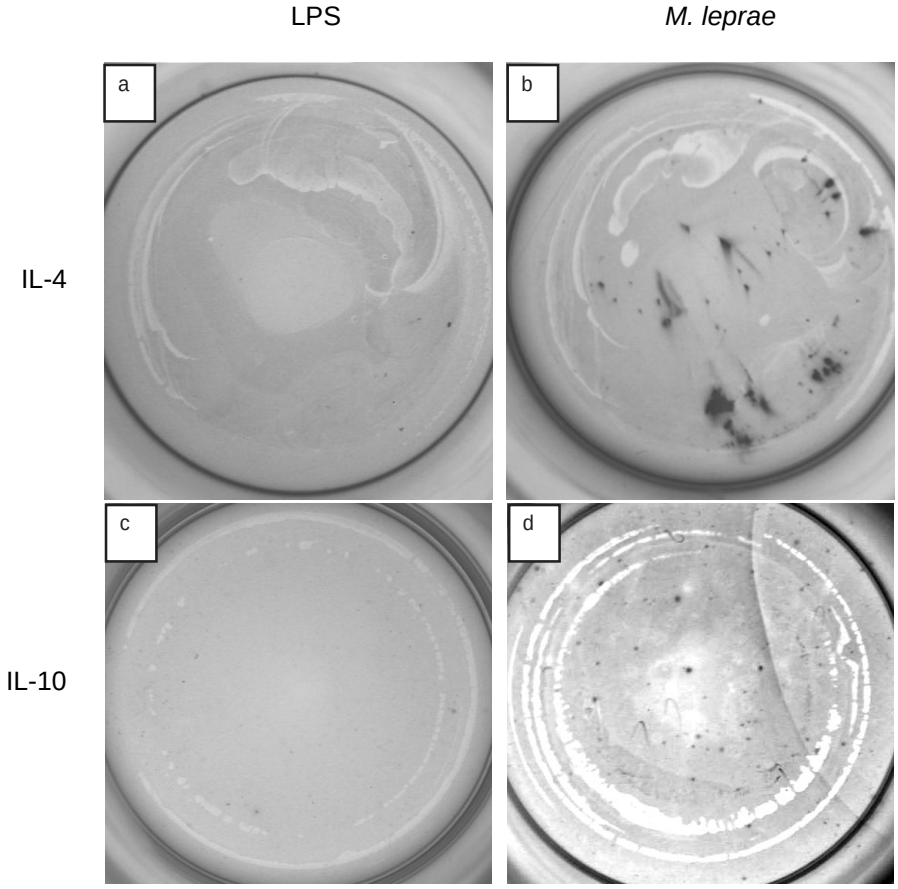

figure 4: ELISPOT analysis of IL-4 and IL-10 producing human Schwann cells, after exposure to LPS (a and c) and M. leprae (b and d). Only few small spots could be observed for IL-4 in case of LPS (a). Both the number and the size of the spots increased dramatically when Schwann cells were exposed to M. leprae (b). Similar results were obtained for IL-10 (c and d).

IL-12 and IL-18 are not produced by human Schwann cell in vitro

To exclude the production of low amounts of the Th1 cytokines IL-12 and IL-18 and to further document the type-2 cytokine phenotype of human Schwann cells, a functional assay was performed using neutralizing antibodies to IL-12 and IL-18. Addition of anti-IL-12 and anti-IL-18 antibodies had no effect on T cell proliferation induced by Schwann cells (figure 7a), confirming that human Schwann cells are unable to produce IL-12 or IL-18 *in vitro* after exposure to *M. leprae*. In contrast, anti-IL-12 and anti-IL-18 antibodies strongly inhibited T cell proliferation when using monocytes as antigen presenting cells, showing that these antibodies are able to inhibit regular Th1 responses (figure 7b). Addition of the rIL-12 or IL-18 to T cell/Schwann cell co-cultures dramatically increased T cell responses to *M. leprae* (figure 7c-d), demonstrating that IL-12 and IL-18 are limiting factors in Schwann cell mediated antigen presentation.

mRNA	primer
IL-10 sense	5'-AAATTTGGTTCTAGGCCGGG-3'
IL-10 antisense	5'-GAGTACAGGGGCATGATATC-3'
β2-m sense	5'-CAGCAGAGAATGGAAAGTC-3'
β2-m antisense	5'-GATCGCTGCTTACATGTCTCG-3'

table 1: PCR primers used for the detection of IL-10 mRNA. β2-microglobulin primers were used as control.

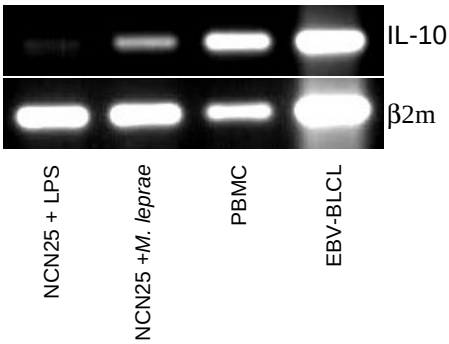

figure 5: RT-PCR analysis of IL-10 mRNA expression by human Schwann cells after exposure to LPS (lane 1) and M. leprae (lane 2). IL-10 mRNA was hardly detectable after a 48 hours period LPS exposure, while M. leprae induced detectable levels of IL-10 mRNA. Levels of β2-microglobulin mRNA were similar under both conditions. PBMC and Epstein-Bar Virus transformed B cells were used as positive controls.

discussion

This study documents the cytokine-profile and -function of *in vitro* cultured human Schwann cells after exposure to *M. leprae*. Schwann cells appeared to produce high levels of IL-6 and IL-8, which are chemoattractants, and IL-4, IL-10, TGF-β and PGE_2, factors involved in regulating helper T cell responses. Production of IL-12 and IL-18, which are involved in augmenting Th1 cellular immunity, could not be detected. Human Schwann cells are likely to play a crucial role in the immunopathogenesis of nerve damage in leprosy. Presentation of *M. leprae* antigens to cytotoxic $CD4^+$ or $CD8^+$ T cells can result in Schwann cell killing in mice (Steinhoff and Kaufmann 1988) and in humans (Spierings *et. al.* chapter 2a). The release of particular cytokines by Schwann cells may have important consequences for the outcome of local immune responses.

Our results indicate that Schwann cells may directly regulate *M. leprae* T cell responses in leprosy lesions. For the first time, we here demonstrate production of human IL-4 by non-hematopoietic cells. Production of IL-4, as well as IL-10, was induced only after exposure of Schwann cells to *M. leprae*, but not to LPS. Moreover, neutralizing IL-4 antibodies strongly enhanced T cell proliferation of a Th1 clone recognizing *M. leprae* antigens presented by human Schwann cells. No such effect was observed when monocytes were used as APC, strongly suggesting that IL-4 production is specifically induced by *M. leprae* in human Schwann cells but not monocytes, and has a direct regulatory role in Th1 responsiveness. IL-4 and IL-10 are known to suppress Th1 responses (Salgame *et al.* 1991; Sieling *et al.* 1993; Moore *et al.* 1993). IL-10 suppresses macrophage functions on various levels, including MHC class II expression (de Waal Malefyt *et al.* 1991; Koppelman *et al.* 1997) and pro-inflammatory cytokine production (Gazzinelli *et al.* 1996; Neyer *et al.* 1997), and has been suggested to be responsible for the latent phase during mycobacterial infection (Murray 1999). Moreover, IL-10 drives the generation of a recently described $CD4^+$ T cell subset, designated regulatory T cells 1 (Tr1), which suppresses antigen-specific immune responses and actively down-regulates a pathological immune response *in vivo* (Groux *et al.* 1997). In this way, *M. leprae* infected Schwann cells may contribute directly to local suppression of Th1 dependent DTH-like cellular immune reactions that may have detrimental consequences for peripheral

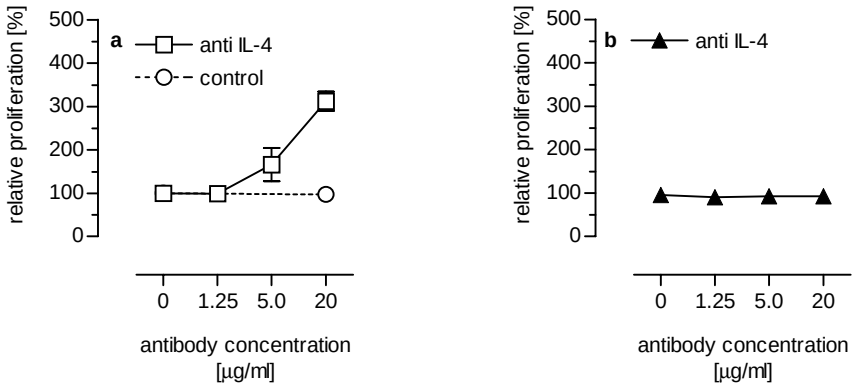

figure 6: Enhancement of M. leprae specific Schwann cell induced T cell proliferation of Th1 clone R1E4 in the presence of neutralizing IL-4 antibodies (a). Antibodies recognizing HLA-DP did not enhance T cell proliferation (open triangles), showing the specific effect of IL-4 antibodies. No enhancement was observed when macrophage were used as APC, excluding a mitogenic effect of IL-4 antibodies (b). T cell proliferation in response to M. leprae in the absence of antibodies were 21000 cpm and 11000 cpm for Schwann cells and macrophages respectively, while medium values were 475 cpm and 113 cpm respectively.

nerve tissue. Human Schwann cells constitutively produce TGF-β. TGF-β attenuates the IL-12 responsiveness of Th1 cells via down-regulation of IL-12Rβ1 and IL-12Rβ2 expression (Gorham *et al.* 1998; Bright and Sriram 1998; Zhang *et al.* 1999), or via inhibition of IL-12 production (Skeen *et al.* 1996). In both cases IFN-γ production is reduced. Furthermore, TGF-β reduces MHC expression (Geiser *et al.* 1993) and suppresses iNOS and NO production (Vodovotz *et al.* 1996). Thus, like IL-4 and IL-10, TGF-β drives immune responses away from a likely detrimental Th1 pathway, such that DTH is suppressed. Similarly, the local release of TGF-β and IL-10 has been proposed to be responsible for the immune privileged status of the eye (D'Orazio and Niederkorn 1998) and may be of importance in reducing Th1 autoreactivity in the central nervous system (Schluesener 1990; Bai *et al.* 1997; Cua *et al.* 1999). Our *in vitro* data using neutralizing IL-4 antibodies strongly suggest that endogenous production of IL-4 by Schwann cells has a similar effects on Th1 responses in leprosy. The inability of Schwann cells to produce IL-12 and IL-18 strengthens the concept that Schwann cells are unable to promote Th1 responses, thus maintaining a Th1 immune privileged environment. Killing of *M. leprae* in the nerves by MDT possibly reduces the production of type 2 cytokines by Schwann cells, thus giving room for detrimental Th1 responses, resulting in enhanced predisposition to reactional episodes as indeed often observed during MDT.

Aside to IL-4 and IL-10, human Schwann cells also produce IL-6, but fail to secrete IL-12 and TNF-α. Thus, the cytokine profile of human Schwann cells strongly resembles that of type-2 dendritic cells (reviewed in (Kalinski *et al.* 1999)). Type-2 dendritic cells prime and polarize T cell responses towards a Th2 phenotype (Kalinski *et al.* 1997). PGE_2 has been found to be a key factor in inducing such type-2 dendritic cells. The secretion of PGE_2 by Schwann cells therefore may also be involved in the induction of a type-2 antigen presenting cell.

IL-6 has been reported to play a crucial role in cellular defense against mycobacteria (Appelberg 1994; Ladel *et al.* 1997) and other parasites (Kopf *et al.* 1994; Dalrymple *et al.* 1995). It is produced in an early stage of mycobacterial infection and inhibits bacterial growth in macrophages (Flesch and Kaufmann 1990; Orme *et al.* 1993). As shown in IL-6 -/- mice, IL-6 also attracts macrophages and T cells to the nervous system (Eugster *et al.* 1998) and might thus play a role in initiating the inflammation in

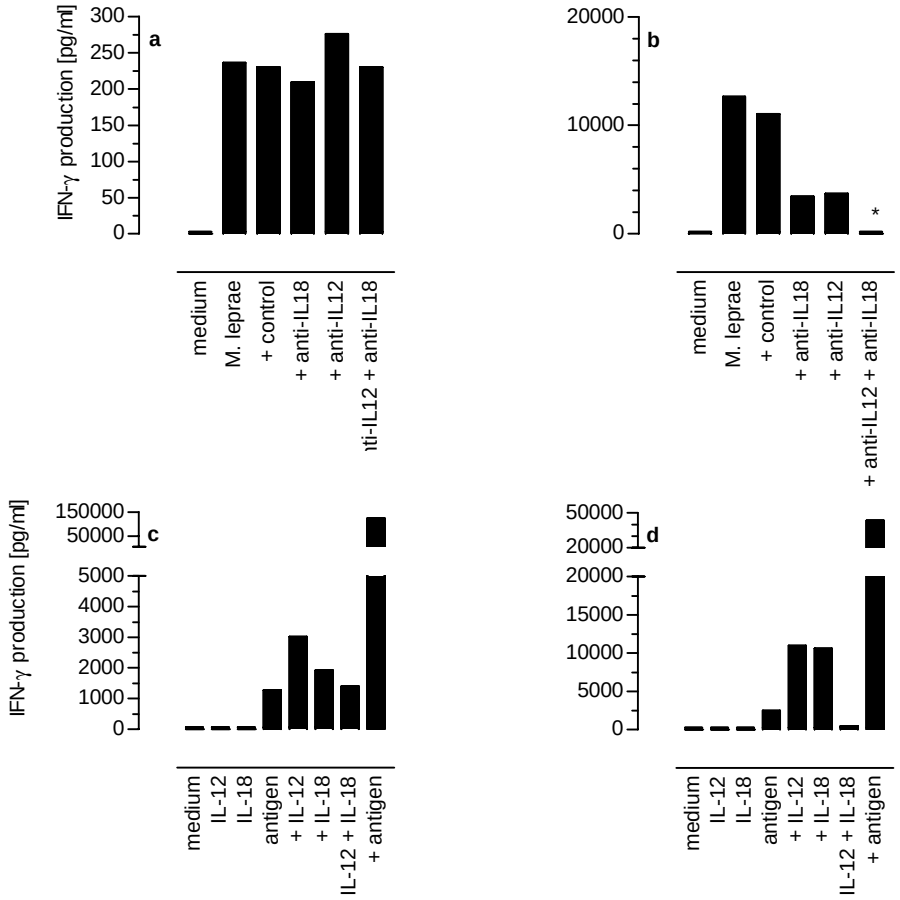

*figure 7: Lack of Schwann cell produced IL-12 and IL-18. (a) IL-12 and IL-18 neutralizing antibodies were unable to inhibit Schwann cell mediated antigen presentation, but strongly inhibit antigen presentation by monocytes as controls (b, * = not determined). (c) Addition of rIL-12, rIL-18 or the combination thereof two increases T cell responses in a similar way as with monocytes (d), showing that IL-12 and IL-18 are limiting factors in Schwann cell mediated antigen presentation.*

neural tissue. Monocytes and $CD4^+$ $CD45RO^+$ T cells are central components of reversal reaction granulomata (Cooper *et al.* 1989). IL-8 is able to recruite these cells and initiate local immune responses (Wilkinson and Newman 1992). The raise in chemokine production after infection of Schwann cells with *M. leprae*, may be an important event in the initiation of inflammatory responses in neural tissue.

The pronounced type-2 cytokine profile of human Schwann cells raises the question why Th1 and not Th2 cells are abundantly present in leprosy neuritis lesions. Even though exposure of Schwann cells to *M. leprae* induces IL-4 and IL-10, *M. leprae* infection of local monocytes and dendritic cells is likely to yield strong IL-12 and IL-18 production, resulting in strong enhancement of Th1 immune responses. Under such circumstances, the production of IL-4 and IL-10 by Schwann cells may be too low to influence this process significantly. It will be of interest to see whether host dependent differences in the production of IL-4 and IL-10 by Schwann cells in response to infection are related to inter-individual differences in susceptibility to nerve damage during leprosy reactions.

In summary, our findings indicate that human Schwann cells are able to up-regulate the production of various cytokines upon exposure to *M. leprae*. Some of the detected cytokines are chemokines that recruit T cells and macrophages. *M. leprae* failed to induce

Schwann cell production of the key type-1 cytokines IL-12 and IL-18, but instead enhanced production of IL-4, IL-10 and PGE_2, products which inhibit Th1 development. TGF-β was produced constitutively. We therefore hypothesize that Schwann cells represent type-2 APC, and that infection with *M. leprae* further enhances this type-2 phenotype. By such a mechanism, Schwann cells may be actively involved in the down-regulation of local DTH like cell mediated immune activation, which is a pronounced feature of leprosy pathology.

chapter 4

T cell subsets expressing Neural Cell Adhesion Molecule: association with antigen independent, MHC unrestricted T cell cytotoxicity in leprosy pathology

Eric Spierings, Tjitske de Boer, Tamara Dekker, Birhane Kaleab, Ben Naafs, William R. Faber, Pranab K. Das, and Tom H. M. Ottenhoff

abstract

Damage of skin and peripheral nerves are major pathological features of leprosy. T cells are believed to play an important role in the pathology of leprosy, but the responsible mechanisms have remained poorly understood. Cytolytic T cells can lyse Schwann cells and other target cells in an MHC restricted, *M. leprae* dependent fashion and may thus contribute to tissue damage in leprosy. However, nerve damage in leprosy has also been observed in the absence of bacilli, suggesting that auto-immune like mechanisms may contribute to the immunopathogenesis of leprosy as well. Here, we have investigated the role of Neural Cell Adhesion Molecule (N-CAM or CD56) in the killing of Schwann cells and other N-CAM positive targets by a human T cell subset that expresses N-CAM. Involvement of N-CAM expressing T cells in leprosy pathology was suggested by the observations that N-CAM expressing T cells could be isolated from inflamed neural tissue, and that antigenic stimulation of these cells with *Mycobacterium leprae* increased both the number of N-CAM$^+$ T cells and their cytolytic activity against N-CAM$^+$ target cells. Paired analyses of peripheral blood samples revealed much lower numbers of N-CAM$^+$ T cells and cytolytic activity in the periphery compared to neuritis lesions. The cytolytic activity of N-CAM$^+$ T cells was antigen independent. Analyses of CD4$^+$ and CD8$^+$ T cell subpopulations revealed that CD8$^+$ T cells were mostly responsible for the observed antigen independent lysis. N-CAM expression was not a stable but rather seemed an acquired characteristic, since it could be modulated *in vitro* on sorted, N-CAM$^+$ cell populations.

In addition, a longitudinal analysis of leprosy patients undergoing active erythema nodosum leprosum (ENL or type 2 leprosy reactions) showed that *M. leprae* stimulation increased N-CAM expression on CD8$^+$ peripheral T lymphocytes only at the time of active ENL. In line with these observations, stimulation with *M. leprae* increased antigen independent lysis of N-CAM positive target cells in close association with the period of active ENL. At the same time, CD8$^+$ N-CAM$^+$ T cells could be visualized in ENL skin lesions

Collectively, the results demonstrate that N-CAM expressing CD8$^+$ T cells can be isolated from nerve lesions of patients with leprosy neuritis, and can also be detected in lesions and peripheral blood of patients with active ENL. These N-CAM$^+$ CD8$^+$ T cells are capable of lysing N-CAM positive targets, including Schwann cells, in an antigen independent, MHC unrestricted fashion, and may thereby contribute to tissue damage in leprosy. These results reveal a novel mechanism of antigen independent, T cell mediated tissue damage, which is likely to play a role in leprosy and possibly other peripheral neuropathies.

introduction

The occurrence of irreversible damage of peripheral nerves is a major complication in leprosy and leprosy reactions (Naafs *et al.* 1976). Although nerve damage manifests itself along the entire leprosy spectrum, it occurs particularly during reactions. Two major types of reactions can be distinguished; Type 1 or Reversal reactions (RR) and type 2 or Erythema Nodosum Leprosum (ENL). Reversal reactions mainly occur in patients in the borderline area of the spectrum. Clinically, these reactions are characterized by increased erythematous infiltration of previously uninflamed lesions. The accompanying influx of lymphocytes is accompanied by strongly increased cell mediated immune response in the lesions (Modlin *et al.* 1983). ENL preferentially occurs in patients on the borderline end of the spectrum and is characterized by typical subcutaneous infiltrations and nodules. Histologically, inflamed ENL tissue shows infiltration of granulocytes and lymphocytes. In contrast to RR, ENL lesions contain lower levels of IL-2 and IFN-γ mRNA, while IL-4, IL-5 and IL-10 mRNA were found to be elevated when compared to RR (Yamamura *et al.* 1992).

Mycobacterium leprae, the causative agent of leprosy, has a high affinity for Schwann cells. It has therefore been suggested that nerve damage is the result of antigen presentation by Schwann cells and subsequent recognition by cytotoxic T cells (Steinhoff and Kaufmann 1988; Spierings *et al.* chapter 2a). However, tissue destruction also occurs in the absence of *M. leprae*, and autoimmune-like mechanisms have been implicated during anti-mycobacterial immune activation. Antigenic cross-reactivity between host and pathogen has been reported, both at the level of antibody responses (Naafs *et al.* 1990; van den Akker *et al.* 1992; Rambukkana *et al.* 1992) as well as at the level of T cell responses (Steinhoff *et al.* 1999). Recently, however, a novel auto-immune like mechanism has been implicated in the pathogenesis of multiple sclerosis. Myelin basic protein was found to induce expression of Neural Cell Adhesion Molecule (N-CAM or CD56) on $CD4^+$ T cells, which subsequently were able to kill N-CAM positive targets in the absence of antigen (Vergelli *et al.* 1996; Antel *et al.* 1998). N-CAM is a molecule expressed by neurons and glial cells in the peripheral and central nervous system (Le Forestier *et al.* 1993). In the immune system, N-CAM expression was originally considered as a marker for NK cells, but has also been detected on T cell lines that can mediate MHC-unrestricted cytotoxicity (Lanier *et al.* 1989; Lu and Negrin 1994). The exact role of N-CAM in T cell mediated cytotoxicity is still unclear. In the nervous system N-CAM molecules can interact homotypically, and it has been hypothesized that N-CAM expressed by effector cells can ligate to N-CAM on target cells (Lanier *et al.* 1989). Formal evidence, however, is still lacking, mostly due to the fact that neutralizing antibodies to N-CAM are not yet available. Since Schwann cells and neurons in the peripheral nervous system also express N-CAM (Le Forestier *et al.* 1993), it is possible that they function as targets for $N\text{-}CAM^+$ T cells.

