RESEARCH PAPER

Lymphocyte antigens targetable by monoclonal antibodies in non-systemic vasculitic neuropathy

Christian Schneider,1 Gilbert Wunderlich,1 Johannes Bleistein,2 Gereon R Fink,1,3 Martina Deckert,4 Anna Brunn,4 Helmar Christoph Lehmann1

ABSTRACT

Objective To identify the most relevant antigens for monoclonal antibodies in lymphocytic infiltrates in non-systemic vasculitic neuropathy (NSVN).

Background Current immunosuppressive treatment for NSVN is insufficient. Monoclonal antibodies might be a treatment option, but the expression profile for targetable antigens on lymphocytic infiltrates in NSVN is unknown.

Methods Sural nerve biopsies from a cohort of patients with NSVN were immunohistochemically studied for the expression of potential candidate antigens in perivascular and intramural lymphocytic infiltrates and correlated with neurological and electrophysiological parameters. 20 patients with treatment naïve NSVN and 5 patients with idiopathic axonal neuropathy were included.

Results The CD52, BAFF and CD49d antigens were expressed in epineurial, perivascular or intramural lymphocytes of all (20/20) patients. CD52 was most prominently expressed in 21.49% of all inflammatory infiltrates. BAFF and CD49d were detected in 11.25% and 10.99% of these lymphocytes, respectively. The CD20, CD25 and CD126 antigens were found less frequently and at low levels only (CD20: 10/20 patients, 5.84% of lymphocytes; CD25: 17/20 patients, 5.22% of lymphocytes; CD126: 3/20 patients, 0.15% of lymphocytes).

Conclusion This is the first study in NSVN that identifies antigens expressed by pathogenic lymphocytes, which are potential targets for future monoclonal antibody treatment. Our data suggest that NSVN is amenable to monoclonal antibodies and, moreover, that targeting CD52 may be particularly promising. Our results strongly warrant future clinical trials in NSVN with monoclonal antibodies.

INTRODUCTION

Non-systemic vasculitic neuropathy (NSVN) is a frequent cause of chronic progressive axonal peripheral neuropathy. Diagnosis is established by nerve biopsy, which demonstrates axonal damage with asymmetric loss of nerve fibres. Perivascular inflammatory infiltrates invade blood vessel walls resulting in haemorrhage and necrosis, followed by vascular obliteration.1 Due to a lack of controlled trials in NSVN, current treatment recommendations are based on expert opinions or the extrapolation of results from treatment trials in systemic vasculitides.2 3 However, the concept that NSVN may constitute a separate disease entity challenges the validity of the latter approach.1 4 It is assumed that NSVN is amenable to immunosuppressive treatment.1 5 So far, treatment regimens include corticosteroids, usually in combination with cyclophosphamide in order to avoid the high relapse rates of corticoid monotherapy. Frequently, this combined immunosuppression needs to be discontinued due to severe side effects such as pneumonia, sepsis or leucopenia.6

The identification of molecules that are pathogenetically relevant and that may be targeted specifically by recombinant monoclonal antibodies opens an exciting novel strategy in the therapy of autoimmune diseases. Such an approach may also yield novel therapeutic concepts for NSVN. So far, monoclonal antibodies directed against cytokines (B cell activating factor, BAFF), cytokine receptors, such as cluster of differentiation (CD)25 and CD126 and other surface molecules(CD20, CD52) are currently tested or have already been approved for treating immune-mediated neurological diseases such as multiple sclerosis.7–11 In contrast, such a strategy has not yet been evaluated in NSVN.

To pave the way for studies with monoclonal antibodies in NSVN, we here characterised the phenotypic profile of inflammatory infiltrates in a series of 20 sural nerve specimens from patients with NSVN. We focused on immunologically relevant molecules for which Food and Drug Administration-approved monoclonal antibodies are available.

Our study identifies CD52 as the most frequently and prominently expressed antigen in NSVN. Thus, the use of antibodies, which target CD52, for the treatment of NSVN warrants further investigation.

