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Mycobacterium leprae-Specific, HLA Class II-Restricted Killing of Human Schwann Cells by CD4+ Th1 Cells: A Novel Immunopathogenic Mechanism of Nerve Damage in Leprosy

Eric Spierings, Tjitske de Boer, Brigitte Wieles, Linda B. Adams, Enrico Marani, and Tom H. M. Ottenhoff*

Peripheral nerve damage is a major complication of reversal (or type-1) reactions in leprosy. The pathogenesis of nerve damage remains largely unresolved, but detailed in situ analyses suggest that type-1 T cells play an important role. Mycobacterium leprae is known to have a remarkable tropism for Schwann cells of the peripheral nerve. Reversal reactions in leprosy are often accompanied by severe and irreversible nerve destruction and are associated with increased cellular immune reactivity against M. leprae. Thus, a likely immunopathogenic mechanism of Schwann cell and nerve damage in leprosy is that infected Schwann cells process and present Ags of M. leprae to Ag-specific, inflammatory type-1 T cells and that these T cells subsequently damage and lyse infected Schwann cells. Thus far it has been difficult to study this directly because of the inability to grow large numbers of human Schwann cells. We now have established long-term human Schwann cell cultures from sural nerves and show that human Schwann cells express MHC class I and II, ICAM-1, and CD80 surface molecules involved in Ag presentation. Human Schwann cells process and present M. leprae, as well as recombinant proteins and peptides to MHC class II-restricted CD4+ T cells, and are efficiently killed by these activated T cells. These findings elucidate a novel mechanism that is likely involved in the immunopathogenesis of nerve damage in leprosy. The Journal of Immunology, 2001, 166: 5883-5888.

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 (1) accompanied by the abundant presence of local CD4+ T cells (1–3) and type-1 cytokines (2, 4–6). In tuberculoid and RR granulomas, cells that express serine esterase, a component of cytotoxic granules, colocalize with CD4+ CD45RO+ memory T cells (2), and analysis of Mycobacterium leprae-reactive T cells confirmed that these T cells indeed produce serine esterase in vitro (7). The induction of cytolytic CD4+ Th1-like cells during mycobacterial infections has been documented extensively (7–10), 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 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-2, which ligates to eβ-dystroglycan receptor-complexes on myelinating Schwann cells (11–13). 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 Ag-specific manner (14), suggesting that murine Schwann cells are susceptible to killing by CD8+ T cells. However, CD4+ T cells, which form the major cellular component of granulomatous leprosy lesions, were not examined.

We now have established human Schwann cell cultures and analyzed their Ag-presenting capacity and their susceptibility to killing by M. leprae-reactive T cells in a human setting. Our data show that human Schwann cells process and present M. leprae to Ag-specific T cells and are subsequently killed during this event. We propose that this could be an important mechanism in nerve damage.

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 (15). Briefly, sural nerve specimens were cut into small pieces and incubated in 85% IMDM, supplemented with 10% lymphokine-activated killer (LAK) cell supernatant, 5% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml PHA (Murex Diagnostics, Dartford, U.K.), in humidified 5% CO2 at
37°C. After 10 days, nonadherent cells were removed and adherent cells propagated in 80% IMDM supplemented with 5% LAK cell supernatant, 10% FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin supplemented with 0.6% glucose.

**Immunostaining of cultured Schwann cells**

Cells were grown on coverslips before indirect immunofluorescence or the invasion by *M. leprae*. The cells were washed twice with PBS(+), fixed with 4% paraformaldehyde for 15 min at room temperature, and then rinsed again three times. To block nonspecific binding, cells were treated with 1% NGS-PBS. To visualize the intracellular protein expression, permeabilization of the cells was performed in the blocking solution containing 1% saponin, 0.1% anti-2′,3′-cyclic nucleotide-3′-phosphodiesterase (CNP) or anti-S100 (β subunit); Sigma, St. Louis, MO) diluted 1:200 was incubated with the cells for 45 min at room temperature in the blocking solution. Then cells were washed in PBS and incubated for 30 min at room temperature with the conjugated Alexa 488 goat anti-mouse IgG (Molecular Probes, Eugene, OR) diluted 1:500 in 1% normal goat serum-PBS. After extensive washing in PBS, coverslips were mounted on 5 μl of Vectashield mounting medium containing 4′,6′-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA) on glass slides, and indirect immunofluorescence was visualized with a fluorescence Microscope (Carl Zeiss, Thornwood, NY) using a 100× oil objective.

