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DNA vaccination efficiently induces antibodies to Nogo-A and does not exacerbate experimental autoimmune encephalomyelitis

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ABSTRACT

Antibodies against the neurite outgrowth inhibitor Nogo-A enhance axonal regeneration following spinal cord injury. However, antibodies directed against myelin components can also enhance CNS inflammation. The present study was designed to assess the efficacy of DNA vaccination for generating antibodies against Nogo-A and to study their pathogenic potential in a mouse model for multiple sclerosis. Mice were immunized by a single i.m. injection of a plasmid expression vector encoding either full length membrane-integral Nogo-A equipped with a signal peptide or two versions of its large N-terminal extramembrane region. The presence of serum antibodies to Nogo-A was measured 4 weeks after injection by ELISA, Western blotting and immunohistochemistry. DNA vaccination efficiently induced production of Nogo-A-specific antibodies that recognized recombinant, intracellular Nogo-A in cell culture but also stained native Nogo-A on the oligodendrocyte surface. Experimental autoimmune encephalomyelitis was induced in DNA-vaccinated mice by immunization with proteolipid peptide (a.a. 139–154). In contrast to vaccination with DNA encoding myelin oligodendrocyte glycoprotein that exacerbates this disease, Nogo-A DNA vaccination did not enhance clinical severity of disease. In summary, DNA vaccination is a simple and efficient method for generating an antibody response to Nogo-A. No pathogenicity was observed even during a full-blown inflammatory response of the central nervous system.

1. Introduction

The extremely limited ability of central nervous system (CNS) axons to regenerate following injury is in part attributed to factors present in CNS myelin that inhibit neurite outgrowth (Schwab, 2002; Schwab and Bartholdi, 1996). One such inhibitor is the myelin-associated protein Nogo-A, which was described simultaneously by three independent groups (Chen et al., 2000; GrandPre et al., 2000; Prinjha et al., 2000). The role of antibodies in neutralizing the inhibitory activity of Nogo-A is well documented, as the inhibition of neurite outgrowth by CNS myelin can be neutralized by both monoclonal and polyclonal Nogo-A-specific antibodies in vitro (Chen et al., 2000). In vivo, Nogo-A-specific antibodies show a strong therapeutic potential after spinal cord injury or stroke where their intrathecal application enhances axonal sprouting and functional recovery (Bregman et al., 1995; Merkler et al., 2001; Wiessner et al., 2003). Recently, these findings were extended to primates in which intrathecal infusion of Nogo-A-specific antibody was shown to enhance motor recovery after spinal lesion (Freund et al., 2006).

Alternatively to the infusion of recombinant antibodies, anti-Nogo-A antibodies may be generated by immunization (Merkler et al., 2003). An effective technique for inducing highly specific antibodies to myelin antigens is DNA vaccination. DNA vaccine delivery systems in many ways mimic the antigenicity of infectious organisms and have become one of the fastest growing fields in vaccine technology. Furthermore, vaccination with DNA encoding the antigen of interest is a technically simple and effective method of generating neutralizing antibodies (Boyer et al., 1997; Lodmell et al., 1998). Because DNA vaccination induces expression of target antigens by host cells, it can result in production of protein with native conformation and post-translational modifications that elicit antibodies of optimal specificity (Attanasio et al., 1997). The efficacy of DNA vaccination was demonstrated in a spinal cord injury model in which vaccination with DNA encoding myelin inhibitors enhanced axonal regeneration (Xu et al., 2004).

Abbreviations: CHO, Chinese hamster ovary; CNS, central nervous system; MBP, myelin basic protein; MOG, myelin oligodendrocyte glycoprotein; PLP, proteolipid protein.