MATERIALS AND METHODS

Clinical and electrophysiological assessment of patients

Clinical data and biopsy specimens from 20 untreated patients with NSVN were analysed retrospectively. All patients fulfilled the diagnostic criteria for pathologically definite NSVN according to the guidelines of the Peripheral Nerve Society.1 There was no evidence for systemic vasculitis, neither clinically nor in laboratory tests. For control, clinical data and biopsy specimens obtained from five patients with chronic idiopathic non-inflammatory axonal neuropathy were included.

In addition, sural nerve biopsies of two patients each suffering from anti-neutrophil cytoplasm antibody-associated systemic vasculitis (AASV) and chronic inflammatory demyelinating polyneuropathy (CIDP), respectively, as well as temporal artery biopsies of two
patients suffering from temporal arteritis have been included. All patients were examined neurologically and electrophysiologically including standard nerve conduction studies. The study protocol was approved by the institutional review board of the University Hospital Cologne, Cologne, Germany (protocol number 16–285).

### Nerve biopsy and immunohistochemical profiling

All patients underwent sural nerve biopsy after obtaining written informed consent. Nerve specimens were subdivided into three parts: one fraction was snap frozen in isopentane (Fluka, Neu-Ulm, Germany), precooled by liquid N2 and stored at −80°C until preparation of 9 µm thick sections; the second fraction was formalin fixed and paraffin embedded to prepare 4 µm thick sections; the third fraction was fixed in 3.9% phosphate buffered glutaraldehyde (Merck, Darmstadt, Germany) for 48 hours, osmicated and embedded in epoxy resin (Serva, Heidelberg, Germany) for preparation of 1 µm semithin sections. Frozen sections were stained with H&E; enzyme histochemistry was performed with Engel’s modified trichrome staining. Semithin sections were stained with 0.1% toluidine blue O (Merck) in 2.5% sodium carbonate (Merck). Formalin-fixed, paraffin-embedded nerve specimen sections were stained with Prussian blue and used for immunohistochemistry employing the avidin–biotin complex technique with appropriate biotinylated secondary antibodies. Immunohistochemical studies were performed on paraffin-embedded specimen sections.

### Immunohistochemical profiling

NSVN sural nerves of all patients (20/20, 100%) harboured epineurial, perivascula or intramural LCA+ inflammatory infiltrates (mean±SD: 10 HPF: 225.8±66.24; see table 2).

Vascular wall damage had led to haemorrhage with hemosiderin deposits in 60% (12/20) of patients. Asymmetric loss of nerve fibres was present in 80% of patients (16/20, figure 1). LCA+ cells were confined to the epineurial tissue of 3/5 control patients and clinical studies showed no distinct clinical, electrophysiological or neuropathological changes compared with patients with short duration of symptoms. Therefore, duration of disease influenced neither disease severity nor the extent of inflammatory infiltrates.

### Statistical analysis

Comparisons between clinical and electrophysiological tests and frequency of inflammatory infiltrates were performed using the Mann-Whitney U test; p values of <0.05 were considered statistically significant.

### RESULTS

#### Patients and clinical studies

Mean age of the patients and controls at the time of biopsy was 67 and 61 years, respectively. The time interval between onset of clinical symptoms and definite diagnosis varied from 1 to 20 years (mean: 4.85 years). 90% of the patients presented with sensory and motor deficits; the remaining patients showed sensory symptoms only. Electrophysiological studies, comprising distal motor latencies, proximal and distal compound muscle action potential amplitudes, and motor nerve conduction velocity of the right tibial or peroneal nerve as well as sensory nerve action potential amplitudes, and sensory nerve conduction velocity of the right sural nerve, revealed severe axonal damage of sensory and motor nerve fibres in all patients (data not shown). Patients with a long history of symptoms showed no distinct clinical, electrophysiological or neuropathological changes compared with patients with short duration of symptoms. Therefore, duration of disease influenced neither disease severity nor the extent of inflammatory infiltrates.

### Immunohistochemical profiling

NSVN sural nerves of all patients (20/20, 100%) harboured epineurial, perivascula or intramural LCA+ inflammatory infiltrates (mean±SD: 10 HPF: 225.8±66.24; see table 2).