**Phenotypic characterization**

Expression of HLA, adhesion, and costimulatory molecules by human Schwann cells was determined by indirect immunofluorescence. Abs recognizing HLA-DP (B7/2), HLA-DQ (SPV-L3), HLA-DR-FITC, CD80-FITC, CD86-PE, ICAM-1-FITC, LFA-1-FITC (Becton Dickinson, Mountain View, CA), and F(ab′)2, (CLB, Amsterdam, The Netherlands) were applied for FACSscan analysis. Cultured cells were incubated with mAbs for 60 min at 4°C. When nonconjugated mAbs were used, samples were subsequently incubated with goat anti-mouse-FITC (Becton Dickinson) for another 60 min at 4°C. When nonconjugated mAbs were used, samples were subsequently incubated with goat anti-mouse-FITC (Becton Dickinson) for another 60 min at 4°C. After extensive washing in PBS, coverslips were mounted on 5 μl of Vectashield mounting medium containing 4′,6′-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA) on glass slides, and indirect immunofluorescence was visualized with a fluorescence microscope (Carl Zeiss, Thornwood, NY) using a 100× oil objective.

**M. leprae preparation**

*M. leprae* was maintained in continuous passage in athymic nu/nu mice (Harlan Sprague-Dawley, Indianapolis, IN) by inoculation of 1 × 10⁶ freshly harvested bacilli into both hind foot pads as described previously (16). Approximately 9 mo after infection, footpad tissue was aseptically harvested and inoculated into a 60 Co irradiator and stored at 4°C (Shepherd, San Fernando, CA). The bacilli were purified by differential centrifugation and enumerated. Absence of contamination of the bacilli preparation (16). Approximately 9 mo after infection, footpad tissue was aseptically harvested bacilli into both hind foot pads as described previously (16). After extensive washing in PBS, coverslips were mounted on 5 μl of Vectashield mounting medium containing 4′,6′-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA) on glass slides, and indirect immunofluorescence was visualized with a fluorescence microscope (Carl Zeiss, Thornwood, NY) using a 100× oil objective.

**T cell clones**

HLA restriction and Ag specificity of CD4⁺ Th1-like clones L10B4, L10C11, R1E4, R3F7, and Rπ15 I-1 have been reported before (7, 17). The epitope recognized by DR11-restricted, 15-kDa protein reactive clone D1B2 (18) has not been described before, and the Ag recognized by the DR11-restricted T cell clone D2C1 has not been determined yet.

**Schwann cell Ag presentation assay**

Human Schwann cells were seeded in 96-well flat-bottom microtiter plates at 2000 cells/well. After 24 h, the cells were washed three times with IMDM and 10% pooled human serum and pulsed with Ags (10⁶ bacilli/well *M. leprae*, 25 μg/ml of sonicated *M. leprae*, 10 μg/ml mycobacterial proteins, or 10 μg/ml peptides) for 40 h. The cells were washed three times and cocultured with 10⁶ T cells for 88 h. During the last 16 h, 0.1 μCi [¹²⁵I]thymidine per well was added. Incorporation of [¹²⁵I]thymidine was measured by liquid scintillation counting. When indicated, mAbs were added in the assay at 1:200 dilution.

