chapter 4

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

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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.

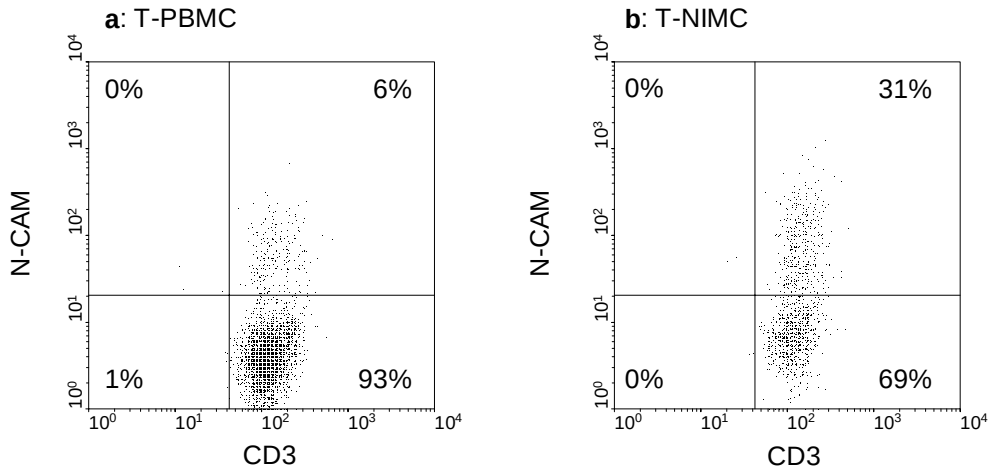

figure 1: Expression of N-CAM on T cells derived from peripheral blood (a) and leprosy neuritis biopsies (b). After antigenic stimulation, all cells were CD3 positive. Neural T cells expressed a significantly higher amount of N-CAM when compared to T cells cultured from peripheral blood.

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-

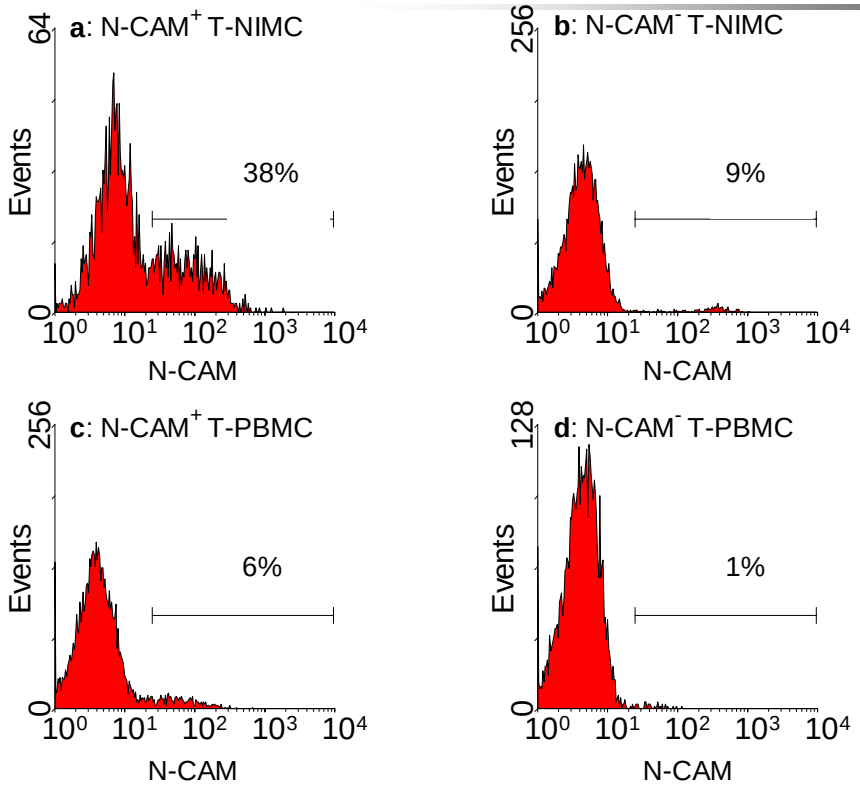

figure 2: Sorting and expansion of N-CAM positive and negative T cells. N-CAM⁺ nerve derived T cells partly lost their N-CAM expression, but a significant number remained positive (a). Small numbers of sorted N-CAM⁻ neural T cells were able to re-express N-CAM (b). Almost all N-CAM⁺ peripheral T cells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