Vascular wall damage had led to haemorrhage with hemosiderin deposits in 60% (12/20) of patients. Asymmetric loss of nerve fibres was present in 80% of patients (16/20, figure 1). LCA+ cells were confined to the epineurial tissue of 3/5 control patients (mean±SD: 1.6±1.52). Epineurial infiltrates consisted predominantly of CD8+ and some CD4+ cells. CD3+ T cells as well as some CD68+ macrophages. Endoneurial lymphocytes corresponded to CD68+ macrophages. In controls, LCA+ cells were absent from vessels.

The sum of immunoreactive and non-immunoreactive lymphocytes in 10 HPFs of the cross-orientated sural nerve specimens for each antigen strongly correlated with the number of lymphocytes of the respective antigens were evaluated for each antigen on the respective slide in 10 HPF.

### Table 1

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Clone</th>
<th>Species</th>
<th>Dilution</th>
<th>Heat-induced epitope retrieval</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>LCA (CD45)</td>
<td>X16/99</td>
<td>Mouse</td>
<td>1:50</td>
<td>Citrate buffer pH 6.0</td>
<td>Leica, Wetzlar, Germany</td>
</tr>
<tr>
<td>CD3</td>
<td>LN10</td>
<td>Mouse</td>
<td>1:200</td>
<td>Target buffer pH 9.0</td>
<td>Leica</td>
</tr>
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<td>CD4</td>
<td>4812</td>
<td>Mouse</td>
<td>1:40</td>
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<td>Biogenex, DCS, Hamburg, Germany</td>
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<td>CB144B</td>
<td>Mouse</td>
<td>1:200</td>
<td>Target buffer pH 9.0</td>
<td>Covance, München, Germany</td>
</tr>
<tr>
<td>CD68</td>
<td>514H12</td>
<td>Mouse</td>
<td>1:1000</td>
<td>Target buffer pH 9.0</td>
<td>Leica</td>
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<td>CD52</td>
<td>YTH43.5</td>
<td>Rat</td>
<td>1:500</td>
<td>Target buffer pH 9.0</td>
<td>Serotec, Puchheim, Germany</td>
</tr>
<tr>
<td>CD25 (IL-2R)</td>
<td>4C9</td>
<td>Mouse</td>
<td>1:25</td>
<td>Target buffer pH 9.0</td>
<td>Leica</td>
</tr>
<tr>
<td>BAFF</td>
<td>Buffy 2</td>
<td>Rat</td>
<td>1:200</td>
<td>Target buffer pH 9.0</td>
<td>Enzo Life Sciences, Lörrach, Germany</td>
</tr>
<tr>
<td>CD20</td>
<td>L26</td>
<td>Mouse</td>
<td>1:300</td>
<td>Citrate buffer pH 6.0</td>
<td>Leica</td>
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<tr>
<td>CD49d (VLA-4)</td>
<td>Polyclonal</td>
<td>Rabbit</td>
<td>1:40</td>
<td>Target buffer pH 9.0</td>
<td>Serotec</td>
</tr>
<tr>
<td>CD126 (IL-6R)</td>
<td>B-R6</td>
<td>Mouse</td>
<td>1:5</td>
<td>Target buffer pH 9.0</td>
<td>Serotec</td>
</tr>
</tbody>
</table>

LCA, leucocyte common antigen; VLA, very late antigen.

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Neuromuscular

Table 2 Total number of epineurial, perivascular or intramural small, mature lymphocytes in 10 HPF in NSVN sural nerve biopsies

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Total number of epineurial, perivascular or intramural small, mature lymphocytes in 10 HPF in NSVN sural nerve biopsies</th>
</tr>
</thead>
<tbody>
<tr>
<td>LCA+</td>
<td>225.75 ± 66.24</td>
</tr>
<tr>
<td>h+CD52+ and h+CD52-</td>
<td>222.3 ± 65.93</td>
</tr>
<tr>
<td>h+CD25+ and h+CD25-</td>
<td>224.75 ± 65.48</td>
</tr>
<tr>
<td>h+BAFF+ and h+BAFF-</td>
<td>217.75 ± 59.34</td>
</tr>
<tr>
<td>h+CD20+ and h+CD20-</td>
<td>223.3 ± 69.99</td>
</tr>
<tr>
<td>h+CD49d+ and h+CD49d-</td>
<td>221.95 ± 70.05</td>
</tr>
<tr>
<td>h+CD126+ and h+CD126-</td>
<td>222.9 ± 69.92</td>
</tr>
</tbody>
</table>

h+, hemalum positive; HPF, high power field; LCA, leucocyte common antigen; NSVN, non-systemic vasculitic neuropathy.