In other experiments, Ag-pulsed Schwann cells were labeled with 40 μCi/ml [⁵¹Cr] (Sodium Chromate; New England Nuclear Life Science Products, Boston, MA) for 2 h 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-100 to determine the spontaneous and maximum [⁵¹Cr] release respectively as described before (8). Cell-free supernatants were collected from the wells after 6 h, and the [⁵¹Cr] release was measured by γ counting. The percentage specific lysis was calculated as follows: percentage specific lysis = [(experimental [⁵¹Cr] release − spontaneous [⁵¹Cr] release)/(maximal [⁵¹Cr] release − spontaneous [⁵¹Cr] release)] × 100%. Spontaneous releases did not exceed 15% of the maximal release. Experiments were performed in quadruplicates.

**Measuring Fas-induced Schwann cell lysis**

Human Schwann cells were labeled with 40 μCi/ml [⁵¹Cr] as described above and subsequently incubated with 1 to 4 μg/ml of the apoptosis-inducing anti-Fas Ab APO-1 (personal gift from Dr. J. P. Medema, Leiden University Medical Center, Leiden, The Netherlands) for 4 h at 37°C. Specific cell lysis was measured and calculated as outlined in the previous section. Simultaneously, induction of apoptosis was analyzed by assessing DNA fragmentation as developed by Nicoletti et al. (19). Jurkat cells were used as positive control in both assays.

**Results**

**Characterization of human Schwann cell cultures**

Schwann cell cultures were established as described above. A typical feature of the cultures was the low cellular division rate, with an average doubling time of 1–2 wk. To verify that the cultures contained Schwann cell, RT-PCR was performed. To demonstrate the purity of the Schwann cell cultures, cells were analyzed by immunostaining with Abs recognizing Schwann cell-specific proteins. All human Schwann cell cultures were positive for CNPase and S-100β by RT-PCR, whereas glial fibrillary acidic protein mRNA was weakly detectable (data not shown). Control fibroblasts and PBMC were negative in RT-PCR for all Schwann cell markers. Immunostaining with S-100β (Fig. 1), CNPase, and glial fibrillary acidic protein mAbs (data not shown) confirmed expression of these Schwann cell markers by all cells in the cultures. Expression of fibroblast marker 5-propylhydroxylase was not detected in any of the Schwann cell cultures (data not shown).

**Phenotypic characterization of Schwann cells**

In Table I and Fig. 2, a and b, it is shown that 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 two of five Schwann cell cultures. Also, CD80 expression was observed (Fig. 2c). Expression of neither LFA-1 nor CD1a/b/c, molecules involved in presentation of nonpeptide Ags (20, 21) could be detected.
Table 1. Schwann cell surface expression of molecules involved in Ag presentation

<table>
<thead>
<tr>
<th>Schwann Cell Lines</th>
<th>HSW</th>
<th>NCN25</th>
<th>LKN118</th>
<th>LKN109</th>
<th>LKN116</th>
</tr>
</thead>
<tbody>
<tr>
<td>HLA-DP</td>
<td>28%</td>
<td>32%</td>
<td>83%</td>
<td>83%</td>
<td>48%</td>
</tr>
<tr>
<td>HLA-DQ</td>
<td>10%</td>
<td>70%</td>
<td>7%</td>
<td>6%</td>
<td>19%</td>
</tr>
<tr>
<td>HLA-DR</td>
<td>98%</td>
<td>93%</td>
<td>99%</td>
<td>94%</td>
<td>98%</td>
</tr>
<tr>
<td>CD1a</td>
<td>20%</td>
<td>3%</td>
<td>3%</td>
<td>2%</td>
<td>6%</td>
</tr>
<tr>
<td>ICAM-1 (CD54)</td>
<td>98%</td>
<td>93%</td>
<td>99%</td>
<td>94%</td>
<td>98%</td>
</tr>
<tr>
<td>LFA-1 (CD11a)</td>
<td>5%</td>
<td>6%</td>
<td>4%</td>
<td>7%</td>
<td>7%</td>
</tr>
<tr>
<td>LFA-3 (CD58)</td>
<td>21%</td>
<td>23%</td>
<td>82%</td>
<td>21%</td>
<td>31%</td>
</tr>
<tr>
<td>CD70</td>
<td>23%</td>
<td>3%</td>
<td>3%</td>
<td>4%</td>
<td>5%</td>
</tr>
</tbody>
</table>