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Although the possibility of using Nogo-A-specific antibodies to promote axonal growth following CNS injury is tantalizing, one major concern is that anti-Nogo-A antibodies may disturb the structural integrity of CNS myelin and even initiate demyelination by antibody-dependent immune effector mechanisms. Indeed, antibodies to myelin components such as the myelin oligodendrocyte glycoprotein (MOG) can be highly pathogenic, causing severe clinical disease and extensive demyelination in animal models, and are implicated in the immunopathogenesis of demyelination in a subset of multiple sclerosis patients (Bourquin et al., 2003; Genain et al., 1999; Kerlero de Rosbo et al., 1990; Linington et al., 1988; Piddlesden et al., 1993). Importantly, the presence of MOG-specific B cells and antibodies in the periphery is benign, and mice fail to develop either spontaneous neurological disease or pathological evidence of demyelination. However, MOG-specific B cells both accelerate and exacerbate experimental autoimmune encephalitis, an autoimmune disease that reproduces many of the clinical and pathological features of multiple sclerosis (Bourquin et al., 2000; Litzenburger et al., 2000).

In this study, we examined the pathogenic potential of Nogo-A-specific antibodies during the clinical course of experimental autoimmune encephalomyelitis induced by an unrelated antigen. To induce a humoral immune response against Nogo-A, mice were vaccinated with plasmid DNA encoding either the N-terminal extramembrane fragment of Nogo-A or the complete amino acid sequence. We show that a single injection of Nogo-A cDNA efficiently induces antibodies to the gene product. Following an encephalitogenic challenge by the proteolipid protein peptide $PLP^{139-154}$, Nogo-A DNA vaccination does not enhance the clinical severity of experimental autoimmune encephalomyelitis. This is in strong contrast to the response induced by MOG-DNA vaccination, which exacerbates the clinical course of disease. These findings provide important information as to the use and safety of DNA vaccination for inducing Nogo-A-specific antibodies.

2. Materials and methods

2.1. Construction of expression plasmids

2.1.1. pSecNogo

An 850 bp 3' fragment of rat *nogo-A* cDNA was amplified by PCR from previously cloned *nogo-A* cDNA (Chen et al., 2000) with primers 5'-gcttagaattgccctgtgac-3' and 5'-gttcgcctcgagaatctgctttgcgcttcaatcc-3' in order to remove the stop codon and insert an XhoI site. The 5' 2.9 kb EcoRI fragment of *nogo-A* and the modified 3' fragment cut with EcoRI and XhoI were inserted into the expression vector pSecTag2C (Invitrogen AG, Basel, Switzerland) digested with EcoRI and XhoI. The construct encodes Nogo-A with an N-terminal Igκ-chain leader sequence and C-terminal myc and His tags. The integrity of the plasmid was verified by restriction analysis and sequencing.

2.1.2. $pNogo_{1-979}$

nogo-A cDNA cloned in the EcoRI site of pBluescript was partially digested with HindII and religated, resulting in the deletion of the last 558 base pairs of *nogo-A* that encode the transmembrane segments. This plasmid containing the truncated *nogo-A* ($nogo_{1-979}$) was digested with BamHI, filled in with Klenow, and digested with ApaI. The resulting fragment was cloned into pSecTag2A (Invitrogen) digested with EcoRV and ApaI. The Igκ leader sequence was deleted by digestion with NheI and SfiI and blunt ends were created with Klenow and T4 DNA polymerase. The plasmid was self-ligated and encodes the extramembrane N-terminal part of Nogo-A, with 3' myc/His tags and without the additional leader sequence.

2.1.3. $pSecNogo_{1-979}$-GPI

The GPI anchor recognition site of Thy1.1 was cut with HindIII and PmeI from pcDNA3.1BDNFmyc-Thy1 (gift of Dr. M. Hoener, Max-Planck Institute, Martinsried), blunt ends were created with Klenow. pSecTag-Nogo-Aext was cut with ApaI and PmeI and blunt ends were created with T4 DNA polymerase. These fragments were ligated and the orientation of the GPI anchor recognition site was checked by restriction analysis. The plasmid encodes the extramembrane N-terminal part of Nogo-A with the 5' Igκ leader sequence and is anchored to the membrane by a GPI anchor. All enzymes were purchased from Roche Diagnostics (Rotkreuz, Switzerland), except PmeI (New England Biolabs GmbH, Frankfurt am Main, Germany). DNA for vaccination was isolated from the transformed *E. coli* strain XL1-Blue with Wizard midiprep kits (Promega, Madison, WI).