of epineurial, perivascular or intramural LCA-positive, small, mature lymphocytes (table 2). CD52 was the most abundantly expressed antigen on epineurial lymphocytes in NSVN biopsies and was present in all specimens (20/20, figure 2, table 3). There was no difference in the CD52 expression profile depending on localisation of lymphocytes in the epineurial tissue, that is, epineurial perivascular or intramural.

Sural nerve biopsies of patients with AASV (2/2) harboured large infiltrates of LCA+ vessel-associated lymphocytes (mean±SD/10 HPF: 445.5±86.76) predominantly consisting of CD3+ T cells. Vessel-associated infiltrates comprised numerous CD52+ lymphocytes (mean±SD/10 HPF: 224.5±34.48; corresponding to 50.39% of all lymphocytes). The CD52 epitope was only weakly expressed on single intramural lymphocytes in the biopsies of two patients with temporal arteritis and completely absent from nerve biopsies of two patients with CIDP (data not shown). Sural nerves of all patients with NSVN (20/20) harboured BAFF+ vessel-associated lymphocytes (table 3). Although 85% (17/20) and 50% (10/20) of the patients with NSVN showed an expression of the CD25 and CD20 antigen, respectively, these antigens were confined to a few lymphocytes (table 3). CD126 was expressed on single perivascular lymphocytes in 15% (3/20) of the patients with NSVN only (table 3). The expression profile of these antigens did not show any correlation neither with clinical nor with electrophysiological parameters.

DISCUSSION

Our data demonstrate that in NSVN, lymphocytes express various surface molecules and cytokines for which therapeutic monoclonal antibodies are available. As such, to the best of our knowledge, this is the first study that provides evidence for the expression of therapeutically targetable surface molecules in NSVN.

At the time point of biopsy, patients exhibited neurological symptoms requiring treatment. These clinical signs of disease activity were associated with the presence of lymphocytic infiltration in the epineurial tissue. Since the inflammatory infiltrates in NSVN are by definition restricted to the peripheral nervous system, our approach of immunophenotyping nerve biopsy specimens is the only one that allows identification of treatment targets. The lack of a correlation between clinical data and antigen expression profiles in the target organ, that is, the peripheral nerve, further emphasises the requirement of nerve biopsy analysis to guide novel concepts for treating NSVN.

Interestingly, the CD52 antigen, which is expressed by a variety of immune cells including T and B cells as well as macrophages, was expressed in all NSVN biopsies. Moreover, CD52 was the most frequently detected antigen (21.49%) in inflammatory infiltrates.

CD52 can be targeted by the humanised monoclonal antibody alemtuzumab, which has been approved for the treatment of patients with multiple sclerosis.7 Our results suggest that CD52 may be a suitable target also in NSVN. Expression of the CD52 antigen was not confined to NSVN but was also detected in...
patients with AASV (this study). In the study by Walsh et al\textsuperscript{14} and in a study with Behçet’s disease\textsuperscript{15} patients were responsive to anti-CD52 treatment. Therefore, such an approach may extend the use of alemtuzumab in patients with AASV, in whom beneficial effects on the overall outcome were suggested.\textsuperscript{14} However, in the latter study, neuropathic symptoms were not specifically assessed.\textsuperscript{14} In other inflammatory conditions, for example, in CIDP (this study and the study by Marsh et al\textsuperscript{16}), temporal arteritis (this study) and inflammatory orbital lesions,\textsuperscript{17} CD52-expressing lymphocytes or response to anti-CD52 treatment were either absent or rare, thus, not supporting anti-CD52 target treatment in these disorders.