One key factor in the induction of N-CAM expression on T cells is likely to be IL-15. Interestingly, IL-15 mRNA has been observed in skin lesions of leprosy patients across the entire leprosy spectrum, showing highest levels in tuberculoid leprosy patients, but also being expressed in lepromatous patients (Jullien *et al.* 1997). This expression of IL-15 was associated with an increased number of N-CAM expressing T cells. Since Schwann cells also express N-CAM, $N\text{-}CAM^+$ T cells may well be involved in autoimmune-like destruction of Schwann cells. We have therefore investigated the involvement of $N\text{-}CAM^+$ T cells in the killing of human Schwann cells and other target cells, as well as the association *in vivo* with RR and ENL as key episodes of immunopathology in leprosy.

materials and methods

patients

Peripheral blood was collected from a total of 11 patients attending the Dermatology Clinic of the AMC, Amsterdam or of the LUMC, Leiden, during follow-up. A healthy BCG vaccinated individual was used as control. Patients were classified according to the Ridley and Jopling classification using clinical and histopathological criteria (Ridley and Jopling 1966). Biopsies from nerves and skin were taken for diagnostic purposes only.

immunohistochemical stainings

Immunohistochemical stainings were performed on frozen biopsy sections (6 μm). Sections were pre-incubated with 0.1 % sodium azide, 0.3 % H_2O_2 to inhibit exogenous peroxidase activity. Primary mouse monoclonal antibodies to N-CAM/CD56 (Leu19), CD3 (Leu4), CD8 (Leu2a) (Becton Dickinson, Mountain View, CA), or IgG1 (DAKO, Glostrup, Denmark) were applied to the specimens, followed by consecutive incubation with horse radish peroxidase labeled rabbit anti-mouse Ig (DAKO) and normal mouse serum (Jackson

table 1: leprosy classification and reactional status of patients used in this study.

patient	classification	leprosy reactions
1	BL	history of ENL, resting
2	BL/LL	ulcerating ENL
3	TT/BT	-
4	BL	history of ENL, resting
5	BT	history of RR, resting
6	sLL	chronic ENL
7	BL/LL	RR + ENL
8	BL/LL	ENL
9	sLL	ENL
10	BT	RR
11	BL	relapse
12	PPD responder	-

Immuno Research Laboratories Inc. West Grove, PA). HRP activity was visualized with 3-amino-9-ethyl carbazole (AEC)(Sigma Aldrich, St. Louis, MO). For double staining, alkaline phosphatease labeled goat anti-mouse antibodies were applied after incubating the samples with N-CAM/CD56 antibodies. CD3 or CD8 staining was applied, using FITC labeled antibodies to CD3 (Becton Dickinson) or CD8 (Clone DK25, Dako), rabbit anti FITC antibodies (DAKO), HRP labeled swine anti-rabbit (Dako) and AEC, while alkaline phosphatase activity was visualized using Fast Blue BB.

cell cultures

The remaining part of the neural biopsies was used to generate nerve infiltrating T cell lines (NIMC). For that purpose, biopsies were cultured on Iscove's Modified Dulbecco's Medium (IMDM) (GIBCO BRL, Grand Island, NY) supplemented with 10% pooled human serum, 20% TCGF (Biotest AG, Dreieich, Germany). Outgrowing T cells were expanded for 10 days in humidified 5% CO_2 at 37°C. As control, PBMC from the same individuals were cultured parallel to the nerve infiltrating T cell lines. For further expansion T cell lines were restimulated with a feeder cell/antigen mixture consisting of Iscove's modified DMEM, supplemented with 10% pooled human serum, 3000 rad irradiated PBMC of 6 random donors (10^6 cells/ml), and *M. leprae* sonicate (Dr. P. J. Brennan, Colorado State University, Fort Collins, CO). After 3 days, TCGF was added to a final concentration of 10%.

phenotypic analysis and sorting

Nerve and peripheral T cell lines were labeled with FITC conjugated anti-CD8, PerCP labeled anti-CD4, and phycoerythrin (PE)-conjugated anti-N-CAM antibodies (Becton Dickinson, Mountain View, CA). After washing three times with PBS, 0.1% BSA, fluorescence intensity was measured by fluorescence activated cell sorter (FACS) analysis. Results were calculated as the percentage of positive cells. For some experiments CD3CD56 positive cells were selected. Sorted cells were cultured for 7 days and analyzed as described above.

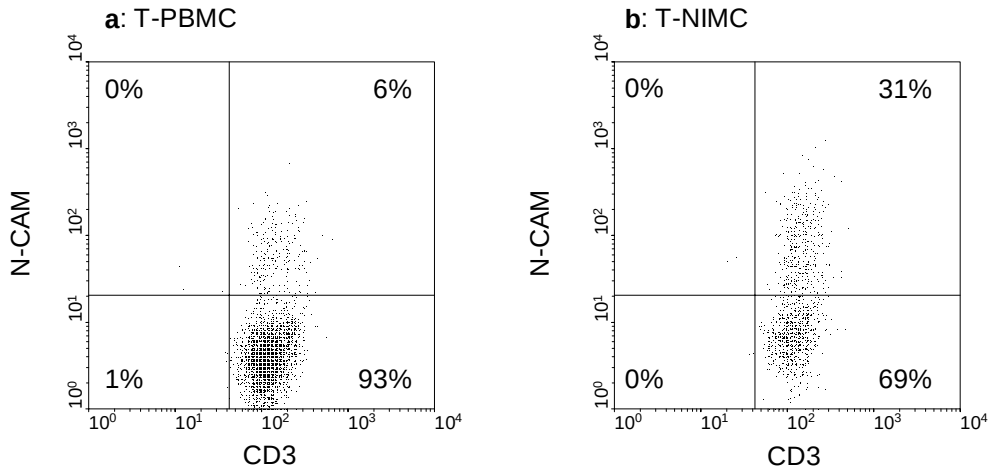

figure 1: Expression of N-CAM on T cells derived from peripheral blood (a) and leprosy neuritis biopsies (b). After antigenic stimulation, all cells were CD3 positive. Neural T cells expressed a significantly higher amount of N-CAM when compared to T cells cultured from peripheral blood.

cytotoxicity assay

Cells from the adherent N-CAM$^+$ astroglioma cell line U251 and human Schwann cell cultures were allowed to adhere for 24 hours in 96 wells flat bottom plates (Greiner GmbH, Solingen, Germany) at a density of 5000/well. N-CAM negative fibroblasts were used as negative control cells in the experiments with a CD8$^+$ polyclonal T cell population. After washing three times with RPMI (GIBCO BRL) plus 10% pooled human serum, the cells were labeled with 40 µCi/ml ^{51}Cr (Sodium Chromate, New England, Boston, USA) for 2 hours at 37°C in a total volume of 100 µl. Unbound ^{51}Cr was removed by washing three times. Freshly cultured effector T cells were added to the targets in a final volume of 200 µl. Target cells were incubated with either medium alone or with 0.5% Triton-X to determine the spontaneous and maximum ^{51}Cr release respectively (Ottenhoff *et al.* 1988). Cell free supernatants were collected from the wells after 6 hours and the ^{51}Cr release was measured by gamma counting. The percentage lysis was calculated as follows: percentage lysis = [(experimental ^{51}Cr release – spontaneous ^{51}Cr release)/(maximal ^{51}Cr release - spontaneous ^{51}Cr release)] x 100%. The spontaneous release did not exceed 20% of the maximal release. Experiments with PBMC were performed in duplicate, while NIMC versus PBMC comparison was assayed in triplicate.

To analyze the contribution of CD4$^+$ and CD8$^+$ T cells in antigen independent cell lysis, T cell lines were stimulated with *M. leprae* and cultured as described above. After 7 days, cells were harvested and incubated with CD4 or CD8 antibodies (Becton Dickinson) in combination with sheep anti-mouse IgG coated Dynabeads® M-450 (Dynal A.S., Oslo, Norway). Depletion was performed according the recommendations of the manufacturer. Resulting fractions were tested in cytotoxicity assays as described above, together with the undepleted T cell line.

N-CAM induction on PBMC

Of each patient 10^6 PBMC per well were stimulated with 1/200 phytohemagglutinin (PHA) (Murex Diagnostics, Dartford, UK), 50 U/ml IL-2, 5 ng/ml IL-15 (R&D Systems, Minneapolis, MN), or 2.5 µg/ml *M. leprae*, in a volume of 1.0 ml. On day 3, 5, and 7, 250 µl cell suspension was collected. Samples were stained with CD4-PerCP, CD8-FITC, and

N-CAM-PE antibodies and analyzed using FACScan as described above. N-CAM expression was scored as the percentage of cells positive within the $CD4^+CD8^-$, $CD4^-CD8^+$, $CD4^-CD8^-$ and $CD4^+CD8^+$ populations. The remainder 250 µl cell suspension was tested in a cytotoxicity assay using U251 as targets at an E:T ratio of 2:1. N-CAM expression was plotted against target lysis for each T cell population.

Schwann cell cultures

Human Schwann cells were isolated from sural nerve biopsies and propagated as described before (van den Berg *et al.* 1995). Briefly, the sural nerve specimens were cut into small pieces and incubated in 85% IMDM, 10% lymphokine activated killer (LAK) cell supernatant (Lamers *et al.* 1992), 5% fetal calf serum (FCS), 100 U/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml PHA. The nerve fragments were incubated in humidified 5% CO_2 at 37°C. After 10 days the non-adherent cells were removed and the adherent cells were propagated in medium consisting of 85% IMDM with 0.6% glucose (Sigma), 10% FCS, and 5% LAK cell supernatant.

ELISA

To test whether human Schwann cells could be a source for IL-15 in neural tissue, 20.000 human Schwann cells per well were seeded into a 24-well flatbottom plate (Greiner). Cells were allowed to adhere for 24 hours at 37°C, 5% CO_2. After replacement of medium with IMDM, 10% FCS, cells were stimulated with LPS (100 ng/ml) or *M. leprae* sonicate (25 µg/ml) and incubated for 48 hours. Supernatants were collected and and assayed in an IL-15 ELISA as described by the manufacturer (R&D Systems, Minneapolis, MN).

data analysis

The correlation between N-CAM expression and target lysis was statistically tested using regression analysis. The statistical significance of the increase in U251 lysis by PBMC of patients with and without reactions was tested with a paired t test. Correlations and differences were considered significant when p values were < 0.05.

results

isolation of N-CAM expressing T cells from leprosy neuritis lesions

T cells were isolated from active neuritis lesions from which biopsies were taken for diagnostic purposes only, as well as from the peripheral blood of the same leprosy patients (n=6, not listed in table 1). Immunostaining of these cultured T cells showed that N-CAM expression was abundant on the population derived from inflamed neural tissue from leprosy patients, while peripheral T cells from the same individuals expressed much lower levels of N-CAM (figure 1). This phenomenon was observed in 5 out of the 6 leprosy patients with neural involvement available for the study, regardless of their leprosy classification. Examination of N-CAM expression by cultured cells demonstrated that N-CAM expression was not a stable phenotype: when either N-CAM positive or N-CAM negative, nerve derived T cell populations were sorted to homogeneity and further expanded in the presence of *M. leprae* and allogeneic antigen presenting cells, $N\text{-}CAM^+$ T cells appeared to loose some N-CAM expression (figure 2a), while some cells from the $N\text{-}CAM^-$ population re-expressed or upregulated N-CAM (figure 2b). In contrast to nerve derived T cells, $N\text{-}CAM^+$ T cells that had been sorted from the peripheral blood from these patients lost N-CAM expression much more rapidly (figure 2c), whereas no expression of N-CAM could be detected in the sorted $N\text{-}CAM^-$ population (figure 2d). Taken together, these results sug-

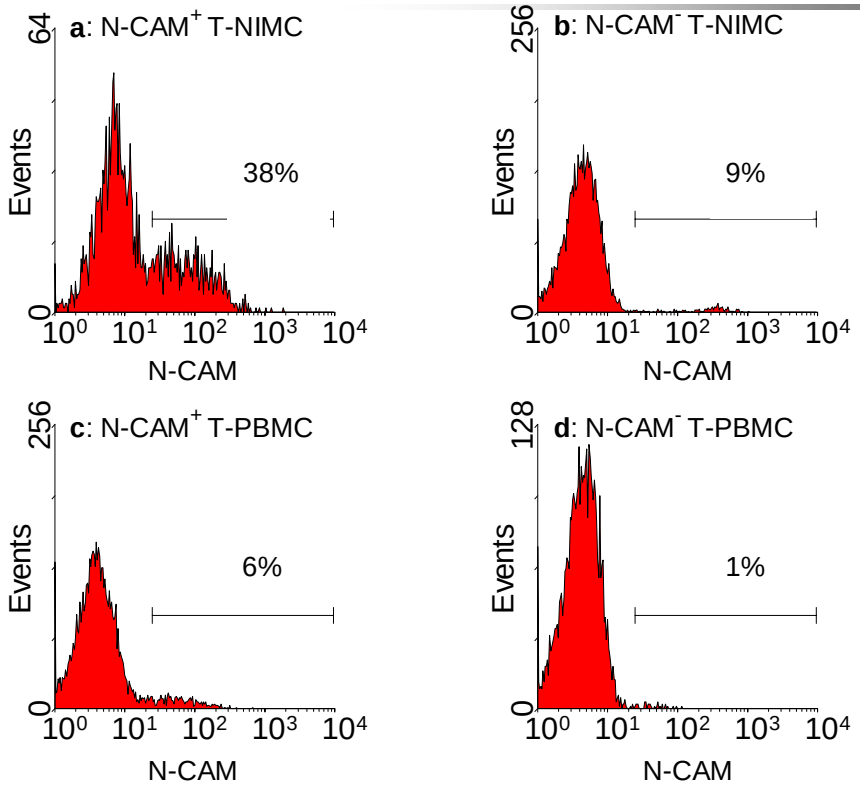

figure 2: Sorting and expansion of N-CAM positive and negative T cells. N-CAM⁺ nerve derived T cells partly lost their N-CAM expression, but a significant number remained positive (a). Small numbers of sorted N-CAM⁻ neural T cells were able to re-express N-CAM (b). Almost all N-CAM⁺ peripheral T cells lost their N-CAM expression after restimulation (c), while the N-CAM⁻ fraction remained negative (d).

gest that (1) leprosy nerve lesions contain T cells that are primed to express N-CAM, (2) N-CAM expression is an acquired and non-permanent phenotype, and (3) *M. leprae* stimulation can modulate N-CAM expression by nerve derived T cells.

lysis of N-CAM positive target cells by N-CAM positive T cells from leprosy neuritis lesions

N-CAM has been implicated as an important molecule in cytotoxicity. To analyze the functional phenotype of N-CAM⁺ T cells derived from leprosy lesions and peripheral blood, T cell lines were stimulated with *M. leprae* and incubated with target cell line U251. U251 as well as *in vitro* cultured human Schwann cells are able to express N-CAM (figure 3a-b). As shown in figure 3c, nerve derived T cell lines displayed strong cytolytic activity towards N-CAM⁺ U251 target cells, whereas peripheral lines killed these targets much less efficiently or not at all. In the presence of conA, however, no difference in lectin dependent lytic potential between peripheral and neural T cell lines could be detected, showing that peripheral and nerve derived T cells possess similar cytotoxic potentials. These results further support the association between N-CAM expression by T cells and their capacity to kill N-CAM⁺ target cells.

Only very low levels of U251 target killing were observed in case of peripheral T cells compared to nerve derived T cells. This suggests that T cells with the capacity to up-regulate N-CAM expression and to kill N-CAM+ target cells, might accumulate or ex-

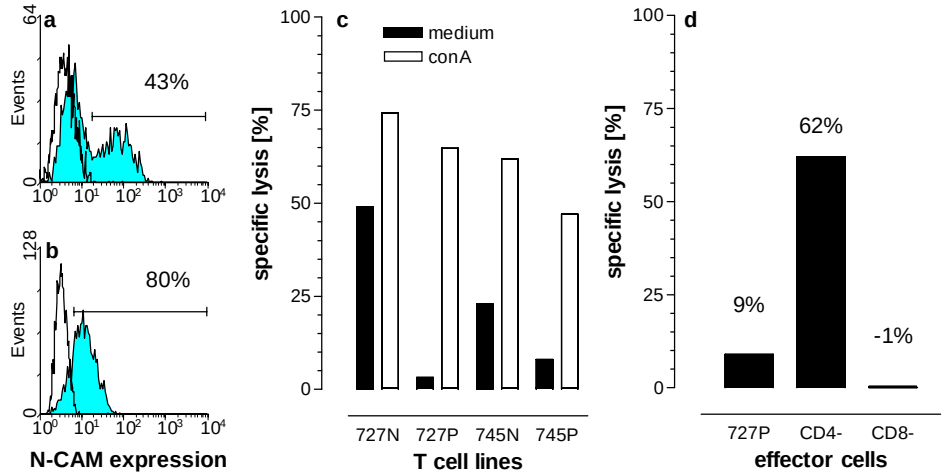

figure 3: Role of N-CAM⁺CD8⁺ T cells in antigen independent, MHC unrestricted target cell killing. Human Schwann cells (a) and U251 (b) express N-CAM on their surface as demonstrated in FACS analyses. Neural (N) and peripheral (P) T cells from two multi-bacillary patients were incubated with ^{51}Cr labeled U251 as described in the materials and methods section (c). No differences in lysis could be observed in the presence of conA (white bars). Neural T cell lines, however, killed U251 more efficiently that peripheral T cell lines (solid bars). Peripheral T cells were depleted for CD4⁺ or CD8⁺ T cells and tested for cytotoxicity (d). Depletion of CD4⁺ cells highly increased U251 killing, while CD8 depletion reduced U251 lysis to below the level of the undepleted cell line. Effector:target ratio was 10:1.

pand in leprosy neuritis lesions and thus may be less frequently found in the circulation. Since N-CAM⁺ CD8⁺ T cells have already been noted in leprosy previously (Kaleab *et al.* 1990a; Jullien *et al.* 1997), we examined whether N-CAM associated lytic activity could be detected in highly purified CD4⁺ or CD8⁺ T cell populations of patients with neural involvement. To this end, T cells (20% CD4⁺, 13% CD8⁺) were enriched for CD4⁺ or CD8⁺ populations by subset depletion (the efficiency of depletion was > 90%). As shown in figure 3d, depletion of CD8⁺ T cells reduced lysis to background levels, while depletion of CD4⁺ T cells significantly enhanced lysis of U251. Ninety-four percent of the CD8⁺ T cells in the undepleted population expressed N-CAM, in contrast to only 6% of the CD4⁺ population.

Collectively, these results suggest that N-CAM⁺ CD8⁺ T cells from leprosy nerve lesions, or purified from the peripheral blood of these patients, are able to kill N-CAM⁺ targets in an antigen independent, MHC unrestricted fashion. These cells may therefore contribute to the antigen independent killing of N-CAM⁺ neural target cells in leprosy lesions.

expression of N-CAM by CD8⁺ T cells during ENL

The data above show that N-CAM is expressed on a population of T cells isolated from leprosy nerve lesions or highly purified from the peripheral blood of these patients and suggest that this high expression correlates with cellular killing of N-CAM⁺ target cells, which may be associated with leprosy pathology. In order to further investigate this correlation between increased lysis and leprosy reactions, peripheral blood samples from a limited number of additional patients with a history of ENL or reversal reactions that had already been collected previously, were studied longitudinally (table 1). PBMC were exposed to various T cell stimulatory agents, including the previously reported N-CAM inducers *M. leprae* and IL-15. For five ENL patients and 3 patients with reversal reactions, T cells could be stimulated and subsequently tested for killing of N-CAM⁺ target U251 and expression of CD4, CD8 and N-CAM. As depicted in figure 4a, a significant increase in expres-

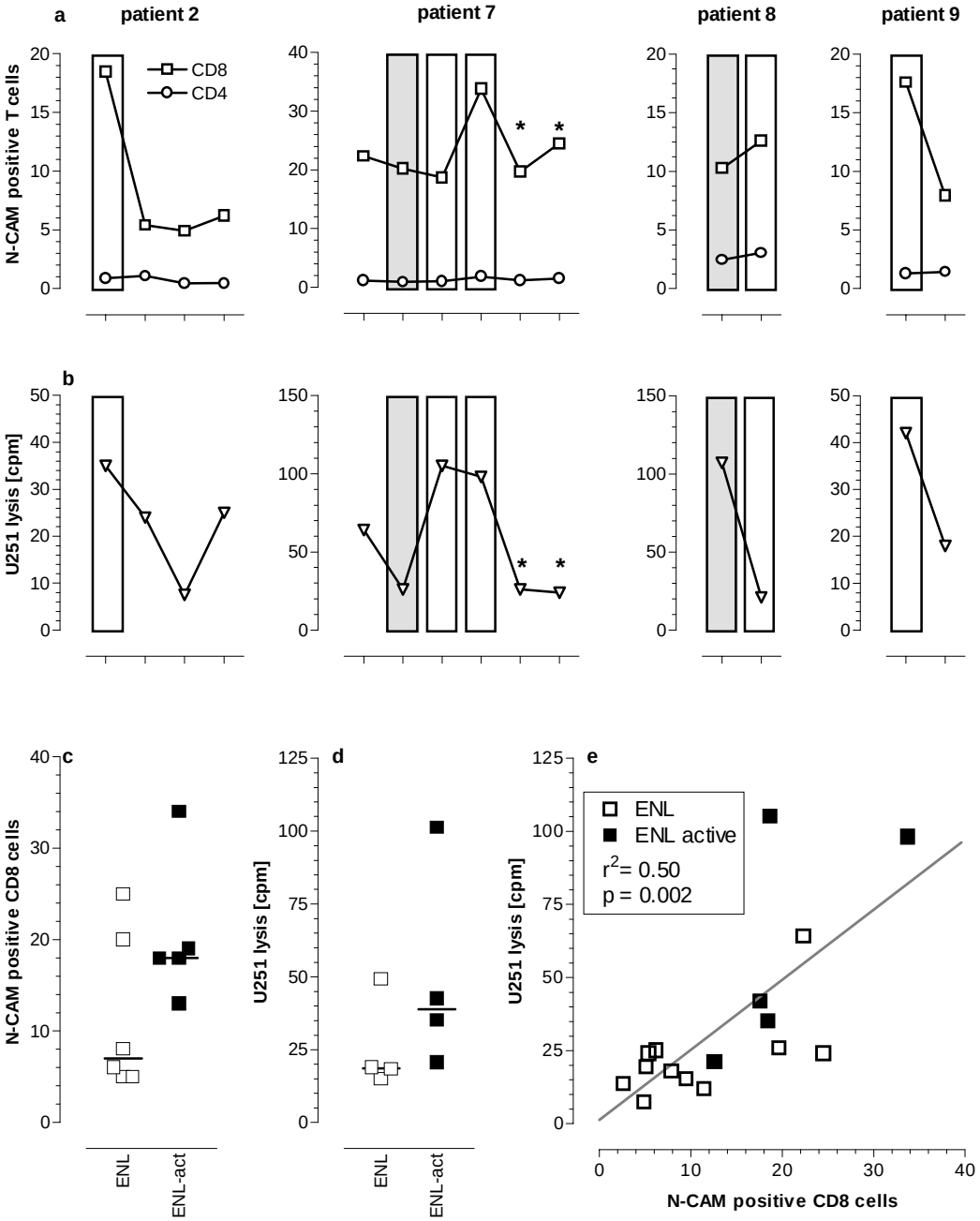

figure 4: Longitudinal analyses of patients with leprosy reactions. a) N-CAM expression on $CD4^+$ and $CD8^+$ T cells after M. leprae stimulation was measured in 4 patients with one or more episodes of active ENL (white bars). Asterices indicate time points at which the patient was under thalidomide treatment and reversal reactions are marked by gray bars. Increased expression was observed during four out of five episodes of active ENL. b) Four out of five episodes of active ENL were accompanied by an increased U251 killing.
M. leprae activated PBMC from leprosy patients with different reactional status yielded differences in N-CAM expression on CD8 cells (c) and U251 killing (d) between ENL in resting phase and clinically active ENL, but neither of these differences were statistically significant. e) N-CAM expression on $CD8^+$ cells from patients with ENL in resting or clinically active phase were plotted against U251 lysis. Samples before the occurrence of ENL were excluded from the analysis. A significant correlation was observed between these two parameters (p=0.002).