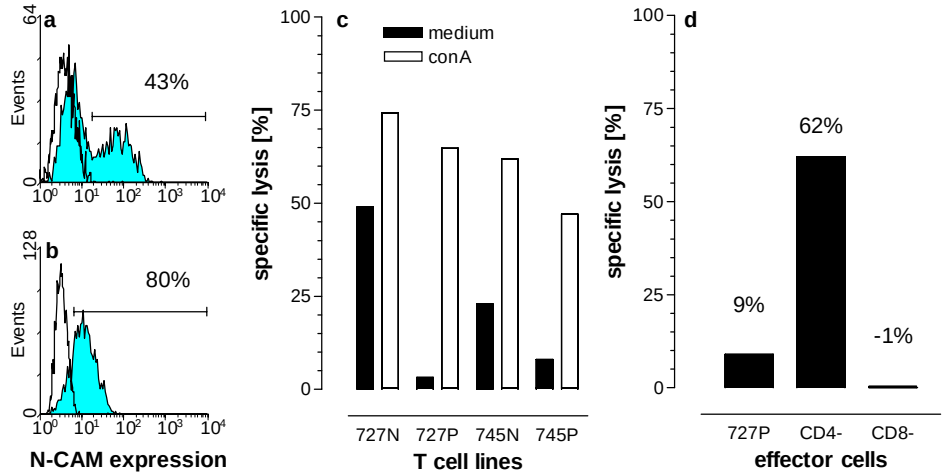

figure 3: Role of N-CAM⁺CD8⁺ T cells in antigen independent, MHC unrestricted target cell killing. Human Schwann cells (a) and U251 (b) express N-CAM on their surface as demonstrated in FACS analyses. Neural (N) and peripheral (P) T cells from two multi-bacillary patients were incubated with ^{51}Cr 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-