2.1.4. pMOG

The full-length MOG coding sequence together with its signal sequence was cloned into the expression vector pcDNA3.1 (Invitrogen) as described previously (Bourquin et al., 2000). The plasmid pcDNA 3.1 was used as control DNA. DNA quality was checked by DNA electrophoresis and concentration was measured via absorption at 260 nm.

2.2. ELISA

Peripheral blood was collected by tail bleeding and, after coagulation, serum was obtained by centrifugation and stored at −20 °C. 96-well vinyl assay plates (Costar, Cambridge, MA) were coated either with 10 μg/ml of a recombinant N-terminal Nogo-A fragment comprising residues 233–940 (Fiedler et al., 2002) or of recombinant myelin oligodendrocyte glycoprotein (Bourquin et al., 2000) or myelin basic protein (both a gift from Dr. F. Kurschus, Max-Planck Institute for Neurobiology). After blocking with 1% (w/v) bovine serum albumin, the assay plates were incubated with the test serum. Specific binding was detected with alkaline phosphatase-conjugated goat anti-mouse IgG, IgG1 or IgG2a (Southern Biotechnology Associates, Birmingham, AL). Signal was developed by the addition of p-nitrophenyl phosphate and the absorption at 405 nm was measured on a MR-4000-ELISA-Reader (Dynatech, Embrach, Germany).

2.3. Cell culture and immunocytochemistry

Stable CHO-Nogo-A cells described previously (Chen et al., 2000) were cultured in MEM-alpha (Gibco, Invitrogen), supplemented with 5% fetal calf serum, 10 mM HEPES and 400 μg/ml G418. For immunocytochemistry, cells cultured on glass coverslips were fixed with 4% PFA, 5% sucrose in PBS for 20 min at room temperature. The cultures were permeabilized with 0.1% Triton X-100 and unspecific binding sites were blocked with 10% fetal calf serum. Mouse antisera were added as primary antibodies to the cells for 1 h, diluted 1:100 in PBS/ 0.1% Triton X-100. The cells were then washed, incubated with goat anti-mouse conjugated with TRITC (Jackson Immunoresearch Laboratories, PA) 1:200, washed and mounted on slides for fluorescence microscopy.

Optic nerve oligodendrocytes isolated from postnatal day 7 rats were cultured as described (Schwab and Caroni, 1988). For cell surface staining, four day-old cultures were incubated with the mouse antisera diluted 1:50 in culture medium for 30 min at room temperature, washed, and fixed. Cells were blocked with 0.1 M maleic acid with 2% (w/v) blocking reagent (Roche, Basel, Switzerland) for 1 h. Secondary goat anti-mouse antibodies conjugated with alkaline phosphatase (Milan Analytica, Lausanne, Switzerland) were used at 1:5000 in 0.1 M maleic acid with 1% (w/v) blocking reagent for 1 h at room temperature. The cultures were washed twice with maleic acid buffer, once with alkaline phosphatase buffer (0.1 M Tris–HCl pH 9.5, 0.1 M NaCl, 5 mM $MgCl_2$) and the staining was developed for 2–3 h at room temperature with 0.175 mg/ml BCIP (Sigma-Aldrich, Germany) and 0.338 mg/ml NBT (Sigma-Aldrich) in alkaline phosphatase buffer.

2.4. Western blot analysis

Mouse cerebellum and CHO-Nogo-A cells were frozen in liquid nitrogen, homogenized in extraction buffer (100 mM Tris–HCL pH 7.6, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.1% Na-deoxycholate) with one protease inhibitor cocktail tablet (Roche Diagnostics) per 10 ml. After centrifugation the protein concentration of the supernatant was determined with Advanced protein assay reagent (Cytoskeleton Inc., Denver, USA) and 20 µg mouse cerebellar lysate or 5 µg CHO-Nogo-A lysate was loaded per lane on 6% polyacrylamide gels. SDS-PAGE and western blot were performed as described (Frank et al., 1998). Blots, cut into one-lane stripes, were blocked with 3% (w/v) Top Block (Juro Supply, Lucerne, Switzerland) and incubated with mouse serum diluted 1:1000 in TBS, 0.2% Tween 20, 1% Top Block. Secondary antibody anti-mouse-HRP was diluted 1:10,000. Blots were developed in Supersignal luminol solution (Pierce Biotechnology, Lausanne) and exposed to X-ray film for 15 s.