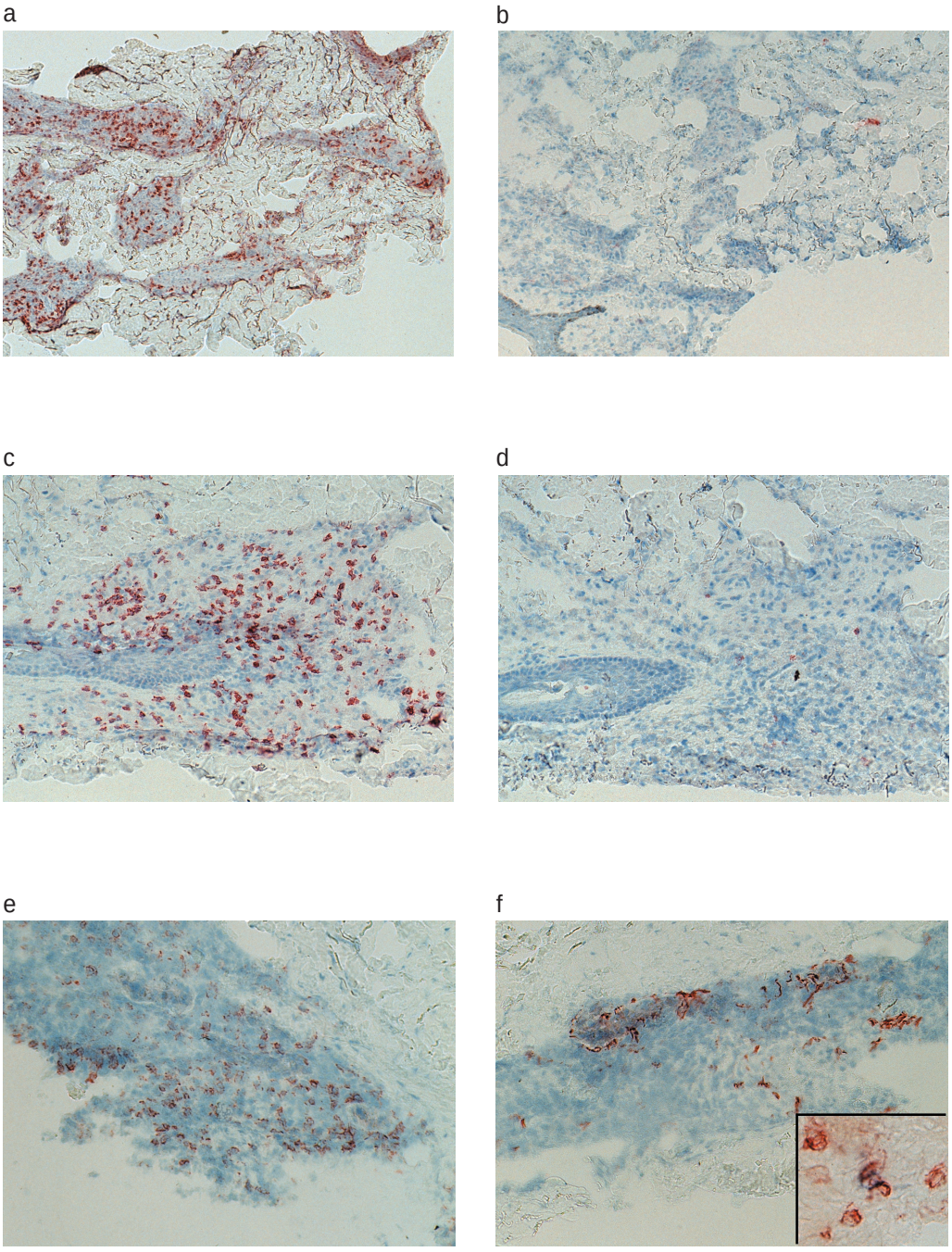

figure 5: A representative photo-micrograph illustration, showing the expression of N-CAM on T cells (a, c, e: CD8 single staining, b, d, f: N-CAM single staining) of patient 7 undergoing ENL via RR. Insert shows N-CAM^{+} CD8^{+} double staining cells in the granuloma.

sion of N-CAM on the $CD8^+$ T cell population could be detected during 4 out of 5 episodes of clinically active ENL. N-CAM expression on $CD8^+$ T cells before and after episodes of active ENL was much lower than during active ENL episodes. In contrast, no such correlation of N-CAM expression with active ENL could be observed in the $CD4^+$ T cell populations.

In parallel, skin biopsies of these leprosy patients taken during or after the occurrence of ENL or reversal reactions, were immunostained for N-CAM and CD8. No N-CAM expression (< 0.01%) could be detected on lesional $CD8^+$ T cells before, and at the time of reversal reaction (figure 5b and 5d), but during clinically active ENL, strong $N\text{-}CAM^+$ expression was detectable (figure 5f). N-CAM expression was observed in all seven patients at the time of clinically active ENL (table 2). The occurrence of leprosy reactions yielded no differences in the number of $CD8^+$ T cells, excluding that the observed increase in N-CAM expression was due only to an increase in the number of $CD8^+$ T cells. Thus, N-CAM expression on lesional $CD8^+$ T cells is observed predominantly during clinically active ENL.

The above observations on nerve derived T cells revealed that increased N-CAM expression was associated with more efficient killing of $N\text{-}CAM^+$ target cells. Therefore, PBMC from patients with and without ENL reactions were exposed to *M. leprae*, and tested for their capability to lyse U251 targets in an antigen independent fashion. Individual results from patients with active ENL are plotted separately, to illustrate that N-CAM expression by $CD8^+$ T cells is increased during 4 out of 5 episodes of active ENL (figure 4a). During four out of five episodes, increased lysis coincided with clinically active ENL (figure 4b). The average N-CAM expression and U251 lysis, as shown in figure 4c and 4d respectively, also tended to be higher at the time of active ENL with overt clinical symptoms when compared to ENL in resting phase, although these differences were not statistically significant. The correlation between N-CAM expression on $CD8^+$ T cells and U251 lysis in ENL patients is plotted in figure 4e. Regression analysis revealed a highly significant correlation between these two parameters ($p=0.002$, $r^2=0.50$), further supporting the notion that $N\text{-}CAM^+$ $CD8^+$ T cells are responsible for the observed antigen independent target lysis.

To further document that $CD8^+$ but not $CD4^+$ T cells are able to up-regulate N-CAM expression in response to *M. leprae*, PBMC of an ENL patient were exposed to various concentrations of *M. leprae*. The results in figure 6a show that *M. leprae* indeed had little to no effect on $CD4^+$ T cells whereas it clearly enhanced N-CAM expression by the $CD8^+$ T cells (from 14% to 20%). A similar analysis was performed on a nerve derived *M. leprae* reactive $CD4^+$ T and a polyclonal $CD8^+$ T cell population. Although $CD4^+$ T cells responded to *M. leprae* in terms of proliferation, no N-CAM induction or U251 lysis could be observed (data not shown). Antigenic stimulation of the $CD8^+$ T cells, however, resulted in increased N-CAM expression (figure 6c). In contrast, IL-2 stimulation of these $CD8^+$ T cells did not result in increased N-CAM expression (figure 6b), showing the *M. leprae* dependency of N-CAM up-regulation on $CD8^+$ T cells. *M. leprae* stimulation and the resulting N-CAM expression was accompanied by enhanced MHC unrestricted, antigen independent lysis of various $N\text{-}CAM^+$ targets, including human Schwann cells and U251 (figure 6d), but not $N\text{-}CAM^-$ fibroblasts (figure 6e). However, $N\text{-}CAM^-$ fibroblasts could be efficiently killed by $N\text{-}CAM^+$ $CD8^+$ T cells in the presence of the lectin concanavalin A (conA), excluding their possible resistance to CTL mediated killing. Thus, these results further support a role for N-CAM in target cell killing in leprosy.

discussion

The functional role of N-CAM expressing T cells is not well understood. Up-regulation of N-CAM expression on $CD4^+$ T cells in response to stimulation with myelin basic protein has been reported in relation to multiple sclerosis (Vergelli *et al.* 1996; Antel *et al.* 1998). We here report the finding of N-CAM expressing, $CD8^+$ rather than $CD4^+$ T cells in

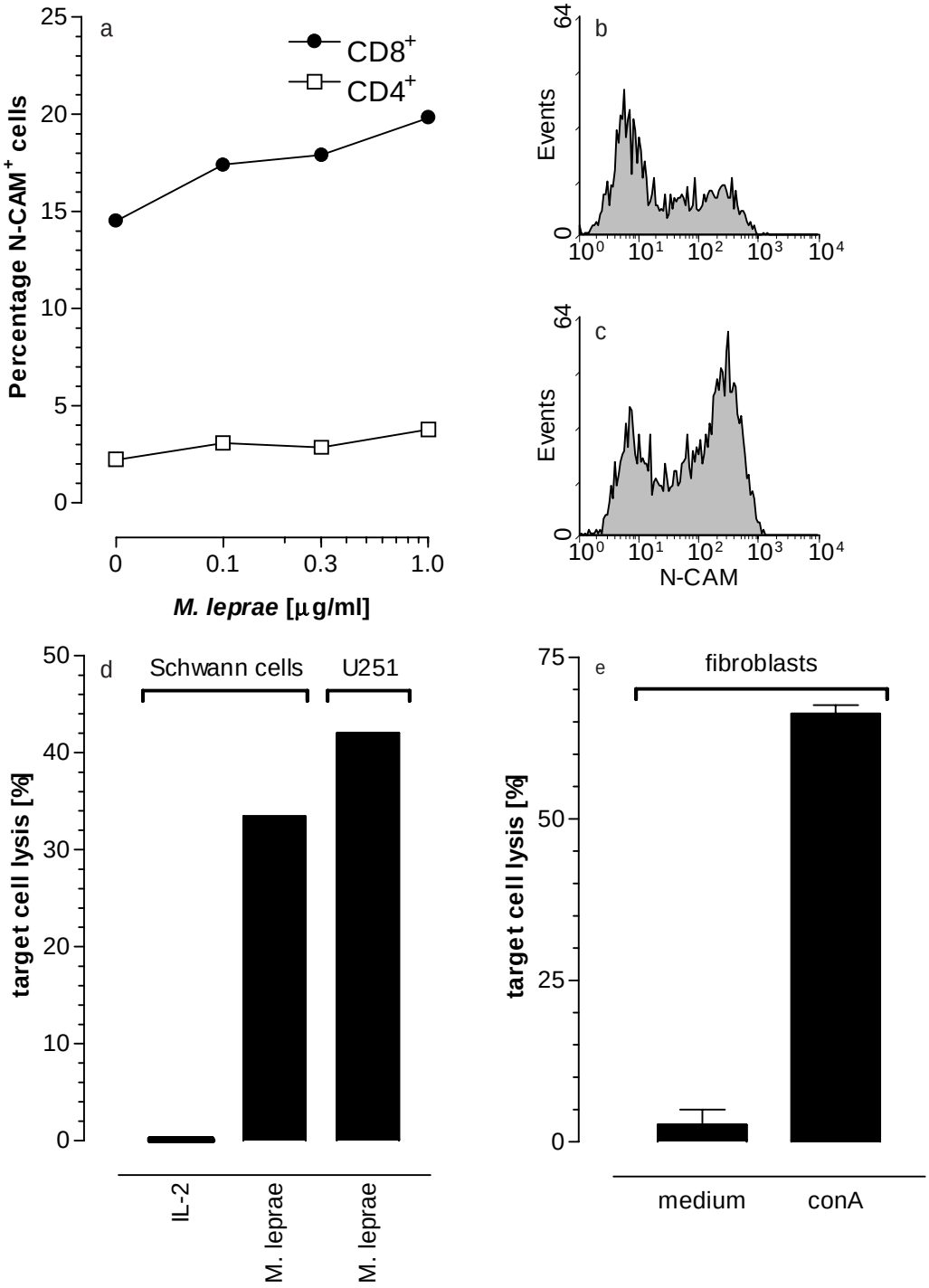

figure 6: Role of M. leprae in the induction of N-CAM expression and U251 lysis. a) PBMC were exposed to different concentrations of M. leprae. M. leprae was found to raise N-CAM expression on CD8⁺ T cells (solid circles), while hardly any effect was observed on CD4⁺ T cells (open squares). N-CAM expression on a polyclonal CD8⁺ T cell line was monitored after expansion with rIL-2 (b) and M. leprae (c). The number of N-CAM expressing cells was significantly higher after expansion with M. leprae when compared to IL-2 expansion. In line with these observations, M. leprae expanded T cells killed human Schwann cells and U251 (E:T ratio 20:1) more efficiently than IL-2 expanded cells (d), while N-CAM⁻ fibroblasts were not lysed at all (e).

relation to leprosy pathology, both in leprosy nerve biopsies and in peripheral blood of leprosy patients with active ENL. Moreover, we show that these N-CAM$^+$ CD8$^+$ T cells are able to kill N-CAM$^+$ target cells, such as U251 and human Schwann cells, in an antigen independent, MHC unrestricted fashion. These findings reveal a novel mechanism of anti-self reactivity which is induced or enhanced by *M. leprae*, yet independent of specific antigen presentation by MHC molecules.

In multiple sclerosis, up-regulation of N-CAM expression resulted in the acquired ability to lyse oligodendrocytes in an antigen independent fashion. Oligodendrocytes fail to express MHC class II, implicating that target cell lysis was also MHC class II independent. This mechanism has been suggested to be of importance in the pathogenesis of multiple sclerosis. An important feature in the induction of N-CAM dependent cytolysis is that neural target cells, in addition to hematopoietic cells as T and NK cells, selectively express N-CAM. N-CAM molecules on target cells have been proposed to interact homotypically with N-CAM on the effector cell (Lanier *et al.* 1989; Nitta *et al.* 1989; Suzuki *et al.* 1991; Palucka *et al.* 1992). Thus, N-CAM expression seems to correlate with inflammatory immunopathology of the central nervous system in multiple sclerosis.

Since leprosy is an inflammatory disease of the peripheral nerve system, we have studied the possible role of N-CAM in the pathology of leprosy. N-CAM$^+$ T cells could be isolated from nerve lesions from 5 out of 6 leprosy patients with neural involvement. A significant fraction of nerve derived T cells was found to express N-CAM, whereas peripheral T cells from these patients did not express N-CAM. The increased number of N-CAM$^+$ cells among nerve derived mononuclear cells correlated with strong, antigen independent, MHC unrestricted lysis of N-CAM$^+$ targets, including Schwann cells. This may be an important observation, because Schwann cell damage is a major feature of nerve destruction in leprosy. Since CD4/CD8 ratios were similar in peripheral and neural T cell lines (data not shown), it is unlikely that the observed difference was due only to a relative increase of the CD8$^+$ T cell population. Thus, T cells from inflamed neural tissue of leprosy patients have an intrinsic capacity to up-regulate N-CAM expression after exposure to *M. leprae*, a phenomenon that may play a significant role in nerve damage in leprosy neuritis.

Also in ENL patients, N-CAM$^+$ CD8$^+$ T cells were observed, in this case among peripheral T cells, but only during active disease and only in response to *M. leprae*. As determined by immunohistochemistry, CD8$^+$ N-CAM$^+$ T cells were clearly present in active ENL skin lesions as well. Interestingly, clinically active ENL also appeared to be associated with a relatively higher killing of U251, in line with the correlation of ENL and N-CAM expression on CD8$^+$ T cells. These observations paralleled the above findings with nerve derived T cells. Longitudinal analyses of patients with reactional episodes demonstrated that lysis of N-CAM$^+$ targets by *M. leprae* stimulated PBMC, and expression of N-CAM on

*table 2: Expression of CD3, CD8, CD68 and N-CAM in inflamed skin during reversal reactions (black) or ENL (blank). The reactional episodes in patients 7 and 8 correspond with those in figure 4a-b. * = less than 0.01%.*

patient:	6	7	7	7	8	8	12	13	14
IgG1	-	-	-	-	-	+/-	-	-	-
CD3	nd	+	++	++	++	++	+++	++	+
CD8	+	++	+	+	+	+	++	++	+
CD68	+++	++	++	++	+++	++	++	++	+
N-CAM	++	+/-	+	++	+/-	+	+	+	+

$CD8^+$ T cells were associated with active episodes in patients with chronic ENL. The observation that enrichment for $CD8^+$ T cells strongly increased U251 killing, while similar enrichment for $CD4^+$ cells reduced lysis to background levels supported these data. The fact that N-CAM^+ T cells were hardly detectable among PBMC of leprosy neuritis patients, might be related to the more systemic nature of ENL as opposed to more localized nerve damage. Alternatively, this may suggest that T cells with the capacity to up-regulate N-CAM expression and to display cytolytic activity, preferentially accumulate or expand in leprosy neuritis lesions and thus may be relatively rare in the circulating T cell pool.

Only one earlier study has reported the expression of N-CAM on T cells in leprosy lesions, both in lepromatous and tuberculoid leprosy, the latter patients having a significantly higher expression (Jullien *et al.* 1997). This study, however, did not include any patients with leprosy reactions or active neuritis. We confirm that N-CAM^+ T cells are indeed hardly detectable in patients on the lepromatous side of the leprosy spectrum, but when these patients undergo episodes of ENL, the number of N-CAM^+ T cells increases. In contrast, hardly any N-CAM^+ T cell could be observed in the skin during reversal reactions. No information is available yet on the *in situ* expression of N-CAM on T cells in active leprosy neuritis lesions. Based on the above isolation of N-CAM+ T cells from such lesions, significant staining is expected. Further studies will be needed to address this issue in relation to nerve damage.

N-CAM expression can be induced by various cytokines, including TGF-β (Stewart *et al.* 1995) and IL-15 (Jullien *et al.* 1997). Interestingly, Schwann cells and neurons produce TGF-β (Unsicker *et al.* 1991). The production of TGF-β by human Schwann cells is constitutive and cannot be enhanced by *M. leprae* or LPS of *Escherichia coli* (Spierings *et al.* chapter 3). TGF-β has also been detected in skin and nerve lesions of leprosy patients (Goulart *et al.* 1996) and could thus be causally related to the high number of N-CAM^+ T cells at the site of inflammation. In addition, IL-15, which is produced by macrophages after exposure to *M. leprae*, has been reported to induce N-CAM expression on T cells (Jullien *et al.* 1997). In this study, IL-15 and *M. leprae* were shown to act in synergy in inducing T cell proliferation. Besides its preference for macrophages, *M. leprae* displays a high affinity for Schwann cells. However, no IL-15 production by Schwann cells after exposure to *M. leprae* or LPS could be detected (data not shown). An open question is whether IL-15 is a necessary intermediate in the N-CAM inducing cascade. N-CAM expression on $CD4^-CD8^+$ T cells after stimulation with *M. leprae* seemed to discriminate active ENL from resting ENL, while IL-15 was unable to do so. This suggests that IL-15 and *M. leprae* use separate mechanisms for the induction of N-CAM. Moreover, purified mycobacterial antigen 85B also up-regulated N-CAM expression on polyclonal $CD8^+$ T cells, suggesting that interactions between antigen presenting cells and T cells are required (data not shown). The exact mechanism by which N-CAM is induced by *M. leprae* remains to be clarified.

N-CAM expression is not a stable feature of T cells derived from inflamed neural tissue. Expansion of N-CAM positively selected cells always resulted in both N-CAM^+ and N-CAM^- populations. However, N-CAM^+ T cells could hardly be found in cultures from PBMC from the same individuals and N-CAM^+ T cells from these cultures and more rapidly lost N-CAM expression. Further stimulation of N-CAM^+ peripheral T cells from these patients with *M. leprae* did not result in an N-CAM^+ population either. We therefore assume that T cells from neuritis lesions have the capacity to up-regulate N-CAM after recognition of *M. leprae* antigens.

Thus, in this study we describe the presence of antigen independent, MHC unrestricted $CD8^+$ N-CAM^+ T cells that display N-CAM associated cytotoxic activity without cross-reacting to microbial or self-antigens. Our results indicate that, in contrast to multiple sclerosis, N-CAM^+ $CD8^+$ cells may be important effector cells that kill N-CAM^+ target cells, including Schwann cells, and as such may contribute to tissue damage in leprosy.

These cells were found in association with leprosy neuritis and active ENL and their lytic capacity may play an important role, not only in the immunopathology of leprosy neuritis and ENL, but also in other peripheral and central neuropathies and perhaps even autoimmune diseases. Tissue specificity would thus not result from molecular mimicry, but rather be determined by tissue tropism of bacteria or viruses and by the expression of N-CAM in these tissues.

chapter 5
the mammalian cell entry 1 (mce1) operon of *Mycobacterium leprae* and *Mycobacterium tuberculosis*

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Tom H. M. Ottenhoff, and Morten Harboe

Microbial Pathogenesis 1999. **27:** 173-177

to G:
home, home again
I like to be there when I can

abstract

The genome project on *Mycobacterium tuberculosis* H37Rv has revealed four mammalian cell entry (*MTmce1-4*) operons putatively involved with entry and survival of mycobacteria in host cells. A homologous operon to the *MTmce1* operon was identified in cosmid B983 of *Mycobacterium leprae*. By comparison with *M. tuberculosis*, several mutations, or sequencing errors, were predicted at specific sites causing frame shifts in the *MLyrbE1A*, *MLyrbE1B* and *MLmce1D* genes. Using targeted sequencing, sequence errors were identified. The corrected *MLmce1* operon sequence appears to be highly homologous to the *MTmce1* operon, and similarly encodes eight potential genes. Thus, both *M. tuberculosis* and *M. leprae mce1* operons may be functional and involved in host cell targeting.

introduction

Arruda *et al.* (Arruda *et al.* 1993) originally described a DNA fragment of *Mycobacterium tuberculosis* that conferred upon a non-pathogenic *Escherichia coli* strain the ability to gain entry into mammalian cells and to survive inside macrophages. The mammalian cell entry *(mce)* gene was subsequently localized in the *M. tuberculosis* H37Rv genome and termed *mce1(Cole et al. 1998)*. Analysis of this genome revealed four copies of *mce*, situated in operons consisting of eight genes. The operons were organized in exactly the same manner with extensive homology, in particular between *mce1* and *mce2*. In each case the genes preceding *mce* coded for two integral membrane proteins (*yrbE*), while *mce* and the following five genes were predicted to encode proteins with signal sequences or hydrophobic stretches at the N-terminus. These sets of proteins, about which little is known, may well be secreted or surface-exposed, which is consistent with the proposed role of *mce* in invasion of host cells.

In *M. tuberculosis* these genes have recently been designated *yrbE1A, yrbE1B, mce1A, mce1B, mce1C, mce1D, mce1E* and *mce1F* (S.T. Cole, personal communication), and correspond to Rv0167, Rv0168, Rv0169 (*mce1*), Rv0170, Rv17l, Rv0172, Rv0173 (*lprK*), and RvO174 (Cole *et al.* 1998). Further*, MTyrbE2A-MTmce2F* correspond to Rv0587-Rv0594, *MTyrbE3A-MTmce3F* to Rv1964-Rv1971, while *MTyrbE4A-MTmce4F* correspond to Rv3501c- Rv3494c. For *M. tuberculosis* and *M. leprae* we use the designations MT or ML to obtain a precise species annotation.

The *mce* gene has been found in strains belonging to the *M. tuberculosis* complex and homologous sequences have been demonstrated in members of the *Mycobacterium avium* complex (*M. avium, M. intracellulare* and *M. scrofulaceum*) (Parker *et al.* 1995).

Searches for similarity with the *Mycobacterium leprae* genome revealed one operon in cosmid B983 containing similar genes lying close together. Detailed analysis revealed extensive homology, while striking differences also occurred in large stretches of DNA indicating the possibility of mutations, or alternatively, sequencing errors in the published sequence of cosmid B983 (Accession no. L78828; GB:MSGB983CS; EMBL: MLB983CS; Authors: Smith D. R. *et al.*, Genome Therapeutics Corporation, Waitham, MA, U.S.A.). In this paper, we describe that these differences have arisen as a result of sequencing errors, as confirmed by targeted resequencing. We discuss the implications thereof concerning evaluation of the homology between the *mce1* operons of *M. tuberculosis* and *M. leprae*.

materials and methods

searching for homology and alignment of sequences

Sequences of *M. tuberculosis* or *M. leprae* proteins and open reading frames were obtained from the Sanger Centre Anonymous FTP site; http://www.sanger.ac.uk/projects, from the EMBL 51+GenBank™ 104 releases, and from Swiss&PIR&Translated release 104 using the BLASTN search tool (Altschul *et al.* 1990). Sequences were analyzed using the DNA*Star programs EditSeq, Protean, MegAlign, GeneMan and MapDraw (DNA*STAR Inc., Madison, WJ.).

Comparisons of proteins were performed using one pair alignments by the Lipman-Pearson method (Lipman and Pearson 1985) (Method parameters: k-tuple 2; Gap Penalty 4; Gap Length Penalty 12). Comparisons of the genes were performed using one pair alignments by the Wilbur-Lipman method (Wilbur and Lipman 1983) or the Martinez/Needleman-Wunsch method (Needleman and Wunsch 1970; Martinez 1983) (Method parameters: Minimum match 10; Gap Penalty 1.10; Gap Length Penalty 0.33).