Because the expression of HLA-DR and ICAM-1 might have been induced by cytokines present in the culture medium, Schwann cells were maintained in the absence of LAK supernatant for 2 wk. Although expression of HLA-DR and ICAM-1 was lower on 2 wk culturing on regular medium (Fig. 2d, time point 0 h), a substantial proportion of the Schwann cells still expressed HLA-DR and ICAM-1. Addition of recombinant IFN-γ restored the initial level of expression within 18 h. Thus, in vitro cultured human Schwann cells express constitutive and IFN-γ-inducible cell surface molecules involved in Ag presentation.

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

One of the Schwann cell cultures expressed HLA-DRB1*1101. T cell clone D1B2 has been reported to recognize an M. leprae protein with a molecular mass of 15 kDa in the context of DR11 (18). To be able to compare recognition of peptides vs 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 Fig. 3.

Human Schwann cells efficiently present Ag to M. leprae-specific type-1 inflammatory T cells

Because Schwann cells are nonprofessional phagocytes that can be infected with M. leprae (14, 22), we investigated whether ex vivo isolated human Schwann cells are capable of processing and presenting exogenous M. leprae Ags to inflammatory type-1 T cells. Varying numbers of Schwann cells ranging from 20 to 2500 cells per well were tested as APC in cultures to which a constant number of mycobacterium-specific CD4+ T cells and Ag was added. As depicted in Fig. 4a, 1000 Schwann cells/well already induced strong Ag-specific T cell proliferation when HLA-DR-matched T cells were added. Although addition of protein Ags directly to the assay induced significant T cell proliferation, preincubation of Schwann cells with Ags for 48 h yielded a 2-fold stronger response (Fig. 4b).

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 cocultured with HLA-DR-matched or -mismatched M. leprae-reactive CD4+, Th1-like clones. As shown in Fig. 4, c and 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). Ag presentation was HLA-DR-restricted, as judged by blocking experiments with HLA-specific Abs (Fig. 5a) and by using HLA-DR-matched and -mismatched T cell/APC combinations.

Ag-specific lysis of Schwann cells by CD4+ Th1-like clones

CD4+ Th1-like cells often display potent cytolytic activity toward a range of host cells, including nonprofessional APC (7–10, 23). Therefore, we investigated whether Schwann cells are susceptible to killing by CD4+ Th1 cells during Ag presentation. As shown in Fig. 6, M. leprae-pulsed Schwann cells are highly susceptible to killing by type-I CD4+ T cell clones from leprosy patients. Killing was Ag dependent and HLA-DR restricted.

FIGURE 2. Surface expression of molecules involved in Ag presentation. Human Schwann cells express both HLA-DR (a, solid profile) and ICAM-1 (b, solid profile). Furthermore, significant expression of the costimulatory molecule CD80 (c) solid profile) was observed, whereas no CD86 (open profile) could be detected. d, Induction of MHC class II and ICAM-1 by IFN-γ. In the absence of IFN-γ (time point 0), HLA-DR (○) and ICAM-1 (□) expression could be observed. Addition of rIFN-γ increased the expression of these membrane molecules, but had no effect on HLA-DR (△) and HLA-DQ (+).

FIGURE 3. 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 with HLA-DR-matched monocytes. The epitope recognized was situated between amino acids 51 and 60.
Although human Schwann cells express Fas (Fig. 7a), apoptosis-inducing Fas Ab APO-1 was not able to induce Schwann cell killing (Fig. 7b), nor did it induce apoptosis (Fig. 7, c and e). In addition to peptide, protein and M. leprae sonicate also are presented efficiently. Schwann cell/T cell combinations used in the latter two experiments are the HLA-DR11-restricted combination HSW/D1B2 (c) and the HLA-DR3-restricted combination NCN25/Rp15 1–1 (d).