2.5. DNA vaccination and induction of experimental autoimmune encephalomyelitis

Female SJL mice (Harlan Winkelmann, Germany) were injected with 100 µg DNA i.m. in physiological solution (1 mg/ml). Four to six weeks after DNA immunization, mice were injected subcutaneously with an emulsion of 100 µg $PLP_{139–154}$ in CFA (Gibco) supplemented with 10 mg/ml inactivated *M. tuberculosis* (H37 RA; Difco Laboratories, Detroit, MI). The animals received an additional i.p. injection of 300 ng *B. pertussis* toxin (List Biological Labs. Inc., Campbell, CA) in 300 µl phosphate-buffered saline on the day of immunization and again 48 h later. Animals were monitored regularly for clinical signs of experimental autoimmune encephalomyelitis (0, no clinical disease; 1, tail weakness; 2, paraparesis; 3, paraplegia; 4, paraplegia with forelimb weakness or paralysis; 5, moribund state or death). Animal studies were approved by the local regulatory agency (Regierung von Oberbayern, Munich, Germany).

2.6. Statistical analysis

Significance was assessed by Student's *T* test (ELISA) or the Mann–Whitney U test (clinical scores). Error bars represent the standard deviation unless otherwise indicated.

3. Results

We investigated the ability of three different plasmid expression vectors encoding Nogo-A to induce an antigen-specific antibody response. The constructs used were $pNogo_{1–979}$, encoding the N-terminal extramembrane fragment of Nogo-A (a.a. 1–979) with C-terminal myc and His tags, pSecNogo, encoding the complete 1163 a.a. of Nogo-A with an N-terminal Igκ chain leader sequence and C-terminal myc and His tags, and $pSecNogo_{1–979}$-GPI, which encodes the N-terminal extramembrane fragment of Nogo-A with the N-terminal Igκ chain leader sequence and a GPI anchor site. Four weeks after a single intramuscular injection with either pSecNogo or $pSecNogo_{1–979}$-GPI, a Nogo-A-specific IgG response was identified by ELISA in the sera of all animals vaccinated. In contrast, only one of four mice vaccinated with $pNogo_{1–979}$ and none of the control DNA-vaccinated animals developed a Nogo-specific antibody response (Fig. 1A). The antibody response developed rapidly, reaching a peak as early as 21 days after vaccination (Fig. 1B). The antibodies induced by vaccination with pSecNogo and $pSecNogo_{1–979}$-GPI were of both the IgG1 and IgG2a isotypes (Fig. 1C), indicative of a mixed Th1/Th2 response to the DNA vaccination. No antibodies to the myelin proteins myelin basic protein (MBP) and myelin oligodendrocyte protein (MOG) were detected after vaccination (Fig. 1D).