PCR and sequencing

From the alignment of B983 with the *mce1* region of *M. tuberculosis,* we predicted regions containing frame shift mutations or sequencing errors in the *MLyrBE1A*, *MLyrbE1B* and *Mlmce1D* genes. Polymerase chain reaction (PCR) primers flanking these regions were designed (Table 1 upper part) and PCR was performed. Amplification of *MLyrbE1A* and *MLyrbE1B* was performed on cosmid B983. For *MLmce1D, M. leprae* DNA was used. Polymerase chain reaction fragments were run on 1.5% agarose, excised, purified and sequenced in forward and reverse direction using the Texas-red labeled primers shown in the lower part of table 1.

table 1. Primers for polymerase chain reaction and sequencing. ***** *BamHI sites were introduced into the forward and reverse primers of mce1D. Modifications are indicated in italics.*

ORF	primer
yrbE1A forward	5'-GCATATATGGTTCCGGCGTGA-3'
yrbE1A reverse	5'-GATAGCGGCTCAGGGCATTAG-3'
yrbE1B forward	5'-GCTGGCTTGGTCGGCTGTTAT-3'
yrbE1B reverse	5'-TACAGCCACGACCAACAAGCC-3'
mce1D forward	5'-*GGTCCT*GGAT*C*CGATCAAGTTCAACTATTTTC-3' *
mce1D reverse	5'-*TACTTA*GGATCCTCACTGGCCTGCTCCTAACTC-3' *
yrbE1A forward	5'-GGGTTGCATTTCTGCCGAC-3'
yrbE1A reverse	5'-GCCCGACACATTCTGCAGG-3'
yrbE1B forward	5'-AAACATGTCGACCGCTGCC-3'
yrbE1B reverse	5'-CCGGCCAAAATCCGAGTAG-3'
mce1D forward	5'-AGTGGGTACAAGGACACGAC-3'
mce1D reverse	5'-CTTGGCGACGGTATAGGCAC-3'

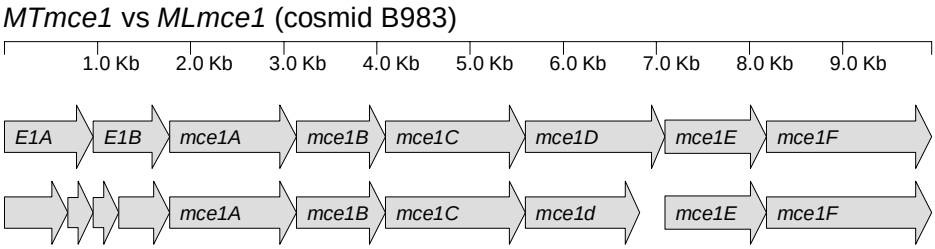

figure 1. Comparison of the deduced open reading frames of the M. tuberculosis mce1 operon (top line) with the M. leprae genes as deduced in the homologous region of cosmid B983 (lower line). The open reading frames of cosmid B983 corresponding to the yrbE1A (E1B) and yrbE1B (E1B) genes were not labeled, while the truncated mce1D gene in cosmid B983 was denoted mce1d.

results

A sequence homology search in the EMBL/GenBank database using the sequence for the *M. tuberculosis mce1* operon identified a homologous region of very similar size in cosmid B983 of *M. leprae* (9918 bp vs 9907 bp). The predicted genes in *M. leprae* did not comply completely, however, with the predicted genes in the *M. tuberculosis mce1* operon. The sequences corresponding to *MTyrbE1A* and *MTyrbE1B* were split in two coding sequences, and the open reading frame corresponding to the *MTmce1D* gene was shorter resulting in a non-coding region until the start of the next gene, *mce1E* (Fig.1). The homologues of the *MTrnce1A*, *MTmce1B*, *MTmce1C*, *MTrnce1E* and *MTrnce1F* genes were comparable in size and organization.

The *MTyrbE1A*, *MTyrbE1B* and *MTmce1D* genes were aligned with corresponding regions in the B983 cosmid using the Wilbur-Lipman or Martinez/Needlman-Wunsch methods. Possible missing bases could be predicted in position 7210 of the cosmid, corresponding to *MTyrbE1A*; in the region 7800-7830 of the cosmid, corresponding to *MTyrbE1B*; and in position 13675 of the cosmid, corresponding to *MTmce1D*. These differences were predicted by the Wilbur-Lipman as well as the Martinez/Needlman-Wunsch methods. In-

table 2. Comparison of the deduced genes and their expressed products in the MLmce1 and MTmce1 operons. [1] = designations of the genes in the mce1 operon of M. tuberculosis H37Rv (Cole et al. 1998). [2] = length of the putative protein in M. tuberculosis; aa = amino acids [3] = length of the putative protein in M. leprae. [4] = Similarity indexes between the proteins (left column) and genes (right column) in the MLmce1 and MTmce1 operons. [5] = Coordinates of the mce genes in B983 after correction of the sequence.

MT genes[1]			len MT[2]	len ML[3]	Similarity[4]		Cosmid B983[5]
yrbE1A	Rv0167		265 aa	267 aa	88.2	76.1	6760..7563
yrbE1B	Rv0168		289 aa	289 aa	82.7	76.6	7567..8436
mce1A	Rv0169	*mce1*	454 aa	441 aa	66.0	53.8	8440..9765
mce1B	Rv0170		346 aa	346 aa	75.1	69.7	6972..10802
mce1C	Rv0171		515 aa	519 aa	71.3	63.2	10799..12358
mce1D	Rv0172		530 aa	531 aa	77.5	69.8	12355..13950
mce1E	Rv0173	*lprK*	390 aa	392 aa	76.2	68.8	13947..15125
mce1F	Rv0174		515 aa	516 aa	76.2	67.8	15119..16669

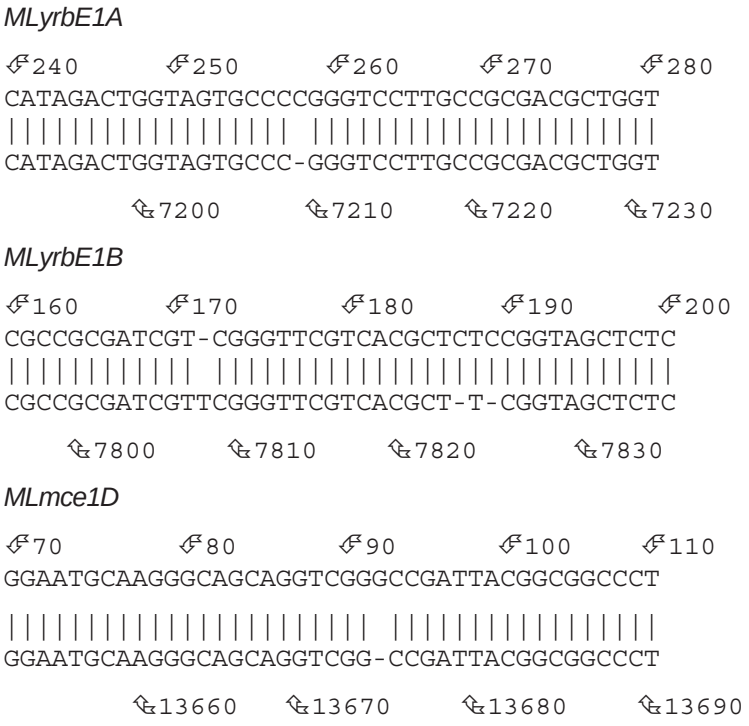

figure 2. Alignments of the partial sequences of the MLyrbE1A gene (accession number AF116212); the MLyrbE1B gene (accession number AF116213); and the MLmcel1D gene (accession number AF096865); with the corresponding parts of the M. leprae B983 cosmid sequence in the lower rows.

troducing extra bases into the predicted positions would give *M. leprae* genes completely corresponding to the *MTyrbE1A*, *MTyrbE1B* and *MTmce1D* genes. To investigate the possibility that either mutations in the *M. leprae* genome had accumulated naturally in the *mce* operon, or that these differences had resulted from possible sequencing errors, primers were designed as shown in table 1, to sequence the regions in question. The resulting sequences confirmed the predictions as being due to sequencing errors (Figure 2).

The B983 cosmid was corrected according to the new sequences, and comparison of the genes in the *M. tuberculosis* and *M. leprae mce1* operons is shown in figure 3 and table 2.

discussion

Mycobacterium tuberculosis and *M. leprae* are relatively closely related microorganisms which contain a number of highly homologous genes. Several genes of secreted *M. tuberculosis* proteins are conserved in *M. leprae* and these genes are similarly organized (Harboe and Wiker 1998). Despite this extensive homology, analyses have revealed the accumulation of many mutations in a large number of *M. leprae* genes compared to the *M. tuberculosis* genome (S. T. Cole, personal communication). For the *mce1* proteins of *M. leprae* and *M. tuberculosis,* the homologies observed varied from 66-88.2% showing that the *mce1* operon is highly conserved in *M. leprae.* For comparison, the 65 kDa heat-

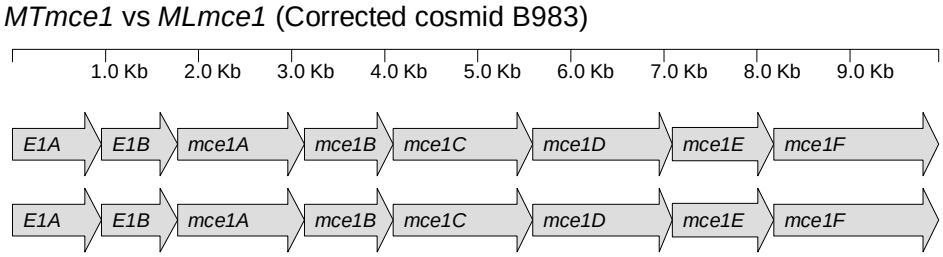

figure 3. Comparison of the deduced open reading frames of the MTmce1 operon (top line) with the M. leprae mce1 operon (lower line) after correction of the MLyrbE1A, MLyrbE1B and MLmce1D genes of cosmid B983. At position 7210 an extra C was inserted in the yrbE1A gene. At position 7898 a T was deleted and two extra Cs were inserted after the Ts in position 7823 and 7824 in the yrbE1B gene. At position 13675 an extra G was inserted in the mce1D gene. The cosmid was 36788bp long before these corrections and with these corrections it would be 36791 bp long.

shock protein (GroEL1) which is a highly conserved protein in phylogeny has a similarity index of 82.6%.

The genome of *M. leprae* is significantly smaller than that of *M. tuberculosis* (2.8 vs 4.4 megabases). Some groups of genes are missing, such as those encoding PGRS (polymorphic GC-rich sequence) (Hermans *et al.* 1992) or MPTR-related (major polymorphic tandem repeats) (Poulet and Cole 1995) peptides. One would expect that non-coding segments are kept at a minimum. Besides the occurrence of natural mutations in *M. leprae,* sequencing errors should also be considered when gaps like that found between *MLmce1D* and *MLmce1E* are encountered. The corrected sequence of B983 gave in fact a slight overlap of 4 bp between *MLmce1D* and *MLmce1E.*

The three examples of genes with the here-identified sequencing errors were discovered by aligning the *M. leprae* sequences with the homologous sequences in *M. tuberculosis.* This shows that the *M. tuberculosis* genome is an important tool for proofreading of *M. leprae* sequences. Thus, systematic use of the complete *M. tuberculosis* genome (Cole *et al.* 1998) for comparison will certainly facilitate the elucidation of the *M. leprae* genome, and will ascertain reliable sequencing results. A comparison of this kind is particularly suited to detect sequencing errors causing frame shifts, which are among the most serious errors encountered. We did not detect other types of sequencing errors, but our data are relatively limited compared to the whole B983 cosmid. Cosmid MLCL622 overlaps at the 3' end of B983 with approximately 12 000 bp. There are several conflicts in this overlap, which shows that it is probably necessary to resequence the complete B983 cosmid.

chapter 6
sulfatide antibodies in leprosy and leprosy reactions

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abstract

Anti-sulfatide antibodies have been reported in various demyelinating peripheral polyneuropathies. We have investigated the diagnostic value of anti-sulfatide antibodies in leprosy. Anti-sulfatide IgM in leprosy patients was not significantly elevated. High anti-sulfatide IgG titers were observed in individuals from endemic areas, irrespective of their leprosy status, while Western European controls were negative. No significant correlation was found between IgM or IgG antibody titers and leprosy classification, although multibacillary patients had higher anti-sulfatide IgM titers than paucibacillary patients. In addition, twenty-three patients developing leprosy reactions were followed longitudinally. Antibody titers in these patients fluctuated slightly during the follow-up period. There was no association with the occurrence of leprosy reactions or treatment. Thus, IgG titers against sulfatides are high in both leprosy patients and healthy controls in endemic areas, whereas such antibodies are not found in Western European controls, suggesting that anti-sulfatide antibodies are induced by environmental factors, such as microorganisms.

introduction

Leprosy is a chronic infectious disease that is caused by *Mycobacterium leprae* (Bloom and Godal 1983). Peripheral nerve damage is a major complication in leprosy and occurs across the entire leprosy spectrum, particularly in patients with acute inflammatory leprosy reactions. Reactional episodes in leprosy are accompanied by increased immune activity. A role for the immune system in the pathology of leprous neuritis has therefore long been suspected. A striking feature of *M. leprae* is its predilection for Schwann cells. Nerve damage could thus result from cellular or humoral immune reactivity towards *M. leprae* located in nerve tissue. Since nerve damage can also occur in uninfected tissue, nonspecific inflammatory or perhaps even autoimmune mechanisms may also play a role. To prevent nerve damage in leprosy, prediction or early detection of neuritis episodes will be crucial.

Loss of nerve function is often associated with demyelination. Antibodies might play an active role in this process. Anti-galactocerebroside antibodies are thought to induce myelin alterations (Roth *et al.* 1985), leading to inhibition of sulfatide synthesis (Fry *et al.* 1974) and demyelination (Saida *et al.* 1979; Sergott *et al.* 1984). These effects can be mimicked by injecting leprosy patients' serum into Swiss white mice (Shetty *et al.* 1985), suggesting that anti-glycolipid antibodies play an active role in the pathogenesis of leprosy neuritis. In sooty mangabey monkeys with experimental leprosy, antibodies towards ceramide, galactocerebroside or asialo-GM_1 were reported to have potential diagnostic value in predicting leprous nerve damage (Cho *et al.* 1993). Antibodies directed to neural glycolipids are also related to leprosy: anti-ceramide (Vemuri *et al.* 1996) and galactocerebroside antibodies (Vemuri and Mukherjee 1991; Vemuri *et al.* 1996) were detected in the majority of leprosy patients, but titers did not differ significantly between patients with and without nerve damage.

Sulfatide is a glycosphingolipid that is expressed as surface determinant of myelin in the central and peripheral nervous system (Dupouey *et al.* 1979). Sulfatide and galactocerebroside play an important role in myelin function and stability (Coetzee *et al.* 1996). Antibodies towards sulfatides have been detected in several neuropathies: eighty-eight percent of insulin dependent diabetes mellitus patients had detectable antibodies to sulfatides (Buschard *et al.* 1993), and anti-sulfatide antibodies have also been found in patients with the Guillain Barré syndrome (Ilyas *et al.* 1991; van den Berg *et al.* 1993) Miller Fisher syndrome (Willison and Veitch 1994) and multiple sclerosis (Ryberg 1978). For serological detection of infection with *M. leprae* various antigens can be used, including phenolic glycolipid-I, lipoarabinomannam, and a number of *M. leprae* specific proteins

(Buchanan 1995). However, no serological assay is available for identification of leprosy reaction or nerve damage. Raised titers of antibodies towards sulfatides and related neural components may be associated with nerve damage. In this study we have therefore measured anti-sulfatide antibody titers in leprosy patients, including patients with type 1 or type 2 leprosy reactions, in order to investigate whether such antibodies might have prognostic or diagnostic value for the detection of leprosy neuritis.

materials and methods

patients

The population studied included 10 multibacillary and 10 paucibacillary patients, 10 household contacts and 10 controls from the Philippines. Multibacillary leprosy patients included all borderline and lepromatous patients with a bacterial index (BI) of at least 2+ on the Ridley scale (Ridley and Jopling 1966) at any one site. Paucibacillary leprosy patients included indeterminate, tuberculoid (TT) and borderline tuberculoid (BT) with BIs < 2+ at any one site. These parameters follow the World Health Organization (WHO) recommendation at the time of collection (World Health Organization Expert Committee on Leprosy 1989). The contacts were persons living in the same household as the multibacillary or paucibacillary cases in the last 3 years. The normal population was composed of persons living in the same community as the patients, presenting other forms of skin diseases but free of clinical signs of leprosy and with no case of leprosy in their households. Five healthy West European controls were included as negative controls, and a group of 10 Guillain Barré Syndrome patients and 5 diabetic patients served as positive controls.

Additionally, sera from 23 leprosy patients were collected longitudinally. The mean follow-up period was 28.3 months. During the follow-up period, 10 of them encountered a type 2 reaction and 9 patients a type 1 reaction. The remaining 4 patients had no reaction during the follow-up period.

The collection of blood for the study was approved by the Leonard Wood Memorial Institutional Review Board (Human Rights Committee) (Cebu, The Philippines) and the Ministry of Health Ethical Committee (Manila, The Philippines). Written informed consent was obtained from all subjects. For collection of sera from the Netherlands, approval was obtained from the Medical Ethical Committee of the Leiden University Medical Center (Leiden, The Netherlands).

enzyme-linked immunoassay (ELISA)

Ninety-six wells flat bottom plates (Greiner GmbH, Solingen, Germany) were coated with sulfatide derived from bovine brain (Sigma Aldrich, St. Louis, MO) dissolved in methanol, overnight at 4°C. The plates were blocked with PBS/1% BSA (Sigma) for 90 minutes at room temperature and washed 5 times with PBS. Sera were titrated twofold ranging from 1/50 to 1/6400 and incubated overnight at 4°C. The wells were washed again 5 times with PBS. Peroxidase conjugated rat anti-human IgG or IgM was diluted 1/1000 in PBS/1% BSA and added to the wells. After incubating 2 hours at room temperature, the wells were washed 3 times with PBS/0.05% Tween and 2 times with PBS. Enzymatic activity was determined in 50 mM citric acid (Merck, Darmstadt, Germany), 100 mM Na_2HPO_4 (Merck) pH 5.0, containing 1mg/ml Phenylenediamine Dichloride (Sigma), 1 µl/ml 30% H_2O_2 (Sigma) The reaction was stopped by adding 10% sodium dodecyl sulfate (Sigma). After 20 minutes the optical density was read at 450 nm.

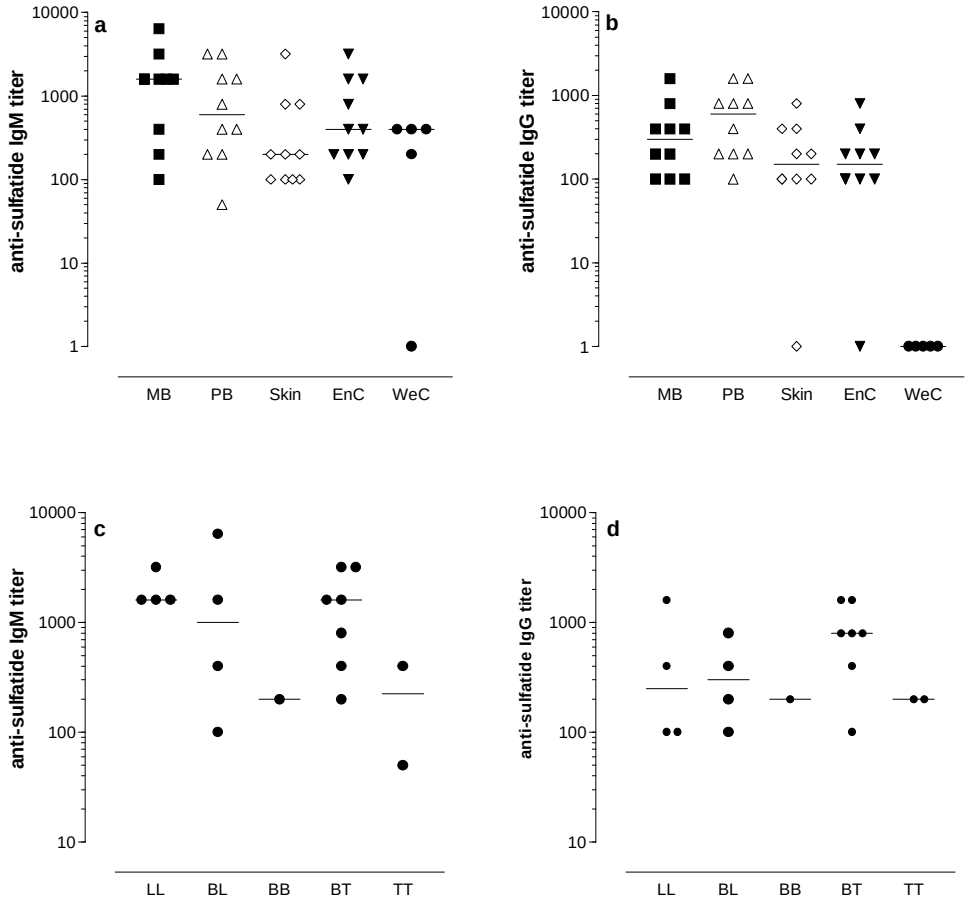

figure 1: Anti-sulfatide IgM (a) and IgG (b) titers in multibacillary (MB), paucibacillary (PB) leprosy patients, endemic healthy contacts (EnC), patients with non-leprosy related skin diseases (Skin) and West European controls (WeC). Median values are plotted for each group. Anti-sulfatide IgM (c) and IgG (d) were plotted against leprosy classification. IgM titers only differed significantly between LL and TT patients (p=0.02). Other differences were not significant.

statistical analysis

The results of the sulfatide ELISA were scored as the highest dilution giving a positive reaction. The titers were transformed logarithmically. Analysis of variance was performed on the results of the different groups. Differences between groups were analyzed with an unpaired two tailed t-test. To analyze the correlation between anti-sulfatide IgM titers and the bacillary index, regression analysis was executed.

results

IgM anti-sulfatide antibodies in untreated leprosy patients

Anti-sulfatide IgM titers in 10 multibacillary and 10 paucibacillary leprosy patients were determined and compared to those in endemic controls with non-leprosy related skin diseases, healthy contacts from the same endemic area and western European controls (figure 1a). Untreated multibacillary patients appeared to have higher anti-sulfatide IgM titers (median: 1600) than paucibacillary patients (median: 600), patients with skin dis-

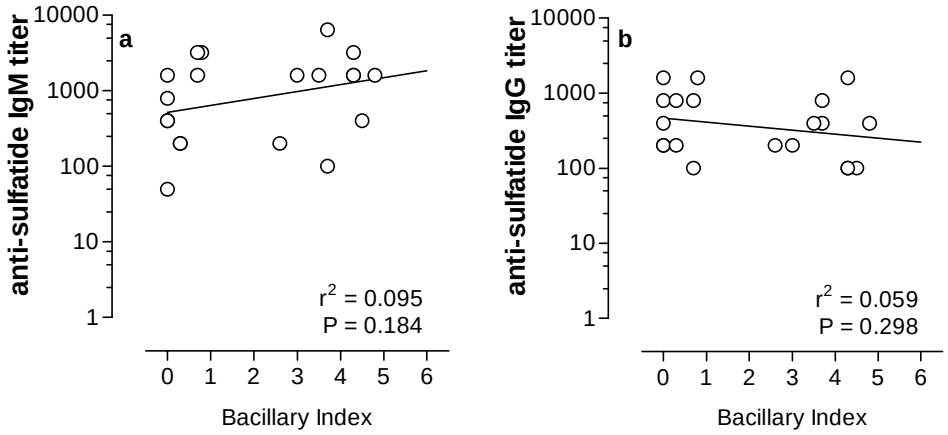

figure 2: Correlation between bacillary index and anti-sulfatide IgM (a) and IgG (b). Regression coefficients and p-values are indicated in each graph.

eases (median: 200) and healthy contacts from the same endemic area (median: 400). Antibody titers in the latter two groups were in the similar range as European healthy controls (median: 400). The differences between the four groups, however, were not statistically significant (p=0.11). However, difference in IgM titers between the different groups of the leprosy spectrum showed significantly lower titers in the tuberculoid group compared to the lepromatous group (figure 1c, p= 0.02).

IgG anti-sulfatide antibodies in untreated leprosy patients

Regarding anti-sulfatide IgG titers (figure 1b), all individuals from the Philippines scored significantly higher than the European controls, which were all negative (p<0.0001). Small differences were found between the four groups from the Philippines. Medians were 300 for the paucibacillary patients, 600 for the multibacillary patients, and 150 for the group with skin diseases and the control group from the same endemic area. These differences were statistically not significant (p=0.09). The differences in IgG titers between the different groups of the leprosy spectrum were not significant either (figure 1d).

correlation between antibody titers and the bacillary index

To examine whether anti-sulfatide antibody titers correlate with the bacterial load of the leprosy patients, anti-sulfatide IgM and IgG titers were plotted against the bacillary index (figure 2). Interestingly, a positive trend was found for IgM but a negative trend for IgG. Both trends failed to read statistical significance (p=0.18 for IgM, p=0.30 for IgG).

anti-sulfatide antibodies and leprosy reactions

Twenty-three patients without any previous history of leprosy reactions were followed longitudinally. In 9 of them a type 1 reversal reaction occurred, 10 patients developed ENL and 4 patients remained free of leprosy reactions during the follow-up period. Anti-sulfatide antibody IgM and IgG titers fluctuated over time (figure 3) in all three groups but did not correlate with either the onset or the occurrence of leprosy reactions. Neither IgM nor IgG titers were affected by therapy (figure 3).