BAFF (11.25\%) and CD49d (10.99\%) were expressed by significant populations of leucocytes. These observations may warrant the application of belimumab, a monoclonal antibody targeting BAFF, which has been used in the therapy of systemic vasculitis. In systemic lupus erythematoses, two phase III studies demonstrated efficacy in reducing overall disease activity; however, frequency of vasculitic neuropathy at baseline and outcome on neuropathic symptoms were not reported.\textsuperscript{18–20}

Furthermore, our data support the idea to apply natalizumab in NSVN, a monoclonal antibody targeting CD49d. Natalizumab is currently used in highly active multiple sclerosis, in which it has been shown to reduce the risk of disease progression and of clinical relapses. In individual patients with CIDP, natalizumab treatment led to an improvement and stabilisation in two and one patient(s), respectively, while disease progressed in one patient.\textsuperscript{21, 22}

The sural nerve of patients suffering from NSVN harboured only few B cells, which is in accordance with a previous study.\textsuperscript{23} In summary, we have, for the first time, identified immune molecules in the sural nerve of patients with NSVN which are potentially targetable. Molecular pathogenesis and the precise contribution of these molecules to NSVN remain to be elucidated. Data suggest that targeting the CD52 antigen, for example, by alemtuzumab, may constitute a promising novel treatment approach in NSVN. Results warrant future controlled trials in NSVN with monoclonal antibodies.

**Correction notice** This paper has been amended since it was published Online First. Owing to a scripting error, some of the publisher names in the references were replaced with ‘BMJ Publishing Group’. This only affected the full text version, not the PDF. We have since corrected these errors and the correct publishers have been inserted into the references.

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**Contributors** CS: Acquisition and analysis of data, drafting the manuscript for content. GW: Study concept, drafting the manuscript for content. JB: Drafting the manuscript for content, data analysis. GFE: Study concept, drafting the manuscript for content. MD: Study concept, drafting the manuscript for content. AB: Acquisition and analysis of data, study concept, drafting the manuscript for content. HCL: study design and concept, drafting the manuscript for content.

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**REFERENCES**


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**Figure 2** Expression of the CD52, CD25, BAFF, CD20, CD49d and CD126 antigens in vasculitic infiltrates of the sural nerve. (A) The sural nerve harbours numerous CD52-positive leucocytes, which have infiltrated the vessel wall and the adjacent epineurium. (B) Vessel-associated CD25-positive leucocytes are present. (C) Numerous BAFF-positive leucocytes have infiltrated the walls of epineurial blood vessels and the epineurium. (D) Only single CD20-positive B cells are present. (E) Some medium-sized vessel walls in the epineurium have been invaded by CD49d-positive cells. (F) Only single CD126-positive leucocytes are present. (A–F) Immunohistochemistry with rat anti-human CD52 (A), mouse anti-human CD25 (B), rat anti-human BAFF (C), mouse anti-human CD20 (D), rabbit anti-human CD49d (E) and mouse anti-human CD126 (F) and slight counterstain with haemalum; original magnification ×400.

**Table 3** Numbers of antigen-expressing perivascular or intramural, epineurial lymphocytes in 10 HPF in NSVN sural nerve biopsies.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Mean</th>
<th>SD</th>
<th>Percent of all lymphocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD52</td>
<td>51.6</td>
<td>38.97</td>
<td>21.49</td>
</tr>
<tr>
<td>CD25</td>
<td>13.1</td>
<td>11.96</td>
<td>5.22</td>
</tr>
<tr>
<td>BAFF</td>
<td>25.85</td>
<td>21.12</td>
<td>11.25</td>
</tr>
<tr>
<td>CD20</td>
<td>12.75</td>
<td>35.13</td>
<td>5.84</td>
</tr>
<tr>
<td>CD49d</td>
<td>26.9</td>
<td>22.89</td>
<td>10.99</td>
</tr>
<tr>
<td>CD126</td>
<td>0.55</td>
<td>1.5</td>
<td>0.15</td>
</tr>
</tbody>
</table>

*HPF, high power field; NSVN, non-systemic vasculitic neuropathy.


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