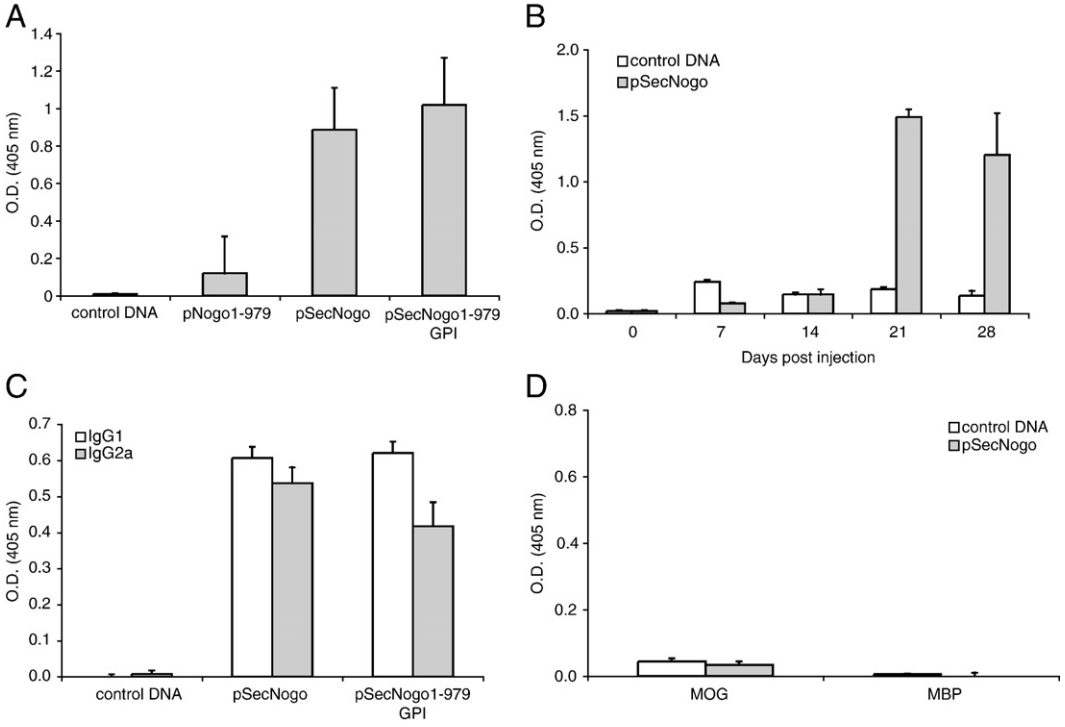

Fig. 1. Vaccination with Nogo-A DNA results in specific antibody production. (A) Nogo-A-specific serum IgG response measured by ELISA 4 weeks after vaccination with $pNogo_{1–979}$ ($n=4$), pSecNogo ($n=5$), $pSecNogo_{1–979}$-GPI ($n=5$) or control DNA ($n=5$). Each animal was tested separately in duplicate. Graph shows the average OD for each group of mice. The difference in O.D. between $pNogo_{1–979}$ and the control vector was not significant. (B) Time course of Nogo-A-specific serum IgG after vaccination with pSecNogo. (C) Nogo-specific serum IgG1 and IgG2a response 4 weeks after vaccination with pSecNogo, $pSecNogo_{1–979}$-GPI or control DNA. (D) Serum IgG response to MOG and MBP 4 weeks after vaccination with pSecNogo. For B–D, serum was pooled from 3 or more mice and assayed in triplicate. All data are representative of 2 independent experiments. Error bars represent standard deviation.

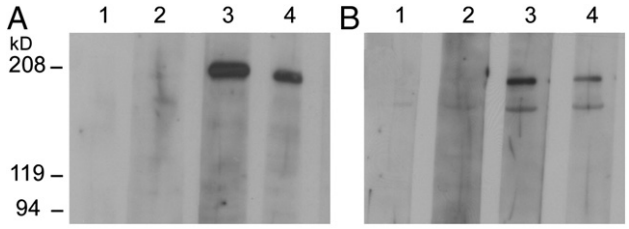

Fig. 2. Antisera of Nogo-A DNA-vaccinated mice detect Nogo-A on Western blot. Blot strips of CHO-Nogo-A (A) and mouse cerebellum (B) lysates were incubated with the diluted mouse antisera. Antisera of mice vaccinated with pSecNogo$_{1-979}$-GPI (lane 3) and pSecNogo (lane 4) recognize both the recombinant rat (A) and the endogenous mouse Nogo-A protein (B) at 190 kD, whereas antisera of mice vaccinated with control DNA (lane 1) and pNogo$_{1-979}$ (lane 2) do not.