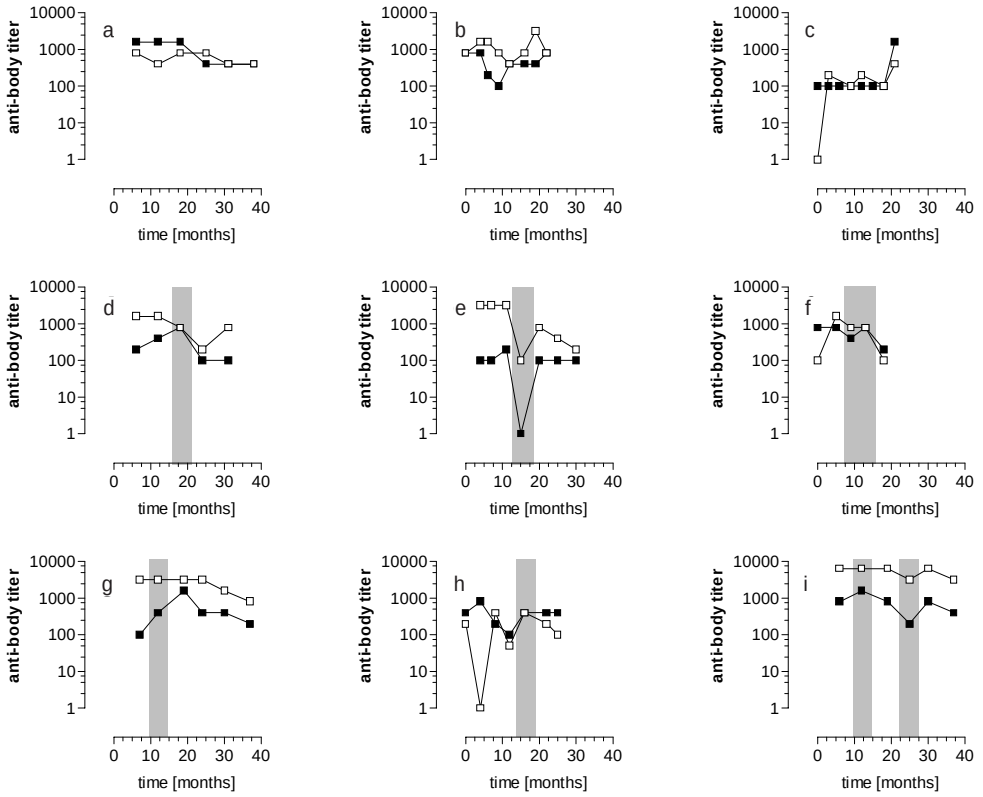

figure 3: Anti-sulfatide antibody titers in reaction free patients (a-c), ENL (d-f) and RR (g-i) patients (3 representative individuals for each group). Reactional episodes are marked in gray. IgM titers are marked with open squares and IgG titers with solid squares.

discussion

In this study, we have investigated the presence of anti-sulfatide antibodies in leprosy with particular emphasis on their association with the occurrence of nerve damage and type 1 or type 2 leprosy reactions. Antibody titers in untreated multi- and paucibacillary patients, healthy contacts, patients with non-leprosy related skin diseases and European controls showed no statistically significant differences in IgG or IgM titers (figure 1a-b). In contrast, anti-sulfatide IgG were absent in European controls and high in diabetic patients and patients with the Guillain Barré syndrome, as expected (data not shown). The differences between western controls and controls from endemic areas might be attributed to the fact that the latter could have had (sub) clinical infections with mycobacteria or other environmental microbes. Such infections might have induced high levels of anti sulfatide antibodies (Luna-Herrera *et al.* 1996). This suggests that the presence of such antibodies is not necessarily associated with nerve inflammation. The results also imply that measurement of anti-sulfatide antibodies is not applicable for early diagnosis, prediction, or monitoring of leprosy reactions in endemic areas. Even though there was a trend towards a correlation between anti-sulfatide IgM titers and bacillary indices, this correlation was not significant.

In a longitudinal analysis, individual anti-sulfatide IgM and IgG titers fluctuated over time. There was no clear association with the onset of leprosy reactions. Anti-sulfatide Ig titers were unaffected by treatment. It has been reported that sooty mangabey monkeys

inoculated intravenously and intracutaneously with *M. leprae* develop anti-ceramide, anti-asialo-GM_1, and anti-galactocerebroside antibodies, 1 to 2 years prior to developing nerve damage (Cho *et al.* 1993). In leprosy patients, anti-sulfatide IgM and IgG titers remained continuously stable over the time period studied (12 to 42 months). The fact that no increase in anti-sulfatide antibody titers could be detected prior to reactional episodes may be attributed to various causes. At the time of diagnosis, 40 to 70% of the leprosy patients already have nerve involvement. Therefore, in these patients raised anti sulfatide Ig titers might already be present before the onset of reactions and thus occur earlier during natural human infection compared to experimental infection of non-human primates. Indeed, experimental infection in the monkey model may not entirely reflect natural infection of the human host and this may strongly influence the titer and type of antibodies. In addition, genetic host factors in leprosy susceptible human hosts may also contribute to these differences. Other neural candidate glycolipids and proteins, like GFAP and S-100 (Thomas and Mukherjee 1990), remain to be studied in order to distinguish between leprosy nerve damage and healthy exposed individuals, as early detection of neural involvement will contribute to prevention of irreversible loss of nerve function.

chapter 7
summary and general discussion

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T cell mediated immune responses to *M. leprae* are essential in protecting the host effectively against developing leprosy. The other side of the coin, however, is the occurrence of collateral damage in the shape of massive destruction of inflamed skin and neural tissue. Tissue damage can be observed in patients across the entire leprosy spectrum. Patients with reactions are particularly at risk. The incidence of reactions has increased dramatically due to the introduction of MDT. A better insight into the mechanisms involved in leprosy reactions and subsequent tissue destruction is needed, in order to prevent permanent disabilities. In this thesis we have defined novel immunological interactions between human Schwann cells, various T cell subsets and *M. leprae* in relation to the immunopathology of leprosy reactions, in particular leprosy neuritis.

Schwann cell invasion

Recently, a molecular explanation for the neurotropism of *M. leprae* has been described, in which the G-domain of the α-chain of extracellular matrix protein laminin2 (LN-α2) plays a central role (Rambukkana *et al.* 1997). LN-α2 functions as a bridge between *M. leprae* and the α/β-dystroglycan complex on Schwann cells (Rambukkana *et al.* 1998). A histone like protein (HLP) has been reported to function as the bacterial receptor for LN-α2 (Shimoji *et al.* 1999). Although these findings enlighten the molecular basis for Schwann cell/*M. leprae* interactions, a number of important questions remain unanswered. *M. leprae* is the only bacillus in the family of mycobacteria that preferentially invades Schwann cells *in vivo*. This fact implies that the mechanism used by *M. leprae* to invade Schwann cells, is based upon specific molecules that are present in *M. leprae* but not other mycobacteria. Regarding HLP, however, highly homologous proteins have been reported in *M. tuberculosis* (Cole *et al.* 1998; Cohavy *et al.* 1999) and other mycobacteria (Lee *et al.* 1998; Pedulla and Hatfull 1998), raising the question why only *M. leprae* expresses such a striking neurotropism. *M. smegmatis* is also able to enter human Schwann cells *in vitro* (chapter 2a). Although invasion seems less efficient than in the case of *M. leprae*, this fact raises some questions regarding the strict *M. leprae* specificity of Schwann cell invasion via HLP/LN-α2 interactions. Differences in expression regarding the amount of protein produced and the localization of the protein, however, may provide an explanation. Originally, prokaryotic HLPs have been localized intracellularly, in close contact with DNA (Broyles and Pettijohn 1986; Bonnefoy and Rouviere-Yaniv 1991). It is therefore surprising to find such a protein on the outer membrane of *M. leprae*. *M. leprae* may possess specific transporter mechanisms to transfer histone like proteins to the cell wall, resulting in a higher concentration of HLP on the outer membrane.

In our hands antibodies to the LN-α2 G domain inhibited Schwann cell/*M. leprae* interaction with 50%, but not antigen presentation (Spierings *et al.* Unpublished). It may well be that the specific interaction between *M. leprae* and Schwann cells via laminin-α2 targets *M. leprae* in neural tissue, thus bringing it in close contact with Schwann cells. However, the actual invasion of Schwann cells may not only involve LN-α2, but also other molecules including Fc-receptors (Vedeler *et al.* 1989), complement receptors (Vedeler *et al.* 1989; Schorey *et al.* 1997), fibronectin (Schorey *et al.* 1995), mannose receptors (Schlesinger 1993). In chapter 6 we describe the presence of an operon that is highly homologous to the *mce1* operon of *M. tuberculosis*. Proteins encoded by these operons have been reported to be involved in host cell entrance (Arruda *et al.* 1993) (Flesselles *et al.* 1999) and may have similar function in *M. leprae*. Functional experiments regarding the functionality of mce proteins in *M. leprae* need to be performed to define their precise role in cellular invasion in general and Schwann cell entrance in particular.

Schwann cells and immune responses

Nerve damage is supposed to be not directly caused by *M. leprae*, but rather the result of immune responses to bacilli present in the nerves. Due to the inability to grow human Schwann cells, studies on immune mediated Schwann cell destruction have been limited to rodent experiments. In these studies, which were not exclusively performed in relation to leprosy but also to other neuropathies, both $CD4^+$ and $CD8^+$ T cells were shown to be capable of interacting with Schwann cells (Wekerle *et al.* 1986; Steinhoff and Kaufmann 1988; Zhang *et al.* 1990; Argall *et al.* 1992; Gold *et al.* 1995). In chapter 2a we addressed this issue using human Schwann cells in combination with human $CD4^+$ T cells, and analyzed proliferative and cytotoxic T cell responses. These studies revealed that *in vitro* cultured untransformed human Schwann cells are well capable of taking up mycobacteria, and process and present them to T cells in an HLA-DR restricted manner. As a consequence of antigen recognition, Schwann cells were killed in this process, thus revealing a novel mechanism of T cell mediated Schwann cell damage.

A point of controversy is the expression of HLA class II on human Schwann cells *in vivo*. Although a number of studies failed to demonstrate expression (Cowley *et al.* 1990; Schmidt *et al.* 1990; Argall *et al.* 1991) other studies reported HLA class II molecules on Schwann cells (Mancardi *et al.* 1988; Mitchell *et al.* 1991). Furthermore, inflammatory cytokines as IFN-γ and TNF-α, as well as Schwann cell invasion by *M. leprae* have been reported to result in up-regulation of MHC class II by rodent Schwann cells (Bergsteinsdottir *et al.* 1992; Atkinson *et al.* 1993; Gold *et al.* 1995). In chapter 2a we demonstrate that *in vitro* cultured human Schwann cells respond accordingly. Thus, although human Schwann cells may not express HLA class II molecules under non-pathological conditions, infection with *M. leprae* and/or the subsequent local immune response probably induce MHC expression by human Schwann cells. In addition to HLA class II molecules, also expression of ICAM-1 was observed both *in vitro* (Gold *et al.* 1995) (chapter 2a) and *in vivo* in relation to leprosy (Sullivan *et al.* 1991), potentially increasing the strength of interaction between T cells and Schwann cells.

In general, TCR-MHC interactions on their own are not sufficient to induce full T cell activation. Co-stimulatory signals via membrane molecules or via secretion of cytokines are required. Cytokines produced by Schwann cells may well influence the outcome of local T cell responses. In this context, we extended our *in vitro* studies to the cytokine production by human Schwann cells after exposure to *M. leprae* (chapter 3). Interestingly, *M. leprae* appeared to induce the production of anti-inflammatory type-2 cytokines as IL-4, IL-10 and PGE_2 and the chemotactic cytokines IL-6 and IL-8. Type-1 inducing cytokines as IFN-γ, IL-12, and IL-18 were not produced. Human Schwann cells exposed to *M. leprae* can therefore be classified as non-professional type-2 antigen presenting cells. Type-2 cytokines are known to suppress type-1 T cells (Salgame *et al.* 1991; Sieling *et al.* 1993; Gong *et al.* 1996; Cua *et al.* 1999), which are strongly associated with reversal reactions and inflammatory immunopathology (Yamamura *et al.* 1991; Yamamura *et al.* 1992; Verhagen *et al.* 1997). The suppressive effect of type-2 cytokines could either be via down-regulating antigen presentation, including HLA class II expression, or via the induction of regulatory T cells (Groux *et al.* 1997). In this way Schwann cells may attempt to maintain their immune privileged status. It is possible that killing of *M. leprae* in the nerves by MDT reduces the production of type 2 cytokines by Schwann cells, thus giving room for detrimental Th1 responses, resulting in enhanced predisposition to reactional episodes as indeed often observed during MDT.

neural T cells and pathology

T cells present in inflamed neural tissue are likely to play an important role in local tissue destruction. In chapter 4, neural T cells were isolated from leprosy patients with neural involvement and compared with similarly generated T cells from peripheral blood of the same individuals. T cell lines from leprosy nerve biopsies differed from T cells in peripheral blood regarding the expression of N-CAM. Although an earlier study BY Jullien *et al.* (Jullien *et al.* 1997) reported that tuberculoid leprosy patients' skin lesions contain $CD3^+$ $N\text{-}CAM^+$ T cells, our work suggested that N-CAM positive T cells were clearly detectable in patients at the time of active ENL, but not in patients with reversal reactions.

We examined the capacity of $N\text{-}CAM^+$ T cells to lyse Schwann cells. Nerve derived T cells efficiently killed $N\text{-}CAM^+$ target cells, including human Schwann cells, in an antigen independent fashion, whereas this was much less efficient in the case of peripheral T cells from the same patients. Lysis was caused by $CD8^+$ and not $CD4^+$ T cells and accompanied by high N-CAM expression on these cells. The mechanism by which T cells up-regulate N-CAM is unclear, but may be regulated by TGF-β (Stewart *et al.* 1995) or IL-15 (Jullien *et al.* 1997). The latter study indeed observed IL-15 production in response to *M. leprae in vitro* and noted a correlation between the number of N-CAM positive T cells in skin lesions and the level of IL-15 mRNA expression. Our findings suggest that $N\text{-}CAM^+$ T cells have cytolytic activity and are specifically able to kill $N\text{-}CAM^+$ target cells, including Schwann cells. $N\text{-}CAM^-$ cells could not be killed. We speculate that this mechanism contributes to the selective killing of Schwann cells in leprosy neuritis.

The question remains whether N-CAM is directly involved in target cell lysis., or whether N-CAM expression is simply a characteristic of NK-like $CD8^+$ T cells. This issue cannot be addressed appropriately at the moment, due to the absence of N-CAM neutralizing antibodies.

Expression of N-CAM may also be of clinical value. Although N-CAM expression was low on peripheral T cells of leprosy patients, stimulation with *M. leprae* induced N-CAM on peripheral $CD8^+$ T cells from ENL patients at the time of active ENL. Moreover, at the same time antigen independent target killing was raised in these patients. A more detailed evaluation of prospectively collected patient materials is needed before these parameters can be implied as possible prognostic factors for ENL.

For the first time, *M. leprae* specific T cells could be isolated and cloned from neural tissue from a patient with a reversal reaction (Spierings *et al.* Unpublished). Analysis of cytokine profiles of these cells confirmed earlier findings in skin lesions: *M. leprae* reactive T cells with a Th1 phenotype are dominantly present during reversal reactions. Whereas T cells isolated from peripheral blood of leprosy patients are generally HLA-DR restricted (Ottenhoff *et al.* 1986b; Le Poole *et al.* 1993; Mutis *et al.* 1993a), a significant number of T cells isolated from this patient was HLA-DP restricted. The small differences between HLA-DP restricted T cells from nerve lesions and peripheral blood were, however, not statistically significant. Since high expression of HLA-DP can be found on human Schwann cells (chapter 2a), HLA-DP restricted T cells may well have functional implications regarding antigen presentation. Analysis of a larger number of reactional patients and functional analysis of these T cells are required in order to evaluate these findings more precisely.

detection of nerve damage

Cellular assays are generally difficult to apply in field situations. Therefore, measurement of serological markers may yield more simple tests for the detection of leprosy reactions or nerve damage. Since the loss of nerve function is often associated with demyelination, we tested whether antibodies to human sulfatide, a myelin component, could be

useful (chapter 6). Antibodies against sulfatides were detectable in leprosy patients of all classification, regardless of the occurrence of reversal reactions or ENL. Furthermore, endemic controls, in contrast to west-European controls, showed positive titers. Sulfatide antibodies are therefore not discriminating between leprosy patients with and without reactions.

Aside to glycolipid components of myelin, neural proteins or, more specific, Schwann cell proteins may be usefull in the early detection of neural involvement. An earlier report suggested the presence of antibodies against a total nerve preparation in leprosy patients (Thomas and Mukherjee 1990). Monoclonal antibodies directed towards GFAP and S-100β recognized proteins of the same size. Direct evidence that these candidate proteins were recognized by serum from leprosy patients is still missing. Using purified S-100β and GFAP, we were unable to confirm these data (Spierings *et al.* Unpublished). Serum of ENL patients, however, specifically interacted with Schwann cell derived proteins of approximately 20 kDa and 50 kDa, the latter being positive in 6 out of 10 cases of ENL. Sera of only 1 out of 9 patients with reversal reactions and 2 out of 21 patients without leprosy reactions contained antibodies to the 50 kDa protein. A human Schwann cell cDNA library has been constructed to characterize these two proteins and to analyze their value in the detection of leprosy reactions (Spierings *et al.* Unpublished).

synopsis

Exposure of Schwann cells to *M. leprae* has multiple consequences. On the one hand, Schwann cells start to produce anti-inflammatory type-2 cytokines, possibly in an attempt to prevent detrimental Th1 responses. On the other hand, Schwann cells may present *M. leprae* antigens to $CD4^+$ Th1 cells which may possess cytotoxic characteristics. The latter results in direct killing of *M. leprae* infected Schwann cells and subsequently in nerve damage. Since Th1 responses are associated with RR, rather than ENL, this mechanism is likely to play a role in the pathogenesis of nerve damage during RR.

T cells isolated from inflamed neural tissue highly express N-CAM, while N-CAM expression was low on T cells isolated from peripheral blood of the same individuals. During clinically active ENL, an increased number of $CD8^+$ T cells expresses N-CAM, in peripheral blood and in skin lesions. This increase is associated with a more efficient antigen independent, MHC unrestricted killing of target cells, including Schwann cells. These T cells may be responsible for Schwann cell destruction in ENL. The precise role of N-CAM in leprosy pathology remains to be elucidated, together with the value of N-CAM expression in predicting activation of ENL.

HLP/LN-α2/DG interactions are likely to play an important role in Schwann cell/*M. leprae* adherence. Regarding the actual cell invasion, the contribution of this interaction remains questionable. *M. leprae* contains an *mce1* operon, which has a high homology with *mce* operons of *M. tuberculosis*. Gene products encoded by these operons have been reported to be involved in cell invasion. Functional data on the mce1 proteins of *M. leprae* is still lacking.

In contrast to monkey models, antibodies directed against sulfatides do not correlate with the occurrence of nerve damage or leprosy reactions. Major problem in this respect is the high antibody titer in healthy controls from endemic areas. The preliminary data regarding antibodies against Schwann cell proteins look promising, although these antibodies are also present long after the occurrence of active ENL. Identification of the 50 kDa protein and testing of larger cohorts are essential to evaluate these findings.

references

Altare, F., Durandy, A., Lammas, D. A., Emile, J. F., Lamhamedi, S., Le Deist, F., Drysdale, P., Jouanguy, E., Doffinger, R., Bernaudin, F., Jeppsson, O., Gollob, J. A., Meinl, E., Segal, A. W., Fischer, A., Kumararatne, D. S., and Casanova, J. L. 1998. Impairment of mycobacterial immunity in human interleukin-12 receptor deficiency. Science **280**: 1432-1435.

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. 1990. Basic local alignment search tool. Journal of Molecular Biology **215**: 403-410.

Anderson, D. J. 1993. Cell and molecular biology of neural crest cell lineage diversification. Curr.Opin.Neurobiol. **3**: 8-13.

Antel, P., McCrea, E., Ladiwala, U., Qin, Q., and Becher, B. 1998. Non-MHC restricted cell-mediated lysis of human oligodendrocytes in vitro: relation with CD56 expression. J.Immunol. **160**: 1606-1611.

Appelberg, R. 1994. Protective role of interferon gamma, tumor necrosis factor alpha and interleukin-6 in *Mycobacterium tuberculosis* and *M. avium* infections. Immunobiology **191**: 520-525.

Argall, K. G., Armati, P. J., King, N. J., and Douglas, M. W. 1991. The effects of West Nile virus on major histocompatibility complex class I and II molecule expression by Lewis rat Schwann cells in vitro. J.Neuroimmunol. **35**: 273-284.

Argall, K. G., Armati, P. J., Pollard, J. D., Watson, E., and Bonner, J. 1992. Interactions between CD4+ T-cells and rat Schwann cells in vitro. 1. Antigen presentation by Lewis rat Schwann cells to P2- specific CD4+ T-cell lines. J.Neuroimmunol. **40**: 1-18.

Arruda, S., Bomfim, G., Knights, R., Huima-Byron, T., and Riley, L. W. 1993. Cloning of an M. tuberculosis DNA fragment associated with entry and survival inside cells. Science **261**: 1454-1457.

Atkinson, P. F., Perry, M. E., Hall, S. M., and Hughes, R. A. 1993. Immunoelectronmicroscopical demonstration of major histocompatibility class II antigen: expression on endothelial and perivascular cells but not Schwann cells in human neuropathy. Neuropathol.Appl.Neurobiol. **19**: 22-30.

Aumailley, M., Gerl, M., Sonnenberg, A., Deutzmann, R., and Timpl, R. 1990. Identification of the Arg-Gly-Asp sequence in laminin A chain as a latent cell-binding site being exposed in fragment P1. FEBS Lett. **262**: 82-86.

Aumailley, M., Gurrath, M., Muller, G., Calvete, J., Timpl, R., and Kessler, H. 1991. Arg-Gly-Asp constrained within cyclic pentapeptides. Strong and selective inhibitors of cell adhesion to vitronectin and laminin fragment P1. FEBS Lett. **291**: 50-54.

Bai, X. F., Shi, F. D., Xiao, B. G., Li, H. L., van der Meide, P. H., and Link, H. 1997. Nasal administration of myelin basic protein prevents relapsing experimental autoimmune encephalomyelitis in DA rats by activating regulatory cells expressing IL-4 and TGF-beta mRNA. J.Neuroimmunol. **80**: 65-75.

Barnes, P. F., Chatterjee, D., Brennan, P. J., Rea, T. H., and Modlin, R. L. 1992. Tumor necrosis factor production in patients with leprosy. Infect.Immun. **60**: 1441-1446.

Barnes, P. F. and Modlin, R. L. 1996. Human cellular immune responses to *Mycobacterium tuberculosis*. Cur.Top.Microbiol.Immunol. **215**: 197-219.

Bergsteinsdottir, K., Kingston, A. E., and Jessen, K. R. 1992. Rat Schwann cells can be induced to express major histocompatibility complex class II molecules in vivo. Journal of Neurocytology **21**: 382-390.

Bergsteinsdottir, K., Kingston, A. E., Mirsky, R., and Jessen, K. R. 1991. Rat Schwann cells produce interleukin-1. J.Neuroimmunol. **34**: 15-23.

Bishop, P. J. and Neumann, G. 1970. The history of the Ziehl-Neelsen stain. Tubercle **51**: 196-206.

Bloom, B. R. and Godal, T. 1983. Selective primary health care: strategies for control of disease in the developing world. V. Leprosy. Rev Infect Dis **5**: 765-780.

Boddingius, J. 1974. The occurrence of *Mycobacterium leprae* within axons of peripheral nerves. Acta Neuropathol.Berl. **27**: 257-270.

Bolin, L. M., Verity, A. N., Silver, J. E., Shooter, E. M., and Abrams, J. S. 1995. Interleukin-6 production by Schwann cells and induction in sciatic nerve injury. J.Neurochem. **64**: 850-858.

Bonnefoy, E. and Rouviere-Yaniv, J. 1991. HU and IHF, two homologous histone-like proteins of Escherichia coli, form different protein-DNA complexes with short DNA fragments. EMBO J. **10**: 687-696.

Boonstra, J. G., Deckers, J. G., Laterveer, J. C., Price, V., van Es, L. A., Bruijn, J. A., Daha, M. R., and van der Woude, F. J. 1997. Pancreas and kidney allograft-infiltrating cells in simultaneous pancreas-kidney transplantation. Transplantation **63**: 1470-1475.

Bright, J. J. and Sriram, S. 1998. TGF-beta inhibits IL-12-induced activation of Jak-STAT pathway in T lymphocytes. J.Immunol. **161**: 1772-1777.