Collectively, these data suggest that human Schwann cells cannot be killed by ligation of surface-expressed Fas and that that Schwann cells can be killed by exogenous ATP. The latter pathway is unlikely to be responsible for Th1-mediated Schwann cell death. It is likely that Schwann cells are susceptible to killing by degranulation of Th1 cells, which is supported by the observation that killing could partly be inhibited by EGTA.

Discussion

By 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, process, and present mycobacterial Ags to MHC class II-restricted CD4\(^+\) CTLs. After Ag presentation, Schwann cells are killed in an Ag-specific fashion by M. leprae-specific, inflammatory CD4\(^+\) T cells. Molecules involved in costimulation and adhesion of T cells were strongly expressed by human Schwann cells and could be up-regulated by
Analysis of leprosy lesions strongly suggests that CD4<sup>+</sup> T cells access the nervous system, thus allowing infiltrating leukocytes to access the nervous system. However, in leprosy reactional episodes, acute inflammatory reactions occur that may lead to damaging of such structures in situ, thus allowing infiltrating leukocytes to access M. leprae-infected Schwann cells. Also, M. leprae infection of Schwann cells may directly lead to loss of complete basal lamina integrity. Thus, it would be conceivable that under inflammatory conditions T cells can access Schwann cells and recognize HLA/peptide complexes presented by the Schwann cell.

Presentation of M. leprae Ags to CD4<sup>+</sup> T cells resulted in both T cell proliferation and T cell-mediated Schwann cell lysis. Cell lysis in general may result from secretion of cytotoxic granules by CTLs or NK cells. Such a mechanism may not only lead to host cell lysis, but also may reduce the viability of intracellular bacteria (32). Other possible mechanisms of cellular killing that have been reported are triggering of purinergic receptors by ATP, or alternatively, the ligation of death receptors such as Fas. However, we show here that ATP-mediated killing is not involved in T cell-mediated Schwann cell lysis, because T cell-dependent killing could not be inhibited by the ATP inhibitor hexokinase. However, hexokinase efficiently prevented ATP-dependent killing of Schwann cells in the absence of T cells, showing that this pathway is functional in human Schwann cells. Similar observations were made for Fas-dependent cell killing. Although Fas is present on human Schwann cells at low levels, as is shown here for the first time, this receptor was shown to be nonfunctional on Schwann cells. Although apoptosis-inducing Fas Abs significantly caused killing of control Jurkat cells, no such cell death could be observed when Schwann cells were incubated with this agent. Thus, these data seem to rule out that Fas is involved in T cell-dependent Schwann cell lysis. Finally, granule-secreting T cells have been demonstrated in leprosy lesions (2, 33). Because our T cells secrete serine esterases (7), and because we find that lysis of Schwann cells could be partly inhibited by the divalent cation chelator EGTA, we assume that Schwann cells are susceptible to granule-mediated killing mediated by cytolytic CD4<sup>+</sup> T cells. Further studies will have to elucidate the precise mechanism of Schwann cell killing and its consequences for intracellular pathogen survival.
Taken together, this study reveals a novel and potentially important mechanism of CD4+ CTL-mediated Schwann cell damage in leprosy. This mechanism is likely to contribute to leprosy nerve damage in vivo, given the predilection of *M. leprae* for Schwann cells, and the dominant role of CD4+ serine esterase+ Th1 cells in leprosy lesions (2). Thus, our data suggest that antagonism of molecular interactions between *M. leprae*, Schwann cells, and inflammatory T cells may provide a rational strategy to prevent Schwann cell and nerve damage in leprosy. Such strategies can now be evaluated by using a system as described in the current study for the first time.

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