On Western blots, antisera from both pSecNogo and pSecNogo$_{1-979}$-GPI vaccinated mice recognized the 190 kD form of recombinant rat Nogo-A expressed in a stably transfected Chinese hamster ovary (CHO) cell line (Fig. 2A), as well as endogenous Nogo-A in mouse cerebellar lysate (Fig. 2B). Serum from mice vaccinated with control DNA or with pNogo1–979 did not recognize either recombinant or endogenous Nogo-A (Fig. 2A, B). Immunohistochemistry of transfected CHO cells revealed that pSecNogo$_{1-979}$-GPI antiserum stained mainly the endoplasmic reticulum where recombinant Nogo-A is principally located in CHO cells (Fig. 3A). Background staining of untransfected CHO cells (Fig. 3A, arrow) was comparable to staining of transfected CHO cells with serum from control DNA-vaccinated mice (Fig. 3B). To assess whether the serum antibodies induced by DNA vaccination could recognize native Nogo-A expressed on the cell surface, we used oligodendrocyte cultures. The cell bodies and the process network of live, unpermeabilized oligodendrocytes can be stained with mAbs specific for inhibitory regions of Nogo-A, but not with control antibodies or with an antibody specific for an intracellular protein (Oertle et al., 2003). Incubation of live, unpermeabilized oligodendrocytes with pSecNogo$_{1-979}$-GPI antiserum led to cell surface staining (Fig. 3C), whereas control antiserum resulted in low background staining (Fig. 3D). This suggests that the anti-Nogo-A antibody response induced by DNA vaccination recognizes epitopes of the native protein exposed at the cell surface in vitro. Interestingly, an attempt to identify linear epitopes on an array of immobilized 15mer peptides as described previously (Zander et al., 2007) using serum obtained from mice vaccinated with the pSecNogo construct failed, suggesting that the immune response was exclusively directed against conformational epitopes.

Antibodies binding to myelin proteins can provide a target for antibody-dependent cell-mediated cytotoxicity and complement-dependent effector mechanisms (Piddlesden et al., 1993; Scolding and Compston, 1991). However, this can only occur in vivo if the blood-brain barrier is disrupted, as in the course of experimental autoimmune encephalomyelitis (Linington et al., 1988; Litzenburger et al., 2000; Schluesener et al., 1987). To assess whether Nogo-DNA vaccination can be encephalitogenic, we monitored clinical signs in mice vaccinated with either pNogo$_{1-979}$, pSecNogo, or pSecNogo$_{1-979}$-GPI. No signs of either neurologic or systemic disease were observed in any of the animals for up to six weeks after DNA vaccination, demonstrating that the immune response induced by DNA vaccination is not pathogenic in healthy recipients during this time. To determine