Broyles, S. S. and Pettijohn, D. E. 1986. Interaction of the Escherichia coli HU protein with DNA. Evidence for

formation of nucleosome-like structures with altered DNA helical pitch. J.Mol.Biol. **187**: 47-60.

Buchanan, T. M. 1995. Serology of leprosy. *In* Leprosy. *Edited by* R.C.Hastings. Churchill Livingstone, Edinburgh pp. 169-176.

Buhrer, S. S., Smits, H. L., Gussenhoven, G. C., van Ingen, C. W., and Klatser, P. R. 1998. A simple dipstick assay for the detection of antibodies to phenolic glycolipid-I of *Mycobacterium leprae*. Am.J.Trop.Med.Hyg. **58**: 133-136.

Buschard, K., Josefsen, K., Horn, T., and Fredman, P. 1993. Sulphatide and sulphatide antibodies in insulin-dependent diabetes mellitus. Lancet **342**: 840-840.

Cadoni, A., Zicca, A., and Mancardi, G. L. 1986. Schwann cell expression of HLA-DR antigen in peripheral neuropathies. Lancet **2**: 1281-1282.

Cho, S. N., Gormus, B. J., Xu, K., Bohm, R. P., Jr., Walsh, G. P., Meyers, W. M., and Kim, J. D. 1993. Serologic responses to nerve antigens in sooty mangabey monkeys with experimental leprosy. Int.J.Lep.Other Mycobact.Dis. **61**: 236-244.

Coetzee, T., Fujita, N., Dupree, J., Shi, R., Blight, A., Suzuki, K., and Popko, B. 1996. Myelination in the absence of galactocerebroside and sulfatide: normal structure with abnormal function and regional instability. Cell **86**: 209-219.

Cohavy, O., Harth, G., Horwitz, M. A., Eggena, M., Landers, C., Sutton, C., Targan, S. R., and Braun, J. 1999. Identification of a novel mycobacterial histone H1 homologue (HupB) as an antigenic target of pANCA monoclonal antibody and serum immunoglobulin A from patients with Crohn's disease. Infect.Immun. **67**: 6510-6517.

Cole, S. T., Brosch, R., Parkhill, J., Garnier, T., Churcher, C., Harris, D., Gordon, S. V., Eiglmeier, K., Gas, S., Barry, C. E., Tekaia, F., Badcock, K., Basham, D., Brown, D., Chillingworth, T., Connor, R., Davies, R., Devlin, K., Feltwell, T., Gentles, S., Hamlin, N., Holroyd, S., Hornsby, T., Jagels, K., and Barrell, B. G. 1998. Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. Nature **393**: 537-544.

Colognato, H., MacCarrick, M., O'Rear, J. J., and Yurchenco, P. D. 1997. The laminin alpha2-chain short arm mediates cell adhesion through both the alpha1beta1 and alpha2beta1 integrins. J.Biol.Chem. **272**: 29330-29336.

Constable, A. L., Armati, P. J., Toyka, K. V., and Hartung, H. P. 1994. Production of prostanoids by Lewis rat Schwann cells in vitro. Brain Res. **635**: 75-80.

Cooper, A. M., Dalton, D. K., Stewart, T. A., Griffin, J. P., Russell, D. G., and Orme, I. M. 1993. Disseminated tuberculosis in interferon gamma gene-disrupted mice. J.Exp.Med. **178**: 2243-2247.

Cooper, C. L., Mueller, C., Sinchaisri, T. A., Pirmez, C., Chan, J., Kaplan, G., Young, S. M., Weissman, I. L., Bloom, B. R., Rea, T. H., and Modlin, R. L. 1989. Analysis of naturally occurring delayed-type hypersensitivity reactions in leprosy by in situ hybridization. J.Exp.Med. **169**: 1565-1581.

Cowley, S. A., Gschmeissner, S. E., Negesse, Y., Curtis, J., and Turk, J. L. 1990. Major histocompatibility complex class II antigen expression in nerves in leprosy; an immunoelectronmicroscopical study. Int.J.Lep.Other Mycobact.Dis. **58**: 560-565.

Cua, D. J., Groux, H., Hinton, D. R., Stohlman, S. A., and Coffman, R. L. 1999. Transgenic interleukin 10 prevents induction of experimental autoimmune encephalomyelitis. J.Exp.Med. **189**: 1005-1010.

D'Orazio, T. J. and Niederkorn, J. Y. 1998. A novel role for TGF-beta and IL-10 in the induction of immune privilege. J.Immunol. **160**: 2089-2098.

Dalrymple, S. A., Lucian, L. A., Slattery, R., McNeil, T., Aud, D. M., Fuchino, S., Lee, F., and Murray, R. 1995. Interleukin-6-deficient mice are highly susceptible to Listeria monocytogenes infection: correlation with inefficient neutrophilia. Infect.Immun. **63**: 2262-2268.

de Jong, R., Altare, F., Haagen, I. A., Elferink, D. G., Boer, T., van Breda, V., Kabel, P. J., Draaisma, J. M., van Dissel, J. T., Kroon, F. P., Casanova, J. L., and Ottenhoff, T. H. M. 1998. Severe mycobacterial and Salmonella infections in interleukin-12 receptor-deficient patients. Science **280**: 1435-1438.

de Waal Malefyt, R., Abrams, J., Bennett, B., Figdor, C. G., and de Vries, J. E. 1991. Interleukin 10(IL-10) inhibits cytokine synthesis by human monocytes: an autoregulatory role of IL-10 produced by monocytes. J.Exp.Med. **174**: 1209-1220.

Deckers, J. G., De Haij, S., van der Woude, F. J., van der Kooij, S. W., Daha, M. R., and van Kooten, C. 1998. IL-4 and IL-13 augment cytokine- and CD40-induced RANTES production by human renal tubular epithelial cells in vitro. J.Am.Soc.Nephrol. **9**: 1187-1193.

Denis, M. 1991. Activated murine natural killer cells control growth of *Mycobacterium lepraemurium* in mouse macrophages; in vitro and in vivo evidence. Int.J.Immunopharmacol. **13**: 881-887.

Dupouey, P., Zalc, B., Lefroit-Joly, M., and Gomes, D. 1979. Localization of galactosylceramide and sulfatide at the surface of the myelin sheath: an immunofluorescence study in liquid medium. Cell.Mol.Biol. **25**: 269-272.

Engvall, E. 1993. Laminin variants: why, where and when? Kidney Int. **43**: 2-6.

references

Engvall, E., Earwicker, D., Haaparanta, T., Ruoslahti, E., and Sanes, J. R. 1990. Distribution and isolation of four laminin variants; tissue restricted distribution of heterotrimers assembled from five different subunits. Cell Regul. **1**: 731-740.

Engvall, E. and Wewer, U. M. 1996. Domains of laminin. J.Cell Biochem. **61**: 493-501.

Eugster, H. P., Frei, K., Kopf, M., Lassmann, H., and Fontana, A. 1998. IL-6-deficient mice resist myelin oligodendrocyte glycoprotein-induced autoimmune encephalomyelitis. Eur.J.Immunol. **28**: 2178-2187.

Flanders, K. C., Ludecke, G., Engels, S., Cissel, D. S., Roberts, A. B., Kondaiah, P., Lafyatis, R., Sporn, M. B., and Unsicker, K. 1991. Localization and actions of transforming growth factor-beta s in the embryonic nervous system. Development **113**: 183-191.

Flesch, I. E. and Kaufmann, S. H. 1990. Stimulation of antibacterial macrophage activities by B-cell stimulatory factor 2 (interleukin-6). Infect.Immun. **58**: 269-271.

Flesselles, B., Anand, N. N., Remani, J., Loosmore, S. M., and Klein, M. H. 1999. Disruption of the mycobacterial cell entry gene of *Mycobacterium bovis* BCG results in a mutant that exhibits a reduced invasiveness for epithelial cells. FEMS Microbiol.Lett. **177**: 237-242.

Flynn, J. L., Goldstein, M. M., Chan, J., Triebold, K. J., Pfeffer, K., Lowenstein, C. J., Schreiber, R., Mak, T. W., and Bloom, B. R. 1995. Tumor necrosis factor-alpha is required in the protective immune response against *Mycobacterium tuberculosis* in mice. Immunity **2**: 561-572.

Flynn, J. L., Goldstein, M. M., Triebold, K. J., Koller, B., and Bloom, B. R. 1992. Major histocompatibility complex class I-restricted T cells are required for resistance to *Mycobacterium tuberculosis* infection. Proc.Natl.Acad.Sci.U.S.A. **89**: 12013-12017.

Ford, A. L., Britton, W. J., and Armati, P. J. 1993. Schwann cells are able to present exogenous mycobacterial hsp70 to antigen-specific T lymphocytes. J.Neuroimmunol. **43**: 151-159.

Francel, P. C., Francel, T. J., Mackinnon, S. E., and Hertl, C. 1997. Enhancing nerve regeneration across a silicone tube conduit by using interposed short-segment nerve grafts. J.Neurosurg. **87**: 887-892.

Fry, J. M., Weissbarth, S., Lehrer, G. M., and Bornstein, M. B. 1974. Cerebroside antibody inhibits sulfatide synthesis and myelination and demyelinates in cord tissue cultures. Science **183**: 540-542.

Gavrilovic, J., Brennan, A., Mirsky, R., and Jessen, K. R. 1995. Fibroblast growth factors and insulin growth factors combine to promote survival of rat Schwann cell precursors without induction of DNA synthesis. Eur.J.Neurosci. **7**: 77-85.

Gazzinelli, R. T., Wysocka, M., Hieny, S., Scharton-Kersten, T., Cheever, A., Kuhn, R., Muller, W., Trinchieri, G., and Sher, A. 1996. In the absence of endogenous IL-10, mice acutely infected with Toxoplasma gondii succumb to a lethal immune response dependent on CD4+ T cells and accompanied by overproduction of IL-12, IFN-gamma and TNF-alpha. J.Immunol. **157**: 798-805.

Geiser, A. G., Letterio, J. J., Kulkarni, A. B., Karlsson, S., Roberts, A. B., and Sporn, M. B. 1993. Transforming growth factor beta 1 (TGF-beta 1) controls expression of major histocompatibility genes in the postnatal mouse: aberrant histocompatibility antigen expression in the pathogenesis of the TGF-beta 1 null mouse phenotype. Proc.Natl.Acad.Sci.U.S.A. **90**: 9944-9948.

Godal, T., Lofgren, M., and Negassi, K. 1972. Immune response to *M. leprae* of healthy leprosy contacts. Int.J.Lep.Other Mycobact.Dis. **40**: 243-250.

Godal, T. and Negassi, K. 1973. Subclinical infection in leprosy. Br.Med.J. **3**: 557-559.

Gold, R., Toyka, K. V., and Hartung, H. P. 1995. Synergistic effect of IFN-gamma and TNF-alpha on expression of immune molecules and antigen presentation by Schwann cells. Cell.Immunol. **165**: 65-70.

Gong, J. H., Zhang, M., Modlin, R. L., Linsley, P. S., Iyer, D., Lin, Y., and Barnes, P. F. 1996. Interleukin-10 downregulates *Mycobacterium tuberculosis*-induced Th1 responses and CTLA-4 expression. Infect.Immun. **64**: 913-918.

Gorham, J. D., Guler, M. L., Fenoglio, D., Gubler, U., and Murphy, K. M. 1998. Low dose TGF-beta attenuates IL-12 responsiveness in murine Th cells. J.Immunol. **161**: 1664-1670.

Goulart, I. M., Figueiredo, F., Coimbra, T., and Foss, N. T. 1996. Detection of transforming growth factor-beta 1 in dermal lesions of different clinical forms of leprosy. Am.J.Pathol. **148**: 911-917.

Groux, H., O'Garra, A., Bigler, M., Rouleau, M., Antonenko, S., de Vries, J. E., and Roncarolo, M. G. 1997. A CD4+ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. Nature **389** : 737-742.

Gulati, A. K. 1995. Immunological fate of Schwann cell-populated acellular basal lamina nerve allografts. Transplantation **59** : 1618-1622.

Haanen, J. B., de Waal Malefyt, R., Res, P. C., Kraakman, E. M., Ottenhoff, T. H. M., de Vries, R. R., and Spits, H. 1991. Selection of a human T helper type 1-like T cell subset by mycobacteria. J.Exp.Med. **174**: 583-592.

Hamerlinck, F. F., Klatser, P. R., Walsh, D. S., Bos, J. D., Walsh, G. P., and Faber, W. R. 1999. Serum neopterin as a marker for reactional states in leprosy. FEMS Immunol.Med.Microbiol. **24**: 405-409.

Hancock, G. E., Cohn, Z. A., and Kaplan, G. 1989. The generation of antigen-specific, major histocompatibility complex- restricted cytotoxic T lymphocytes of the CD4+ phenotype. Enhancement by the cutaneous administration of interleukin 2. J.Exp.Med. **169**: 909-919.

Hansen, G. A. 1874. Undersogelser angaende spedalskhedens ansager. Norsk Magasin for Laegevitenskapen **4**: 1-88.

Harboe, M. and Wiker, H. G. 1998. Secreted proteins of *Mycobacterium leprae*. Scand.J.Immunol. **48**: 577-584.

Hermans, P. W., van Soolingen, D., and van Embden, J. D. 1992. Characterization of a major polymorphic tandem repeat in *Mycobacterium tuberculosis* and its potential use in the epidemiology of *Mycobacterium kansasii* and *Mycobacterium gordonae*. J.Bacteriol. **174**: 4157-4165.

Hunter, S. W. and Brennan, P. J. 1981. A novel phenolic glycolipid from *Mycobacterium leprae* possibly involved in immunogenicity and pathogenicity. J.Bacteriol. **147**: 728-735.

Hunter, S. W., Fujiwara, T., and Brennan, P. J. 1982. Structure and antigenicity of the major specific glycolipid antigen of *Mycobacterium leprae*. J.Biol.Chem. **257**: 15072-15078.

Hussain, R., Lucas, S. B., Kifayet, A., Jamil, S., Raynes, J., Uqaili, Z., Dockrell, H. M., Chiang, T. J., and McAdam, K. P. 1995. Clinical and histological discrepancies in diagnosis of ENL reactions classified by assessment of acute phase proteins SAA and CRP. Int.J.Lep.Other Mycobact.Dis. **63**: 222-230.

Ilyas, A. A., Mithen, F. A., Dalakas, M. C., Wargo, M., Chen, Z. W., Bielory, L., and Cook, S. D. 1991. Antibodies to sulfated glycolipids in Guillain-Barre syndrome. J.Neurol.Sci. **105**: 108-117.

Janson, A. A., Klatser, P. R., van der Zee, R., Cornelisse, Y. E., de Vries, R. R., Thole, J. E., and Ottenhoff, T. H. M. 1991. A systematic molecular analysis of the T cell-stimulating antigens from *Mycobacterium leprae* with T cell clones of leprosy patients. Identification of a novel *M. leprae* HSP 70 fragment by *M. leprae*-specific T cells. J.Immunol. **147**: 3530-3537.

Jarlier, V. and Nikaido, H. 1994. Mycobacterial cell wall: structure and role in natural resistance to antibiotics. FEMS Microbiol.Lett. **123**: 11-18.

Jessen, K. R., Brennan, A., Morgan, L., Mirsky, R., Kent, A., Hashimoto, Y., and Gavrilovic, J. 1994. The Schwann cell precursor and its fate: a study of cell death and differentiation during gliogenesis in rat embryonic nerves. Neuron **12**: 509-527.

Jessen, K. R. and Mirsky, R. 1991. Schwann cell precursors and their development. Glia **4**: 185-194.

Jouanguy, E., Altare, F., Lamhamedi, S., Revy, P., Emile, J. F., Newport, M. J., Levin, M., Blanche, S., Seboun, E., Fischer, A., and Casanova, J. L. 1996. Interferon-gamma-receptor deficiency in an infant with fatal bacille Calmette-Guerin infection. N.Engl.J.Med. **335**: 1956-1961.

Jullien, D., Sieling, P. A., Uyemura, K., Mar, N. D., Rea, T. H., and Modlin, R. L. 1997. IL-15, an immunomodulator of T cell responses in intracellular infection. J.Immunol. **158**: 800-806.

Kaleab, B. Mycobacteria induced cytotoxic T cells in leprosy. Leiden University Hospital.

Kaleab, B., Kiessling, R., van Embden, J. D., Thole, J. E., Kumararatne, D. S., Pisa, P., Wondimu, A., and Ottenhoff, T. H. M. 1990b. Induction of antigen-specific CD4+ HLA-DR-restricted cytotoxic T lymphocytes as well as nonspecific nonrestricted killer cells by the recombinant mycobacterial 65-kDa heat-shock protein. Eur.J.Immunol. **20**: 369-377.

Kaleab, B., Ottenhoff, T. H. M., Converse, P., Halapi, E., Tadesse, G., Rottenberg, M., and Kiessling, R. 1990a. Mycobacterial-induced cytotoxic T cells as well as nonspecific killer cells derived from healthy individuals and leprosy patients. Eur.J.Immunol. **20**: 2651-2659.

Kalinski, P., Hilkens, C. M., Snijders, A., Snijdewint, F. G., and Kapsenberg, M. L. 1997. IL-12-deficient dendritic cells, generated in the presence of prostaglandin E2, promote type 2 cytokine production in maturing human naive T helper cells. J.Immunol. **159**: 28-35.

Kalinski, P., Hilkens, C. M., Wierenga, E. A., and Kapsenberg, M. L. 1999. T-cell priming by type-1 and type-2 polarized dendritic cells: the concept of a third signal. Immunol.Today **20**: 561-567.

Kamijo, R., Le, J., Shapiro, D., Havell, E. A., Huang, S., Aguet, M., Bosland, M., and Vilcek, J. 1993. Mice that lack the interferon-gamma receptor have profoundly altered responses to infection with Bacillus Calmette-Guerin and subsequent challenge with lipopolysaccharide. J.Exp.Med. **178**: 1435-1440.

Kaneko, H., Yamada, H., Mizuno, S., Udagawa, T., Kazumi, Y., Sekikawa, K., and Sugawara, I. 1999. Role of tumor necrosis factor-alpha in Mycobacterium-induced granuloma formation in tumor necrosis factor-alpha-deficient mice. Lab Invest **79**: 379-386.

Kaplan, G., Kiessling, R., Teklemariam, S., Hancock, G. E., Sheftel, G., Job, C. K., Converse, P., Ottenhoff, T. H. M., Becx-Bleumink, M., Dietz, M., and Cohn, Z. A. 1989. The reconstitution of cell-mediated immunity in the cutaneous lesions of lepromatous leprosy by recombinant interleukin 2. J.Exp.Med. **169**: 893-907.

Keeley, R., Atagi, T., Sabelman, E., Padilla, J., Kadlcik, P., Agras, J., Eng, L., Wiedman, T. W., Nguyen, K., Sudekum, A., and Rosen, J. 1993. Synthetic nerve graft containing collagen and synthetic Schwann cells

improves functional, electrophysiological, and histopathological parameters of of peripheral nerve regeneration. Rest.Neurol.Neurosci. **5**: 353-366.

Khanolkar-Young, S., Rayment, N., Brickell, P. M., Katz, D. R., Vinayakumar, S., Colston, M. J., and Lockwood, D. N. 1995. Tumour necrosis factor-alpha (TNF-alpha) synthesis is associated with the skin and peripheral nerve pathology of leprosy reversal reactions. Clin.Exp.Immunol. **99** : 196-202.

Kiefer, R., Streit, W. J., Toyka, K. V., Kreutzberg, G. W., and Hartung, H. P. 1995. Transforming growth factor-beta 1: a lesion-associated cytokine of the nervous system. Int.J.Dev.Neurosci. **13**: 331-339.

Kindler, V., Sappino, A. P., Grau, G. E., Piguet, P. F., and Vassalli, P. 1989. The inducing role of tumor necrosis factor in the development of bactericidal granulomas during BCG infection. Cell **56**: 731-740.

Kingston, A. E., Bergsteinsdottir, K., Jessen, K. R., van der Meide, P. H., Colston, M. J., and Mirsky, R. 1989. Schwann cells co-cultured with stimulated T cells and antigen express major histocompatibility complex (MHC) class II determinants without interferon-gamma pretreatment: synergistic effects of interferon-gamma and tumor necrosis factor on MHC class II induction. Eur.J.Immunol. **19**: 177-183.

Kopf, M., Baumann, H., Freer, G., Freudenberg, M., Lamers, M., Kishimoto, T., Zinkernagel, R., Bluethmann, H., and Kohler, G. 1994. Impaired immune and acute-phase responses in interleukin-6-deficient mice. Nature **368**: 339-342.

Koppelman, B., Neefjes, J. J., de Vries, J. E., and de Waal Malefyt, R. 1997. Interleukin-10 down-regulates MHC class II alphabeta peptide complexes at the plasma membrane of monocytes by affecting arrival and recycling. Immunity **7**: 861-871.

Kumararatne, D. S., Stober, C., Harvey, C. J., Kendrick, N., and Lammas, D. A. 1996. Heterogeneity of ATP receptor expression on human monocyte-derived macrophages.Third International Conference on the Pathogenesis of Mycobacterial Infections , 102.

Ladel, C. H., Blum, C., Dreher, A., Reifenberg, K., Kopf, M., and Kaufmann, S. H. 1997. Lethal tuberculosis in interleukin-6-deficient mutant mice. Infect.Immun. **65**: 4843-4849.

Ladel, C. H., Daugelat, S., and Kaufmann, S. H. 1995. Immune response to *Mycobacterium bovis* bacille Calmette Guerin infection in major histocompatibility complex class I- and II-deficient knock-out mice: Contribution of CD4 and CD8 T cells to acquired resistance. Eur.J.Immunol. **25**: 377-384.

Lal, H., Jain, V. K., Mittal, R. A., Chaudhary, S. D., and Saini, V. 1993. Detection of antibodies to phenolic glycolipid by ELISA in leprosy patients. Indian J.Lepr. **65**: 95-99.

Lalvani, A., Brookes, R., Wilkinson, R. J., Malin, A. S., Pathan, A. A., Andersen, P., Dockrell, H. M., Pasvol, G., and Hill, A. V. 1998. Human cytolytic and interferon gamma-secreting CD8+ T lymphocytes specific for *Mycobacterium tuberculosis*. Proc.Natl.Acad.Sci.U.S.A. **95**: 270-275.

Lamers, C. H., van de Griend, R. J., Braakman, E., Ronteltap, C. P., Benard, J., Stoter, G., Gratama, J. W., and Bolhuis, R. L. 1992. Optimization of culture conditions for activation and large- scale expansion of human T lymphocytes for bispecific antibody- directed cellular immunotherapy. Int.J.Cancer **51**: 973-979.

Lammas, D. A., Stober, C., Harvey, C. J., Kendrick, N., Panchalingam, S., and Kumararatne, D. S. 1997. ATP-induced killing of mycobacteria by human macrophages is mediated by purinergic P2Z(P2X7) receptors. Immunity **7**: 433-444.

Lanier, L. L. 1998. NK cell receptors. Ann.Rev.Immunol. **16**: 359-393.

Lanier, L. L., Testi, R., Bindl, J., and Phillips, J. H. 1989. Identity of Leu-19 (CD56) leukocyte differentiation antigen and neural cell adhesion molecule. J.Exp.Med. **169**: 2233-2238.

Laochumroonvorapong, P., Wang, J., Liu, C. C., Ye, W., Moreira, A. L., Elkon, K. B., Freedman, V. H., and Kaplan, G. 1997. Perforin, a cytotoxic molecule which mediates cell necrosis, is not required for the early control of mycobacterial infection in mice. Infect.Immun. **65**: 127-132.

Le Douarin, N., Dulac, C., Dupin, E., and Cameron-Curry, P. 1991. Glial cell lineages in the neural crest. Glia **4**: 175-184.

Le Forestier, N., Lescs, M. C., and Gherardi, R. K. 1993. Anti-NKH-1 antibody specifically stains unmyelinated fibres and non- myelinating Schwann cell columns in humans. Neuropathol.Appl.Neurobiol. **19**: 500-506.

Le Poole, I. C., Mutis, T., van den Wijngaard, R. M., Westerhof, W., Ottenhoff, T. H. M., de Vries, R. R., and Das, P. K. 1993. A novel, antigen-presenting function of melanocytes and its possible relationship to hypopigmentary disorders. J.Immunol. **151**: 7284-7292.

Lee, B. H., Murugasu-Oei, B., and Dick, T. 1998. Upregulation of a histone-like protein in dormant *Mycobacterium smegmatis*. Mol.Gen.Genet. **260**: 475-479.