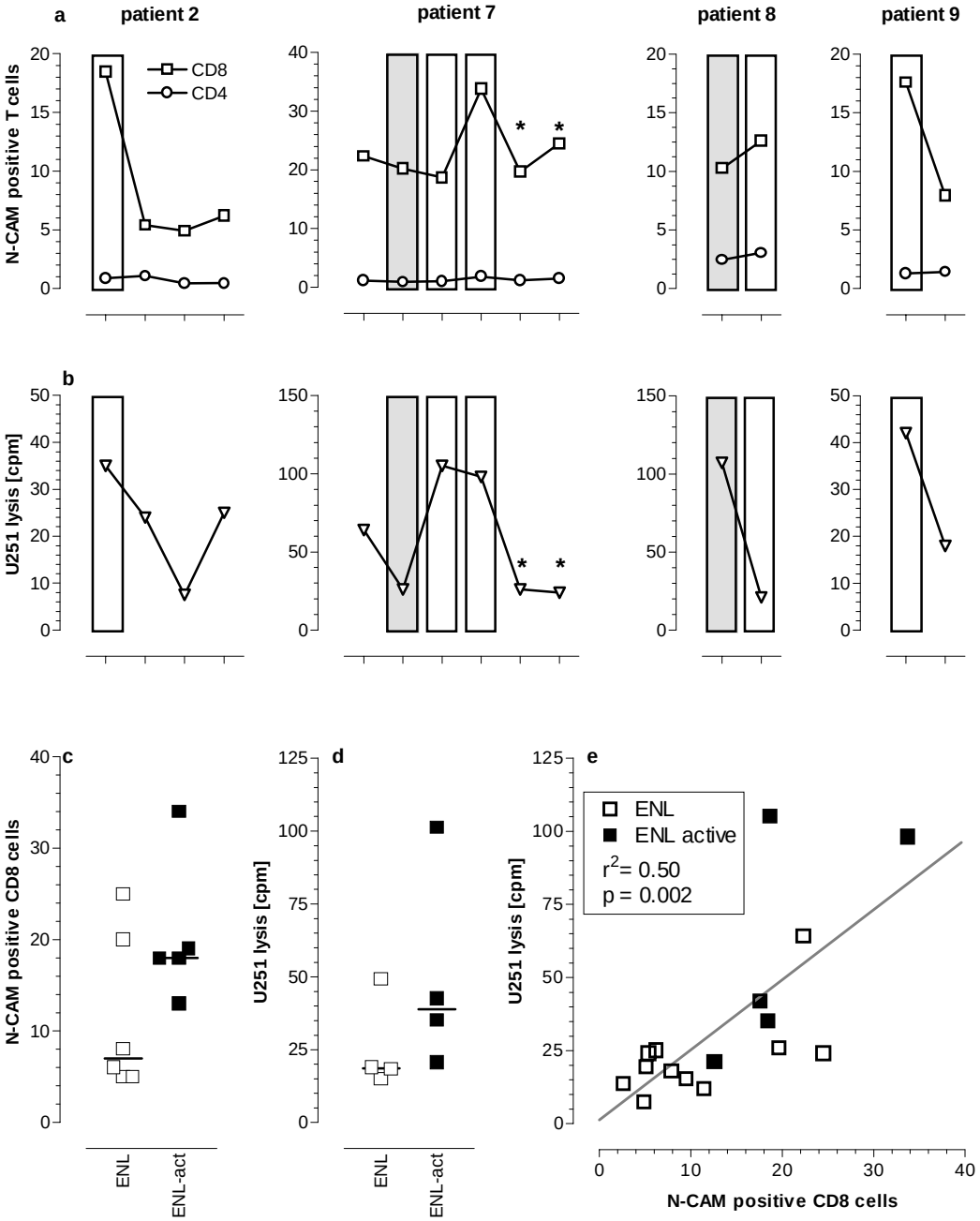

figure 4: Longitudinal analyses of patients with leprosy reactions. a) N-CAM expression on $CD4^+$ and $CD8^+$ T cells after M. leprae stimulation was measured in 4 patients with one or more episodes of active ENL (white bars). Asterices indicate time points at which the patient was under thalidomide 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).

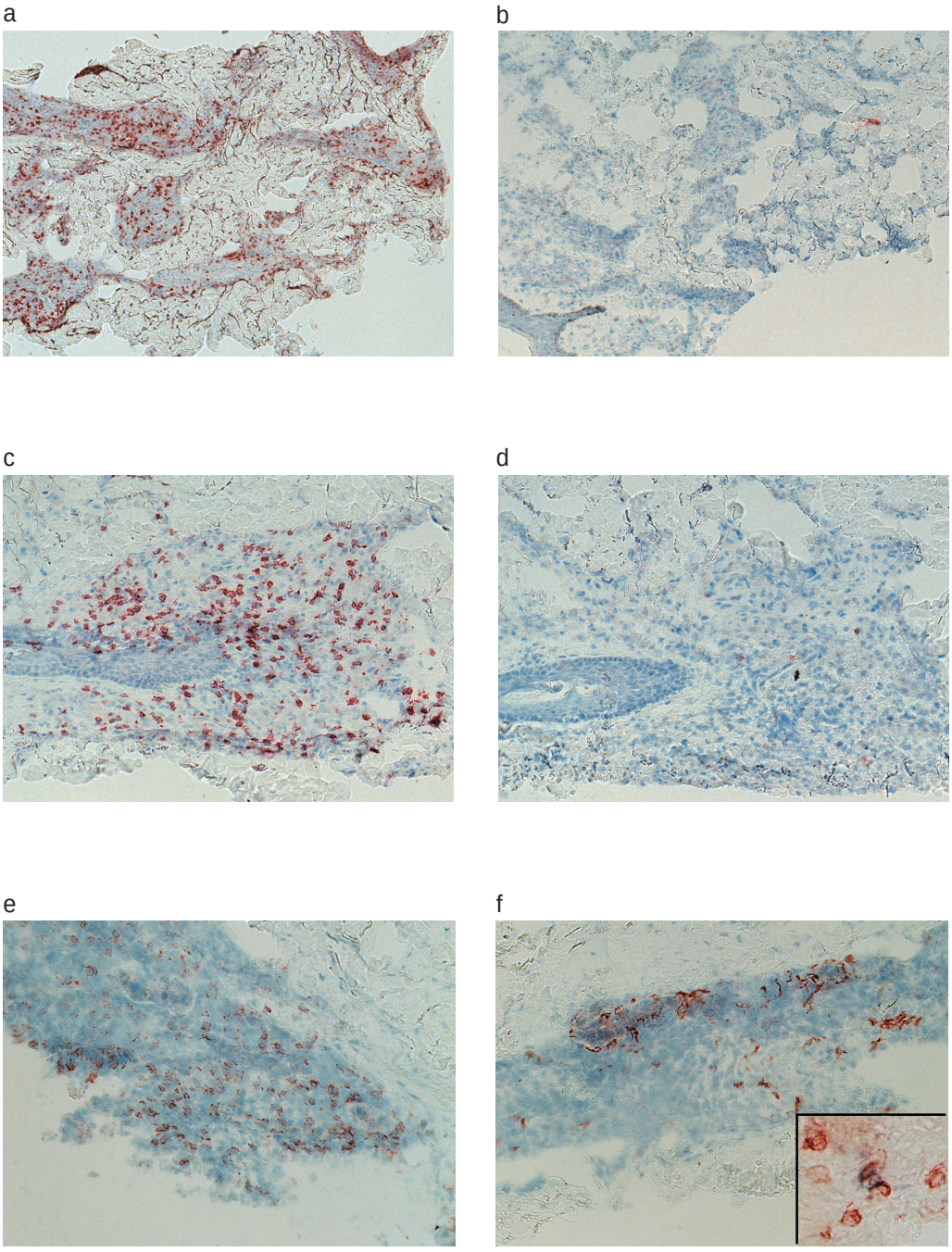

figure 5: A representative photo-micrograph illustration, showing the expression of N-CAM on T cells (a, c, e: CD8 single staining, b, d, f: N-CAM single staining) of patient 7 undergoing ENL via RR. Insert shows N-CAM^{+} CD8^{+} double staining cells in the granuloma.