Leivo, I. and Engvall, E. 1988. Merosin, a protein specific for basement membranes of Schwann cells, striated muscle, and trophoblast, is expressed late in nerve and muscle development. Proc.Natl.Acad.Sci.U.S.A. **85**: 1544-1548.

Lienhardt, C. and Fine, P. E. 1994. Type 1 reaction, neuritis and disability in leprosy. What is the current epidemiological situation? Lepr.Rev. **65**: 9-33.

Lipman, D. J. and Pearson, W. R. 1985. Rapid and sensitive protein similarity searches. Science **227**: 1435-1441.

Ljunggren, H. G. and Karre, K. 1985. Host resistance directed selectively against H-2-deficient lymphoma variants. Analysis of the mechanism. J.Exp.Med. **162**: 1745-1759.

Lockwood, D. N., Vinayakumar, S., Stanley, J. N., McAdam, K. P., and Colston, M. J. 1993. Clinical features and outcome of reversal (type 1) reactions in Hyderabad, India. Int.J.Lep.Other Mycobact.Dis. **61** : 8-15.

Lu, P. H. and Negrin, R. S. 1994. A novel population of expanded human CD3+CD56+ cells derived from T cells with potent in vivo antitumor activity in mice with severe combined immunodeficiency. J.Immunol. **153**: 1687-1696.

Luna-Herrera, J., Arce-Paredes, P., and Rojas-Espinosa, O. 1996. Antibodies to phenolic glycolipid-I and sulfatide-I in leprosy and tuberculosis. Int.J.Lep.Other Mycobact.Dis. **64**: 327-329.

Magram, J., Sfarra, J., Connaughton, S., Faherty, D., Warrier, R., Carvajal, D., Wu, C. Y., Stewart, C., Sarmiento, U., and Gately, M. K. 1996. IL-12-deficient mice are defective but not devoid of type 1 cytokine responses. Ann.N.Y.Acad.Sci. **795**: 60-70.

Mancardi, G. L., Cadoni, A., Zicca, A., Schenone, A., Tabaton, M., de Martini, I., and Zaccheo, D. 1988. HLA-DR Schwann cell reactivity in peripheral neuropathies of different origins. Neurology **38**: 848-851.

Martinez, H. M. 1983. An efficient method for finding repeats in molecular sequences. Nucleic Acids Res. **11**: 4629-4634.

Mazzaccaro, R. J., Stenger, S., Rock, K. L., Porcelli, S. A., Brenner, M. B., Modlin, R. L., and Bloom, B. R. 1998. Cytotoxic T lymphocytes in resistance to tuberculosis. Adv.Exp.Med.Biol. **452**: 85-101.

Mitchell, G. W., Williams, G. S., Bosch, E. P., and Hart, M. N. 1991. Class II antigen expression in peripheral neuropathies. J.Neurol.Sci. **102**: 170-176.

Mizutani, Y. and Yoshida, O. 1994. In vitro enhancement of natural killer cell activity by BCG and the antagonistic inhibition of the susceptibility of K562 cells to lysis by peripheral blood lymphocytes in patients with urinary bladder tumor. Int.J.Urol. **1**: 49-56.

Modlin, R. L., Gebhard, J. F., Taylor, C. R., and Rea, T. H. 1983. In situ characterization of T lymphocyte subsets in the reactional states of leprosy. Clin.Exp.Immunol. **53**: 17-24.

Molloy, A., Laochumroonvorapong, P., and Kaplan, G. 1994. Apoptosis, but not necrosis, of infected monocytes is coupled with killing of intracellular bacillus Calmette-Guerin. J.Exp.Med. **180**: 1499-1509.

Moore, K. W., O'Garra, A., de Waal Malefyt, R., Vieira, P., and Mosmann, T. R. 1993. Interleukin-10. Ann.Rev.Immunol. **11**: 165-190.

Mosmann, T. R., Cherwinski, H. M., Bond, M. W., Giedlin, M. A., and Coffman, R. L. 1986. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. J.Immunol. **136**: 2348-2357.

Murray, P. J. 1999. Defining the requirements for immunological control of mycobacterial infections. Trends Microbiol. **7**: 366-372.

Murwani, R., Hodgkinson, S., and Armati, P. J. 1996. Tumor necrosis factor alpha and interleukin-6 mRNA expression in neonatal Lewis rat Schwann cells and a neonatal rat Schwann cell line following interferon gamma stimulation. J.Neuroimmunol. **71**: 65-71.

Mutis, T., Cornelisse, Y. E., Datema, G., van den Elsen, P. J., Ottenhoff, T. H. M., and de Vries, R. R. 1994. Definition of a human suppressor T-cell epitope. Proc.Natl.Acad.Sci.U.S.A. **91**: 9456-9460.

Mutis, T., Cornelisse, Y. E., and Ottenhoff, T. H. M. 1993a. Mycobacteria induce CD4+ T cells that are cytotoxic and display Th1-like cytokine secretion profile: heterogeneity in cytotoxic activity and cytokine secretion levels. Eur.J.Immunol. **23**: 2189-2195.

Mutis, T., Kraakman, E. M., Cornelisse, Y. E., Haanen, J. B., Spits, H., de Vries, R. R., and Ottenhoff, T. H. M. 1993b. Analysis of cytokine production by Mycobacterium-reactive T cells. Failure to explain *Mycobacterium leprae*-specific nonresponsiveness of peripheral blood T cells from lepromatous leprosy patients. J.Immunol. **150**: 4641-4651.

Naafs, B. 1989. Reactions in leprosy. *In* Biology of Mycobacteria. *Edited by* C.Ratledge, J.Stanford, and J.M.Grange. Academic Press Ltd., London pp. 359-403.

Naafs, B. 1996. Bangkok Workshop on Leprosy Research. Treatment of reactions and nerve damage. Int.J.Lep.Other Mycobact.Dis. **64**: S21-S28.

Naafs, B., Kolk, A. H., Chin, A. L. R., Faber, W. R., van Dijk, G., Kuijper, S., Stolz, E., and van Joost, T. 1990. Anti-*Mycobacterium leprae* monoclonal antibodies cross-react with human skin: an alternative explanation for the immune responses in leprosy. J.Invest Dermatol. **94**: 685-688.

Naafs, B., Pearson, J. M., and Baar, A. J. 1976. A follow-up study of nerve lesions in leprosy during and after reaction using motor nerve conduction velocity. Int.J.Lepr.Other Mycobact.Dis. **44**: 188-197.

references

Naafs, B. and Wheate, H. W. 1978. The time interval between the start of anti-leprosy treatment and the development of reactions in borderline patients. Lepr.Rev. **49**: 153-157.

Nagata, S. and Golstein, P. 1995. The Fas death factor. Science **267**: 1449-1456.

Narayanan, R. B., Girdhar, A., Girdhar, B. K., and Malaviya, G. N. 1990. Immunohistological analysis of nerve granulomas in neuritic leprosy. Int.Arch.Allergy Appl.Immunol. **92**: 50-55.

Narayanan, R. B., Laal, S., Sharma, A. K., Bhutani, L. K., and Nath, I. 1984. Differences in predominant T cell phenotypes and distribution pattern in reactional lesions of tuberculoid and lepromatous leprosy. Clin.Exp.Immunol. **55**: 623-628.

Needleman, S. B. and Wunsch, C. D. 1970. A general method applicable to the search for similarities in the amino acid sequence of two proteins. J.Mol.Biol. **48**: 443-453.

Newport, M. J., Huxley, C. M., Huston, S., Hawrylowicz, C. M., Oostra, B. A., Williamson, R., and Levin, M. 1996. A mutation in the interferon-gamma-receptor gene and susceptibility to mycobacterial infection. N.Engl.J.Med. **335**: 1941-1949.

Neyer, L. E., Grunig, G., Fort, M., Remington, J. S., Rennick, D., and Hunter, C. A. 1997. Role of interleukin-10 in regulation of T-cell-dependent and T-cell- independent mechanisms of resistance to Toxoplasma gondii. Infect.Immun. **65**: 1675-1682.

Nitta, T., Yagita, H., Sato, K., and Okumura, K. 1989. Involvement of CD56 (NKH-1/Leu-19 antigen) as an adhesion molecule in natural killer-target cell interaction. J.Exp.Med. **170**: 1757-1761.

Nomizu, M., Song, S. Y., Kuratomi, Y., Tanaka, M., Kim, W. H., Kleinman, H. K., and Yamada, Y. 1996. Active peptides from the carboxyl-terminal globular domain of laminin alpha2 and Drosophila alpha chains. FEBS Lett. **396**: 37-42.

Noordeen, S. K. 1994. Elimination of leprosy as a public health problem. Int.J.Lep.Other Mycobact.Dis. **62**: 278-283.

Noordeen, S. K. 1995. Eliminating leprosy as a public health problem; why the optimism is justified. Int.J.Lep.Other Mycobact.Dis. **63**: 559-566.

Oddo, M., Renno, T., Attinger, A., Bakker, T., MacDonald, H. R., and Meylan, P. R. 1998. Fas ligand-induced apoptosis of infected human macrophages reduces the viability of intracellular *Mycobacterium tuberculosis*. J.Immunol. **160**: 5448-5454.

Orme, I. M., Roberts, A. D., Griffin, J. P., and Abrams, J. S. 1993. Cytokine secretion by CD4 T lymphocytes acquired in response to *Mycobacterium tuberculosis* infection. J.Immunol. **151**: 518-525.

Ottenhoff, T. H. M., Elferink, D. G., Klatser, P. R., and de Vries, R. R. 1986a. Cloned suppressor T cells from a lepromatous leprosy patient suppress *Mycobacterium leprae* reactive helper T cells. Nature **322**: 462-464.

Ottenhoff, T. H. M., Kaleab, B., van Embden, J. D., Thole, J. E., and Kiessling, R. 1988. The recombinant 65-kD heat shock protein of *Mycobacterium bovis* Bacillus Calmette-Guerin/*M. tuberculosis* is a target molecule for CD4+ cytotoxic T lymphocytes that lyse human monocytes. J.Exp.Med. **168**: 1947-1952.

Ottenhoff, T. H. M., Neuteboom, S., Elferink, D. G., and de Vries, R. R. 1986b. Molecular localization and polymorphism of HLA class II restriction determinants defined by *Mycobacterium leprae*-reactive helper T cell clones from leprosy patients. J.Exp.Med. **164**: 1923-1939.

Palucka, A. K., Porwit, A., and Reizenstein, P. 1992. A supportive role of neural cell adhesion molecule (NCAM) in adhesion between leukaemic blasts and cytotoxic lymphocytes. Scand.J.Immunol. **35**: 399-406.

Parker, S. L., Tsai, Y. L., and Palmer, C. J. 1995. Comparison of PCR-generated fragments of the *mce* gene from *Mycobacterium tuberculosis*, *M. avium*, *M. intracellulare*, and *M. scrofulaceum*. Clin.Diagn.Lab Immunol. **2**: 770-775.

Pearson, J. M. and Ross, W. F. 1975. Nerve involvement in leprosy—pathology, differential diagnosis and principles of management. Lepr.Rev. **46**: 199-212.

Pedulla, M. L. and Hatfull, G. F. 1998. Characterization of the mIHF gene of *Mycobacterium smegmatis*. J.Bacteriol. **180**: 5473-5477.

Pessolani, M. C., Marques, M. A., Antonio, V. L., Sarno, E. N., and Brennan, P. J. 1999. Characterization of *Mycobacterium leprae* laminin binding proteins: A candidate adhesin involved in bacteria attachment to Schwann cells.Fourth International Conference on the Pathogenesis of Mycobacterial Infections , 130.

Piontek, G. E., Taniguchi, K., Ljunggren, H. G., Gronberg, A., Kiessling, R., Klein, G., and Karre, K. 1985. YAC-1 MHC class I variants reveal an association between decreased NK sensitivity and increased H-2 expression after interferon treatment or in vivo passage. J.Immunol. **135**: 4281-4288.

Pizzo, P., Murgia, M., Zambon, A., Zanovello, P., Bronte, V., Pietrobon, D., and Di Virgilio, F. 1992. Role of P2z purinergic receptors in ATP-mediated killing of tumor necrosis factor (TNF)-sensitive and TNF-resistant L929 fibroblasts. J.Immunol. **149**: 3372-3378.

Pollard, J. D., McCombe, P. A., Baverstock, J., Gatenby, P. A., and McLeod, J. G. 1986. Class II antigen expression and T lymphocyte subsets in chronic inflammatory demyelinating polyneuropathy. J.Neuroimmunol.

13: 123-134.

Poulet, S. and Cole, S. T. 1995. Characterization of the highly abundant polymorphic GC-rich-repetitive sequence (PGRS) present in *Mycobacterium tuberculosis*. Arch.Microbiol. **163** : 87-95.

Rambukkana, A., Das, P. K., Krieg, S., Young, S., Le Poole, I. C., and Bos, J. D. 1992. Mycobacterial 65,000 MW heat-shock protein shares a carboxy-terminal epitope with human epidermal cytokeratin 1/2. Immunology **77**: 267-276.

Rambukkana, A., Salzer, J. L., Yurchenco, P. D., and Tuomanen, E. I. 1997. Neural targeting of *Mycobacterium leprae* mediated by the G domain of the laminin-alpha2 chain. Cell **88**: 811-821.

Rambukkana, A., Yamada, H., Zanazzi, G., Mathus, T., Salzer, J. L., Yurchenco, P. D., Campbell, K. P., and Fischetti, V. A. 1998. Role of alpha-dystroglycan as a Schwann cell receptor for *Mycobacterium leprae*. Science **282**: 2076-2079.

Restrepo, L. M., Barrera, L. F., and Garcia, L. F. 1990. Natural killer cell activity in patients with pulmonary tuberculosis and in healthy controls. Tubercle **71**: 95-102.

Richardus, J. H., Finlay, K. M., Croft, R. P., and Smith, W. C. 1996. Nerve function impairment in leprosy at diagnosis and at completion of MDT: a retrospective cohort study of 786 patients in Bangladesh. Lepr.Rev. **67**: 297-305.

Ridley, D. S. and Jopling, W. H. 1966. Classification of leprosy according to immunity. A five-group system. Int.J.Lep.Other Mycobact.Dis. **34**: 255-273.

Roche, P. W., Theuvenet, W. J., and Britton, W. J. 1991. Risk factors for type-1 reactions in borderline leprosy patients. Lancet **338**: 654-657.

Romagnani, S. 1991. Human Th1 and Th2 subsets: doubt no more. Immunol.Today **12**: 256-257.

Rook, G. A., Attiyah, R. A., and Foley, N. 1989. The role of cytokines in the immunopathology of tuberculosis, and the regulation of agalactosyl IgG. Lymphokine Res. **8**: 323-328.

Rose, P. and Waters, M. F. 1991. Reversal reactions in leprosy and their management. Lepr.Rev. **62**: 113-121.

Roth, G. A., Roytta, M., Yu, R. K., Raine, C. S., and Bornstein, M. B. 1985. Antisera to different glycolipids induce myelin alterations in mouse spinal cord tissue cultures. Brain Res. **339**: 9-18.

Ryberg, B. 1978. Multiple specificities of antibrain antibodies in multiple sclerosis and chronic myelopathy. J.Neurol.Sci. **38**: 357-382.

Said, G. and Hontebeyrie-Joskowicz, M. 1992. Nerve lesions induced by macrophage activation. Research in Immunology **143**: 589-999.

Saida, K., Saida, T., Brown, M. J., and Silberberg, D. H. 1979. In vivo demyelination induced by intraneural injection of anti- galactocerebroside serum: a morphologic study. Am.J.Pathol. **95**: 99-116.

Salgame, P., Abrams, J. S., Clayberger, C., Goldstein, H., Convit, J., Modlin, R. L., and Bloom, B. R. 1991. Differing lymphokine profiles of functional subsets of human CD4 and CD8 T cell clones. Science **254**: 279-282.

Sampaio, E. P., Kaplan, G., Miranda, A., Nery, J. A., Miguel, C. P., Viana, S. M., and Sarno, E. N. 1993. The influence of thalidomide on the clinical and immunologic manifestation of erythema nodosum leprosum. J.Infect.Dis. **168**: 408-414.

Schall, T. J., Bacon, K., Toy, K. J., and Goeddel, D. V. 1990. Selective attraction of monocytes and T lymphocytes of the memory phenotype by cytokine RANTES. Nature **347**: 669-671.

Schlesinger, L. S. 1993. Macrophage phagocytosis of virulent but not attenuated strains of *Mycobacterium tuberculosis* is mediated by mannose receptors in addition to complement receptors. J.Immunol. **150**: 2920-2930.

Schluesener, H. J. 1990. Transforming growth factors type beta 1 and beta 2 suppress rat astrocyte autoantigen presentation and antagonize hyperinduction of class II major histocompatibility complex antigen expression by interferon-gamma and tumor necrosis factor-alpha. J.Neuroimmunol. **27**: 41-47.

Schmidt, B., Stoll, G., Hartung, H. P., Heininger, K., Schafer, B., and Toyka, K. V. 1990. Macrophages but not Schwann cells express Ia antigen in experimental autoimmune neuritis. Ann.Neurol. **28**: 70-77.

Schorey, J. S., Carroll, M. C., and Brown, E. J. 1997. A macrophage invasion mechanism of pathogenic mycobacteria. Science **277**: 1091-1093.

Schorey, J. S., Li, Q., McCourt, D. W., Bong Mastek, M., Clark-Curtiss, J. E., Ratliff, T. L., and Brown, E. J. 1995. A *Mycobacterium leprae* gene encoding a fibronectin binding protein is used for efficient invasion of epithelial cells and Schwann cells. Infect.Immun. **63**: 2652-2657.

Schulze, B., Mann, K., Poschl, E., Yamada, Y., and Timpl, R. 1996. Structural and functional analysis of the globular domain IVa of the laminin alpha 1 chain and its impact on an adjacent RGD site. Biochem.J. **314 (Pt 3)**: 847-851.

Sergott, R. C., Brown, M. J., Silberberg, D. H., and Lisak, R. P. 1984. Antigalactocerebroside serum demyelinates

optic nerve in vivo. J.Neurol.Sci. **64**: 297-303.

Shetty, V. P., Mistry, N. F., and Antia, N. H. 1985. Serum demyelinating factors and adjuvant-like activity of *Mycobacterium leprae*: possible causes of early nerve damage in leprosy. Lepr.Rev. **56**: 221-227.

Shimoji, Y., Ng, V., Matsumura, K., Fischetti, V. A., and Rambukkana, A. 1999. A 21-kDa surface protein of *Mycobacterium leprae* binds peripheral nerve laminin-2 and mediates Schwann cell invasion. Proc.Natl.Acad.Sci.U.S.A. **96**: 9857-9862.

Siddiqui, M. R., Meisner, S., Fisher, S., Balakrishnan, K., Pitchappan, R. M., Ghei, S., Narayan, N. P., Siraraman, D., and Hill, A. V. 1999. A genome scan for major leprosy susceptibility genes.Fourth International Conference on the Pathogenesis of Mycobacterial Infections , 13.

Sieling, P. A., Abrams, J. S., Yamamura, M., Salgame, P., Bloom, B. R., Rea, T. H., and Modlin, R. L. 1993. Immunosuppressive roles for IL-10 and IL-4 in human infection. In vitro modulation of T cell responses in leprosy. J.Immunol. **150**: 5501-5510.

Sieling, P. A., Chatterjee, D., Porcelli, S. A., Prigozy, T. I., Mazzaccaro, R. J., Soriano, T., Bloom, B. R., Brenner, M. B., Kronenberg, M., Brennan, P. J., and Modlin, R. L. 1995. CD1-restricted T cell recognition of microbial lipoglycan antigens. Science **269**: 227-230.

Skeen, M. J., Miller, M. A., Shinnick, T. M., and Ziegler, H. K. 1996. Regulation of murine macrophage IL-12 production. Activation of macrophages in vivo, restimulation in vitro, and modulation by other cytokines. J.Immunol. **156**: 1196-1206.

Skoff, A. M., Lisak, R. P., Bealmear, B., and Benjamins, J. A. 1998. TNF-alpha and TGF-beta act synergistically to kill Schwann cells. J.Neurosci.Res. **53**: 747-756.

Smith, W. C. 1997. We need to know what is happening to the incidence of leprosy. Lepr.Rev. **68**: 195-200.

Steinhoff, U., Brinkmann, V., Klemm, U., Aichele, P., Seiler, P., Brandt, U., Bland, P. W., Prinz, I., Zugel, U., and Kaufmann, S. H. 1999. Autoimmune intestinal pathology induced by hsp60-specific CD8 T cells. Immunity. **11**: 349-358.

Steinhoff, U. and Kaufmann, S. H. 1988. Specific lysis by CD8+ T cells of Schwann cells expressing *Mycobacterium leprae* antigens. Eur.J.Immunol. **18**: 969-972.

Stenger, S., Hanson, D. A., Teitelbaum, R., Dewan, P., Niazi, K. R., Froelich, C. J., Ganz, T., Thoma-Uszynski, S., Melian, A., Bogdan, C., Porcelli, S. A., Bloom, B. R., Krensky, A. M., and Modlin, R. L. 1998. An antimicrobial activity of cytolytic T cells mediated by granulysin. Science **282**: 121-125.

Stenger, S., Mazzaccaro, R. J., Uyemura, K., Cho, S., Barnes, P. F., Rosat, J. P., Sette, A., Brenner, M. B., Porcelli, S. A., Bloom, B. R., and Modlin, R. L. 1997. Differential effects of cytolytic T cell subsets on intracellular infection. Science **276**: 1684-1687.

Stewart, H. J. S., Rougon, G., Dong, Z. P., Dean, C., Jessen, K. R., and Mirsky, R. 1995. TGF-beta s upregulate NCAM and L1 expression in cultured Schwann cells, suppress cyclic AMP-induced expression of 04 and galactocerebroside, and are widely expressed in cells of the Schwann cell lineage in vivo. Glia **15**: 419-436.

Street, N. E., Schumacher, J. H., Fong, T. A., Bass, H., Fiorentino, D. F., Leverah, J. A., and Mosmann, T. R. 1990. Heterogeneity of mouse helper T cells. Evidence from bulk cultures and limiting dilution cloning for precursors of Th1 and Th2 cells. J.Immunol. **144**: 1629-1639.

Sugawara, I., Yamada, H., Kaneko, H., Mizuno, S., Takeda, K., and Akira, S. 1999. Role of interleukin-18 (IL-18) in mycobacterial infection in IL-18-gene- disrupted mice. Infect.Immun. **67**: 2585-2589.

Sullivan, L., Sano, S., Pirmez, C., Salgame, P., Mueller, C., Hofman, F., Uyemura, K., Rea, T. H., Bloom, B. R., and Modlin, R. L. 1991. Expression of adhesion molecules in leprosy lesions. Infect.Immun. **59**: 4154-4160.

Suneetha, L. M., Satish, P. R., Suneetha, S., Job, C. K., and Balasubramanian, A. S. 1997. *M. leprae* binds to a 28-30-kDa phosphorylated glycoprotein of rat peripheral nerve. Int.J.Lep.Other Mycobact.Dis. **65**: 352-356.

Suzuki, N., Suzuki, T., and Engleman, E. G. 1991. Evidence for the involvement of CD56 molecules in alloantigen-specific recognition by human natural killer cells. J.Exp.Med. **173**: 1451-1461.

Takeda, K., Tsutsui, H., Yoshimoto, T., Adachi, O., Yoshida, N., Kishimoto, T., Okamura, H., Nakanishi, K., and Akira, S. 1998. Defective NK cell activity and Th1 response in IL-18-deficient mice. Immunity. **8**: 383-390.

Taub, D. D., Proost, P., Murphy, W. J., Anver, M., Longo, D. L., van Damme, J., and Oppenheim, J. J. 1995. Monocyte chemotactic protein-1 (MCP-1), -2, and -3 are chemotactic for human T lymphocytes. J.Clin.Invest. **95**: 1370-1376.

Thole, J. E., Janson, A. A., Cornelisse, Y. E., Schreuder, G. M., Wieles, B., Naafs, B., de Vries, R. R., and Ottenhoff, T. H. M. 1999. HLA-class II-associated control of antigen recognition by T cells in leprosy: a prominent role for the 30/31-kDa antigens. J.Immunol. **162**: 6912-6918.

Thomas, B. M. and Mukherjee, R. 1990. Antineural antibodies in sera of leprosy patients. Clin.Immunol.Immunopathol. **57**: 420-429.

Timpl, R. and Brown, J. C. 1994. The laminins. Matrix Biol. **14**: 275-281.