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

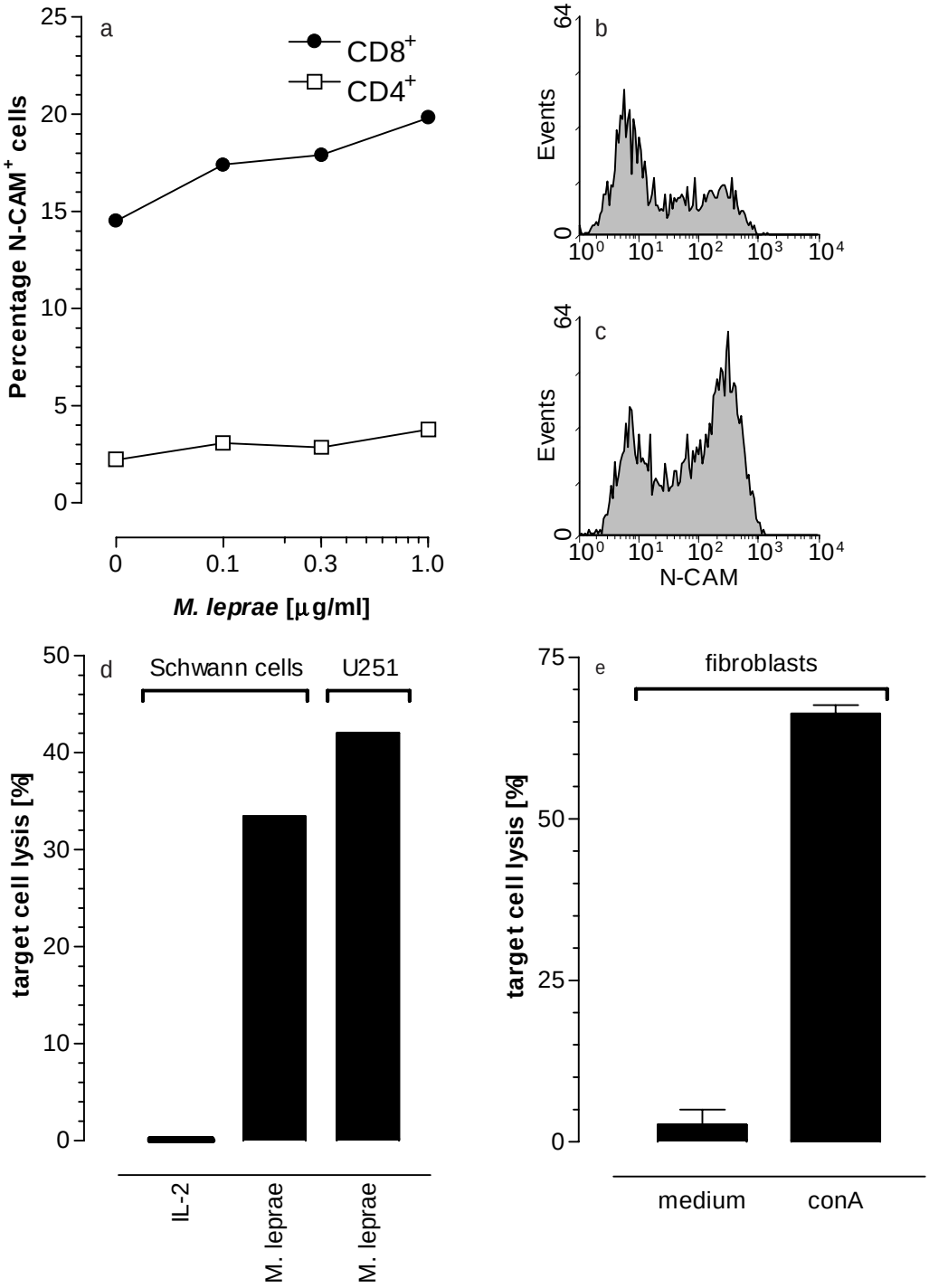

figure 6: Role of M. leprae in the induction of N-CAM expression and U251 lysis. a) PBMC were exposed to different concentrations of M. leprae. M. leprae was found to raise N-CAM expression on CD8⁺ T cells (solid circles), while hardly any effect was observed on CD4⁺ T cells (open squares). N-CAM expression 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.