Turka, L. A., Goodman, R. E., Rutkowski, J. L., Sima, A. A., Merry, A., Mitra, R. S., Wrone-Smith, T., Toews, G., Strieter, R. M., and Nickoloff, B. J. 1995. Interleukin 12: a potential link between nerve cells and the immune response in inflammatory disorders. Mol.Med. **1**: 690-699.

Unsicker, K., Flanders, K. C., Cissel, D. S., Lafyatis, R., and Sporn, M. B. 1991. Transforming growth factor beta isoforms in the adult rat central and peripheral nervous system. Neuroscience **44**: 613-625.

van Brakel, W. H., Khawas, I. B., and Lucas, S. B. 1994. Reactions in leprosy: an epidemiological study of 386 patients in west Nepal. Lepr.Rev. **65**: 190-203.

van den Akker, T. W., Naafs, B., Kolk, A. H., Glopper-van der Veer, E., Chin, R. A., Lien, A., and van Joost, T. 1992. Similarity between mycobacterial and human epidermal antigens. Br.J.Dermatol. **127**: 352-358.

van den Berg, L. H., Bar, P. R., Sodaar, P., Mollee, I., Wokke, J. J., and Logtenberg, T. 1995. Selective expansion and long-term culture of human Schwann cells from sural nerve biopsies. Ann.Neurol. **38**: 674-678.

van den Berg, L. H., Lankamp, C. L., de Jager, A. E., Notermans, N. C., Sodaar, P., Marrink, J., de Jong, H. J., Bar, P. R., and Wokke, J. H. 1993. Anti-sulphatide antibodies in peripheral neuropathy. J.Neurol.Neurosurg.Psychiatry **56**: 1164-1168.

van den Berg, R. H., Faber-Krol, M. C., Sim, R. B., and Daha, M. R. 1998. The first subcomponent of complement, C1q, triggers the production of IL-8, IL-6, and monocyte chemoattractant peptide-1 by human umbilical vein endothelial cells. J.Immunol. **161**: 6924-6930.

van der Meide, P. H., Groenestein, R. J., de Labie, M. C., Heeney, J. L., Pala, P., and Slaoui, M. 1995. Enumeration of lymphokine-secreting cells as a quantitative measure for cellular immune responses in rhesus macaques. J.Med.Primatol. **24**: 271-281.

van der Pouw Kraan TC, Boeije, L. C., Smeenk, R. J., Wijdenes, J., and Aarden, L. A. 1995. Prostaglandin-E2 is a potent inhibitor of human interleukin 12 production. J.Exp.Med. **181**: 775-779.

Vedeler, C. A., Nilsen, R., and Matre, R. 1989. Localization of Fc gamma receptors and complement receptors CR1 on human peripheral nerve fibres by immunoelectron microscopy. J.Neuroimmunol. **23**: 29-33.

Vemuri, N. and Mukherjee, R. 1991. Immunoreactivity of nerve lipid antigens in leprosy. J.Clin.Lab.Anal. **5**: 157-161.

Vemuri, N., Viera, L. M., Taneja, K. K., Gangal, S. V., and Mukherjee, R. 1996. Antisphingolipid antibodies in the sera of leprosy patients. Lepr.Rev. **67**: 95-103.

Vergelli, M., Le, H., van Noort, J. M., Dhib-Jalbut, S., McFarland, H., and Martin, R. 1996. A novel population of CD4+CD56+ myelin-reactive T cells lyses target cells expressing CD56/neural cell adhesion molecule. J.Immunol. **157**: 679-688.

Verhagen, C. E., van der Pouw Kraan TC, Buffing, A. A., Chand, M. A., Faber, W. R., Aarden, L. A., and Das, P. K. 1998. Type 1- and type 2-like lesional skin-derived *Mycobacterium leprae*- responsive T cell clones are characterized by coexpression of IFN- gamma/TNF-alpha and IL-4/IL-5/IL-13, respectively. J.Immunol. **160**: 2380-2387.

Verhagen, C. E., Wierenga, E. A., Buffing, A. A., Chand, M. A., Faber, W. R., and Das, P. K. 1997. Reversal reaction in borderline leprosy is associated with a polarized shift to type 1-like *Mycobacterium leprae* T cell reactivity in lesional skin: a follow-up study. J.Immunol. **159**: 4474-4483.

Vodovotz, Y., Geiser, A. G., Chesler, L., Letterio, J. J., Campbell, A., Lucia, M. S., Sporn, M. B., and Roberts, A. B. 1996. Spontaneously increased production of nitric oxide and aberrant expression of the inducible nitric oxide synthase in vivo in the transforming growth factor beta 1 null mouse. J.Exp.Med. **183**: 2337-2342.

Wekerle, H., Schwab, M., Linington, C., and Meyermann, R. 1986. Antigen presentation in the peripheral nervous system: Schwann cells present endogenous myelin autoantigens to lymphocytes. Eur.J.Immunol. **16**: 1551-1557.

Wierenga, E. A., Snoek, M., de Groot, C., Chretien, I., Bos, J. D., Jansen, H. M., and Kapsenberg, M. L. 1990. Evidence for compartmentalization of functional subsets of CD2+ T lymphocytes in atopic patients. J.Immunol. **144**: 4651-4656.

Wilbur, W. J. and Lipman, D. J. 1983. Rapid similarity searches of nucleic acid and protein data banks. Proc.Natl.Acad.Sci.U.S.A. **80**: 726-730.

Wilkinson, P. C. and Newman, I. 1992. Identification of IL-8 as a locomotor attractant for activated human lymphocytes in mononuclear cell cultures with anti-CD3 or purified protein derivative of *Mycobacterium tuberculosis*. J.Immunol. **149**: 2689-2694.

Willison, H. J. and Veitch, J. 1994. Immunoglobulin subclass distribution and binding characteristics of anti-GQ1b antibodies in Miller Fisher syndrome. J.Neuroimmunol. **50**: 159-165.

Wolfe, S. A., Tracey, D. E., and Henney, C. S. 1977. BCG-induced murine effector cells. II. Characterization of natural killer cells in peritoneal exudates. J.Immunol. **119**: 1152-1158.

Woolley, A. L., Hollowell, J. P., and Rich, K. M. 1990. Fibronectin-laminin combination enhances peripheral

nerve regeneration across long gaps. Otolaryngol.Head Neck Surg. **103**: 509-518.

World Health Organization 1995. Progress towards the elimination of leprosy as a public health problem. Part I. Wkly Epidemiol.Rec. **70**: 177-182.

World Health Organization 1997. Progress towards leprosy elimination. Wkly Epidemiol.Rec. **72**: 165-172.

World Health Organization Expert Committee on Leprosy 1989. Clinical aspects of leprosy related control. World Health Organ.Tech.Rep.Ser. **768**: 13-19.

Wu, C., Ferrante, J., Gately, M. K., and Magram, J. 1997. Characterization of IL-12 receptor beta1 chain (IL-12Rbeta1)-deficient mice: IL-12Rbeta1 is an essential component of the functional mouse IL- 12 receptor. J.Immunol. **159**: 1658-1665.

Xing, Z., Wang, J., Croitoru, K., and Wakeham, J. 1998. Protection by CD4 or CD8 T cells against pulmonary *Mycobacterium bovis* bacillus Calmette-Guerin infection. Infect.Immun. **66**: 5537-5542.

Yamada, H., Denzer, A. J., Hori, H., Tanaka, T., Anderson, L. V., Fujita, S., Fukuta-Ohi, H., Shimizu, T., Ruegg, M. A., and Matsumura, K. 1996. Dystroglycan is a dual receptor for agrin and laminin-2 in Schwann cell membrane. J.Biol.Chem. **271**: 23418-23423.

Yamamura, M., Uyemura, K., Deans, R. J., Weinberg, K., Rea, T. H., Bloom, B. R., and Modlin, R. L. 1991. Defining protective responses to pathogens: cytokine profiles in leprosy lesions. Science **254**: 277-279.

Yamamura, M., Wang, X. H., Ohmen, J. D., Uyemura, K., Rea, T. H., Bloom, B. R., and Modlin, R. L. 1992. Cytokine patterns of immunologically mediated tissue damage. J.Immunol. **149**: 1470-1475.

Zanovello, P., Bronte, V., Rosato, A., Pizzo, P., and Di Virgilio, F. 1990. Responses of mouse lymphocytes to extracellular ATP. II. Extracellular ATP causes cell type-dependent lysis and DNA fragmentation. J.Immunol. **145**: 1545-1550.

Zhang, M., Gong, J. H., Presky, D. H., Xue, W., and Barnes, P. F. 1999. Expression of the IL-12 receptor beta 1 and beta 2 subunits in human tuberculosis. J.Immunol. **162**: 2441-2447.

Zhang, Y. P., Porter, S., and Wekerle, H. 1990. Schwann cells and myasthenia gravis. Preferential uptake of soluble and membrane-bound AChR by normal and immortalized Schwann cells, and immunogenic presentation to AChR-specific T line lymphocytes. Am.J.Pathol. **136**: 111-122.

samenvatting

Lepra is een infectieuze ziekte die veroorzaakt wordt door besmetting met de bacterie *Mycobacterium leprae*. Naast het binnendringen van professionele fagocyterende cellen uit het afweersysteem, heeft de bacterie een hoge affiniteit voor Schwann cellen. Deze cellen hebben hun celmembraan strak om perifere zenuwen gerold en vormen hiermee lipoprotein complexen, ook wel myeline genoemd. Myeline verhoogt de stroomgeleiding door de zenuw en biedt tevens bescherming tegen allerlei schadelijke factoren. In het centrale zenuwstelsel wordt deze rol vervuld door oligodendrocyten.

Het merendeel van de mensen dat met *M. leprae* in contact komt, is in staat een beschermende immuunrespons te ontwikkelen en de bacterie zonder verdere complicaties te verwijderen. Degenen die echter niet in staat zijn een dergelijke afweer op te wekken, ontwikkelen lepra. Diverse verschijningsvormen van de ziekte kunnen onderscheiden worden. Aan de ene zijde van het zogenaamde lepra spectrum zijn de lepromateuze patiënten te vinden. Deze patiënten vertonen een zeer diffuus ziektebeeld, genereren geen goede cel gemedieerde immuun respons en zijn niet in staat de bacterie te elimineren. Aan de andere zijde van het spectrum bevinden zich de tuberculoïde patiënten. Deze groep ontwikkelt een sterke T cel gemedieerde immuunreactie tegen *M. leprae*, waardoor slechts weinig tot geen bacteriën zijn te vinden in deze patiënten. Tussen deze twee extremen bevinden zich de zogenaamde borderline patiënten.

Sinds enige tijd is er een effectieve behandeling van de infectie mogelijk, de zogenaamde multiple drug therapie (MDT), een cocktail van antibiotica. MDT heeft geresulteerd in een verlaging van het aantal geregistreerde mensen met lepra. Het aantal nieuwe gevallen van infectie is echter gelijk gebleven. Een belangrijk bijkomend probleem is het verhoogde vóórkomen van lepra reacties. Dit zijn episodes van een verhoogde immuniteit. Patiënten met reacties lopen echter een verhoogde kans op zenuwschade, de belangrijkste complicatie bij lepra. Om onherstelbare schade aan zenuwen en een levenslange handicap te voorkomen is het van belang een beter inzicht te krijgen in de processen die ten grondslag liggen aan dit fenomeen. Het doel van dit proefschrift was dieper inzicht te verkrijgen in de rol die T cellen en Schwann cellen spelen in het onstaan van zenuwschade tijdens lepra.

Omdat menselijke Schwann cellen tot op heden niet te kweken waren, is het onderzoek naar de mechanismen achter zenuwschade in het verleden uitgevoerd met Schwann cellen van knaagdieren. In hoofdstuk 2a is getracht humane Schwann cellen te isoleren en de bevindingen in muis en rat te verifiëren. Humane Schwann cellen blijken in staat te zijn *M. leprae* op te nemen en het vervolgens te presenteren via MHC klasse II moleculen. De daaropvolgende herkenning door $CD4^+$ T cellen met cytotoxische eigenschappen leidt tot destructie van de Schwann cell. Dit mechanisme speelt mogelijk een rol in het veroorzaken van zenuwschade tijdens lepra, in het bijzonder gedurende reversal reacties, waarbij het aantal $CD4^+$ T cellen dat *M. leprae* herkent, verhoogd is.

Naast het presenteren van *M. leprae* antigenen aan T cellen, oefenen Schwann cellen mogelijk nog op andere wijzen invloed uit op lokale afweerreacties. In hoofdstuk 3 is geanalyseerd welke cytokines, signaalstoffen tussen cellen, geproduceerd worden door Schwann cellen na blootstelling aan *M. leprae*. Deze inventarisatie bracht aan het licht dat Schwann cellen niet in staat zijn zogenaamde type-1 cytokines te produceren, maar wel een aantal type-2 cytokines maken. Aanwezigheid van type-1 cytokines resulteert normaliter in een type-1 T cel respons, welke in geval van lepra naast bescherming ook verondersteld wordt betrokken te zijn bij weefselschade. Type-2 cytokines daarentegen, zijn in staat een dergelijke respons te onderdrukken. Door de productie van type-2 cytokines en de afwezigheid van type-1 cytokines, lijken Schwann cellen te proberen zich te bescher-

men tegen deze schadelijke type-1 responsen. Mogelijk verklaart dit ook waarom reacties juist optreden tijdens behandeling van de infectie: het wegnemen van *M. leprae* zou de type-2 cytokine productie door Schwann cellen kunnen verlagen, waardoor type-1 cytokines de overhand krijgen. Het is denkbaar dat deze verschuiving richting type-1 één van de oorzaken is van een schadelijke type-1 T cel respons.

Zenuwdestructie wordt ook waargenomen in zenuwen die niet geïnfecteerd zijn met *M. leprae*. Daarom wordt verondersteld dat ook auto-immuun-achtige mechanismen een rol spelen in het veroorzaken van zenuwschade tijdens lepra. Een dergelijk fenomeen is beschreven voor multiple sclerosis, waar T cellen na stimulatie met een bepaald eiwit, het Neurale Cel Adhesie Molecuul (N-CAM) tot expressie brengen. Zoals de naam al zegt, komt dit molecuul ook voor in zowel het centrale, als het perifere zenuwstelsel, waar N-CAM op oligodendrocyten of Schwann cellen een interactie aangaat met N-CAM op zenuwcellen. Een dergelijke interactie wordt ook verondersteld plaats te vinden tussen N-CAM$^+$ T cellen enerzijds en Schwann cellen of oligodendrocyten anderzijds, resulterend in een tegen de Schwann cel of oligodendrocyt gerichte cytotoxiciteit. In hoofdstuk 4 is onderzocht of een dergelijk mechanisme een rol speelt bij lepra. N-CAM$^+$ T cellen zijn inderdaad aanwezig in huidbiopten van lepra patiënten enkel en alleen tijdens episodes van klinisch actieve ENL. Ook zijn dergelijke cellen te isoleren uit ontstoken zenuwweefsel, terwijl zij nauwelijks aanwezig zijn in perifeer bloed van deze patiënten. De verhoogde expressie gaat samen met een verhoogde antigeen- en MHC onafhankelijke cytotoxie van N-CAM$^+$ cellen. Deze cytotoxiciteit blijkt veroorzaakt te worden door de CD8$^+$ T cel populatie. Deze resultaten suggereren een nieuw mechanisme van zenuwschade in lepra waarbij N-CAM$^+$ CD8$^+$ T cellen een belangrijke rol spelen.

Bij het binnendringen van Schwann cellen door *M. leprae* speelt de α2 keten van laminine-2 een cruciale brugfunctie tussen de bacterie en de Schwann cel. Eén receptor op de Schwann cel is het α/β dystroglycaan complex. Als receptor op *M. leprae* is een eiwit gevonden met een molecuul gewicht van 21 kDa. Gelijksoortige eiwitten zijn echter ook aanwezig in andere mycobacteriën, zodat de vraag reist waarom deze andere bacteriën niet een gelijke affiniteit vertonen voor Schwann cellen en of dit mechanisme inderdaad van cruciaal belang is in de invasie van gastheer cellen. In hoofdstuk 5 is gekeken of *M. leprae* een homoloog bezit van de in *Mycobacterium tuberculosis* geïdentificeerde *mce1* (mammalian cell entry 1) eiwitten, waarvan bekend is dat het actief betrokken is bij cel invasie. Het feit dat dit eiwit voorheen niet gevonden werd in *M. leprae*, bleek te berusten op fouten in de DNA sequentie. Het *mce1* operon bleek ook in *M. leprae* volledig aanwezig te zijn en was op gelijke wijze georganiseerd. Mogelijk spelen deze eiwitten dus ook in *M. leprae* een rol tijdens cel invasie.

Er zijn weinig goede markers voor vroegtijdige detectie van lepra reacties en zenuwschade. Kandidaat markers zouden antilichamen tegen zenuwcomponenten kunnen zijn. Een eerdere studie suggereerde dat twee Schwann cell specifieke eiwitten, S-100β en GFAP, specifiek herkend worden door lepra patiënten met zenuwontstekingen. Glashard bewijs hiervoor ontbreekt echter. In hoofdstuk 6 is bekeken of de aanwezigheid van antilichamen tegen sulfatiden, een myeline component, correleert met het optreden van lepra reacties. Dergelijke antilichamen bleken aanwezig in vrijwel alle leprapatiënten, ongeacht classificatie of aanwezigheid van reacties, terwijl ze niet te detecteren waren in gezonde west-europese controles. Gezonde controles afkomstig uit gebieden waar lepra endemisch is, hadden echter een zelfde hoeveelheid antilichamen als in lepra patiënten werd gevonden. Het meten van antilichamen tegen sulfatiden is dus niet bruikbaar voor het detecteren van lepra, lepra reacties of zenuwschade bij lepra.

Samenvattend kunnen we concluderen dat humane Schwann cellen een belangrijke rol lijken te spelen in het ontstaan van zenuwschade na infectie met *M. leprae*. Schwann cellen zijn namelijk in staat T cel gemedieerde immuunresponsen op gang te brengen.

Wat betreft het verwijderen van de bacterie is deze respons een gunstige. Een nadelig neveneffect is echter vernietiging van de Schwann cellen en aantasting van de zenuwen. Mogelijk probeert de Schwann cel zich tegen dergelijke schadelijke processen te beschermen door de productie van cytokines die deze responsen kunnen onderdrukken. Daarnaast zouden, met name tijdens ENL, auto-immuun-achtige mechanismen een rol kunnen spelen, waarbij N-CAM$^+$ CD8$^+$ T cellen een cruciale positie in zouden kunnen nemen. De exacte rol van deze cellen en het mechanisme dat deze cellen genereert dient nog opgehelderd te worden.

abbreviations

APC	antigen presenting cells
ATP	adenosine triphosphate
BI	bacillary index
BB	borderline leprosy
BCG	bacille Calmette-Guerin
BL	borderline lepromatous leprosy
BT	borderline tuberculoid leprosy
CMI	cell mediated immunity
CNPase	2',3'-cyclic nucleotide-3'-phosphohydrolase
CNS	central nervous system
cpm	counts per minute
CR	complement receptor
DG	dystroglycan
DTH	delayed type hypersensitivity
ENL	erythema nodosum leprosum
E:T	effector:target
FCS	fetal calf serum
GFAP	glial fibrillary protein
HLA	human leukocyte antigen
HLP	histone like protein
IFN	interferon
ICAM	intercellular adhesion molecule
Ig	immunoglobulin
IL	interleukin
LAK	lymphokine activated killer
LL	leprosmatous leprosy
LN	laminin
LPS	lipopolysaccharide
MDT	multiple drug therapy
MHC	major histocompatibility complex
NIMC	nerve infiltrating mononuclear cells
NK	natural killer
PCR	polymerase chain reaction
PBMC	peripheral blood mononuclear cells
N-CAM	neural cell adhesion molecule
mce	mammalian cell entry
MCP	monocyte chemotactic factor
PGE_2	prostaglandin E_2
PNS	peripheral nervous system
RANTES	regulated upon activation, normally T cell expressed and presumably secreted
RR	reversal reaction
TCR	T cell receptor
TGF	transforming growth factor
Th	T-helper
TNF	tumor necrosis factor
Tr	T-regulatory
TT	tuberculoid leprosy

nawoord

Een kort woord voor iedereen die op enigerlei wijze heeft bijgedragen aan de tot stand koming van dit proefschrift, en veel ruimte om daar een persoonlijk woord aan toe te voegen.

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A short word for everyone who contributed to this thesis, and a lot of space to add a personal word to it.

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Eric

curriculum vitae

De auteur van dit proefschrift werd op geboren op 9 oktober 1969 te Rosmalen en groeide daar ook op. Na het behalen van het VWO diploma aan het Gymnasium Bernrode te Heeswijk-Dinther, begon hij in 1989 de studie Gezondheidswetenschappen (nu Biomedische Wetenschappen) aan de Rijksuniversiteit Leiden. Tijdens zijn studie volgde hij stages aan de afdelingen Oncologie, Heelkunde, en Immunohaematologie en Bloedbank van het toenmalige Academisch Ziekenhuis Leiden. Na een hoofdvakstage op het gebied van lepra (Dr. B. Wieles, Dr. J. E. R. Thole en Dr. T. H. M. Ottenhoff, afdeling Immunohaematolgie en Bloedbank) en een stage op het gebied van tuberculose (Prof. Dr. M. Harboe, Institutt for Generell- og Revmatisk Immunologi, Oslo, Noorwegen), werd in 1995 het doctoraal diploma behaald. Aangetrokken door de complexiteit van de ziekte lepra, begon hij in datzelfde jaar aan het promotie onderzoek dat geleid heeft tot dit proefschrift. Het onderzoek vond plaats onder de inspirerende begeleiding van Dr. T. H. M. Ottenhoff aan de afdeling Immunohaematolgie en Bloedbank van het Leids Universitair Medisch Centrum. Sinds 16 maart 2000 is hij werkzaam als post-doc bij Prof. Dr. E. A. J. M. Goulmy aan dezelfde afdeling op het gebied van minor histocompatibiliteits antigenen.

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publications

Wieles, B., Spierings, E., van Noort, J., Naafs, B., Offringa, R., and Ottenhoff, T. H. M. 1995. Molecular characterization and T-cell-stimulatory capacity of *Mycobacterium leprae* antigen T5. Infect.Immun. **63**: 4682-4685.

Ottenhoff, T. H. M., Spierings, E., Nibbering, P. H., and de Jong, R. 1997. Modulation of protective and pathological immunity in mycobacterial infections. Int.Arch.Allergy Appl.Immunol. **113**: 400-408.

Ottenhoff,T. H. M., Spierings, E., Nibbering, P. H., de Jong, R., and de Vries, R. R. 1998. Immunology and immunopathology of mycobacterial infections. *In* Opportunistic intracellular bacteria and immunity. *Edited by* L. J. Paradise. Plenum Press, New York pp. 91-106.

Wiker, H. G., Michell, S. L., Hewinson, R. G., Spierings, E., Nagai, S., and Harboe, M. 1999. Cloning, expression and significance of MPT53 for identification of secreted proteins of *Mycobacterium tuberculosis*. Microb.Pathog. **26**: 207-219.

Wiker, H. G., Spierings, E., Kolkman, M. A., Ottenhoff, T. H. M., and Harboe, M. 1999. The mammalian cell entry operon 1 (*mce1*) of *Mycobacterium leprae* and *Mycobacterium tuberculosis*. Microb.Pathog. **27**: 173-177.

Spierings, E., de Vlieger, M., Brand, A., Klatser, P. R., and Ottenhoff, T. H. M. 1999. Antibodies to sulfatide in leprosy and leprosy reactions. Am.J.Trop.Med.Hyg. **61**: 495-499.

Spierings, E., Vleggeert-Lankamp, C. L. A. M., Marani, E., Thomeer, R. T. W. M., and Ottenhoff,T.H.M. 2000. Allorecognition of artificial nerve guides filled with human Schwann cells: an *in vitro* pilot study. Transplantation **69**: 455-456.

Spierings, E., de Boer, T., Zulianello, L., Adams, L. B.,Marani, E., and Ottenhoff, T. H. M. *Mycobacterium leprae* specific, HLA class II restricted killing of human Schwann cells by $CD4^+$ Thelper-1 cells: a novel immunopathogenic mechanism of nerve damage in leprosy. submitted for publication.

Spierings E., de Boer, T., Zulianello, L., and Ottenhoff, T. H. M. Novel mechanisms in the immunopathogenesis of leprosy nerve damage: the role of Schwann cells, T cells, and *Mycobacterium leprae*. submitted for publication.