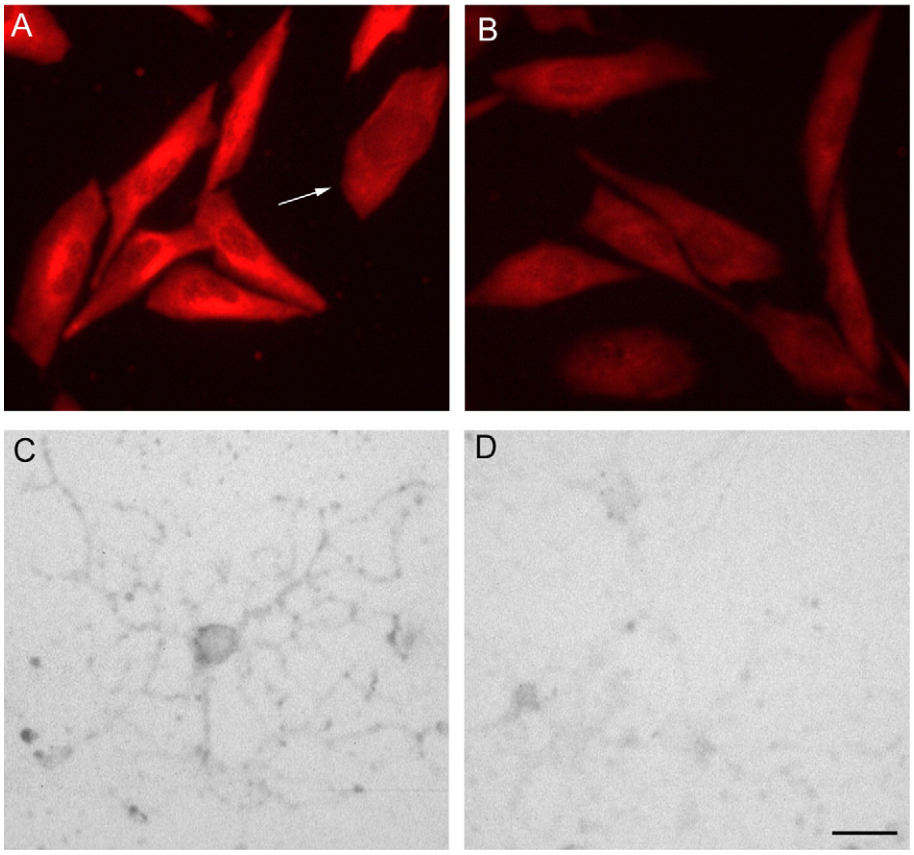

Fig. 3. Antisera of Nogo-A DNA-vaccinated mice detect cell surface and intracellular Nogo-A. A and B: CHO-Nogo-A and CHO-WT cells were plated mixed 1:1, permeabilized with TX-100 and stained with mouse antisera. Antisera of mice vaccinated with pSecNogo$_{1-979}$-GPI specifically stained CHO-Nogo-A cells with only low background staining of CHO-WT cells (arrow) (A). Background staining was comparable to that of cells stained with antisera of mice vaccinated with the control vector (B). C and D: Live, unpermeabilized oligodendrocytes were incubated with antisera and secondary antibody and assayed for alkaline phosphatase activity. Antisera of mice vaccinated with pSecNogo$_{1-979}$-GPI recognize endogenous Nogo-A at the cell surface of oligodendrocytes (C), whereas incubation of cells with antisera of control mice resulted in background staining (D). Scale bar 7.5 (m (A and B) and 16.5 (m (C and D).

whether this was also the case following disruption of the blood-brain barrier, we investigated the influence of Nogo-A-DNA vaccination on the clinical course of experimental autoimmune encephalomyelitis induced 4 weeks after DNA vaccination by the encephalitogenic myelin proteolipid protein (PLP) peptide 139–154. In SJL/J mice this immunization protocol results in a relapsing–remitting form of experimental autoimmune encephalomyelitis associated with inflammation and demyelination in the CNS (Tuohy et al., 1988).

As reported previously, vaccination with a DNA expression vector encoding murine MOG four to six weeks before the induction of experimental autoimmune encephalomyelitis greatly enhances disease severity (Bourquin et al., 2000). Strikingly, this was not the case when experimental autoimmune encephalomyelitis was induced in mice with a pre-existing Nogo-A-specific response. In mice vaccinated with either pSecNogo or pSecNogo$_{1-979}$-GPI, the course of disease was similar to that observed in animals vaccinated with the control vector (Fig. 4). The onset of experimental autoimmune encephalomyelitis occurred on average 10 days p.i. and the acute peak of disease followed 2–3 days later, after which the mice developed a chronic, relapsing–remitting disease course. In contrast, in mice vaccinated with DNA encoding murine MOG, disease onset occurred 2–3 days earlier than in control mice and the clinical course of experimental autoimmune encephalomyelitis was far more severe (Fig. 4).

4. Discussion

Vaccination with DNA encoding the antigen of interest is an effective technique for inducing highly specific antibodies. Here we show that a single vaccination with DNA constructs encoding either full length membrane-integral Nogo-A equipped with a signal peptide or its large N-terminal extramembrane region induces systemic production of Nogo-A-specific antibodies as early as 21 days after injection. The serum antibodies recognized recombinant, intracellular Nogo-A in cell culture but also stained native Nogo-A on the oligodendrocyte surface. Vaccination with DNA encoding an intracellular truncated form of Nogo-A did not result in production of anti-Nogo-A antibodies. This is in accordance with other DNA vaccination studies reporting that antibody responses induced by DNA encoding an intracellular form of an antigen are generally lower than responses induced by DNA encoding either the secreted or plasma membrane-bound form of the antigen (Drew et al., 2000; Inchauspe et al., 1997; Lewis et al., 1999). One advantage of DNA vaccination is the induction of long-term B cell responses, as shown for vaccination with MOG-DNA for which specific antibodies were still detectable 5 months after immunization (Bourquin et al., 2000).

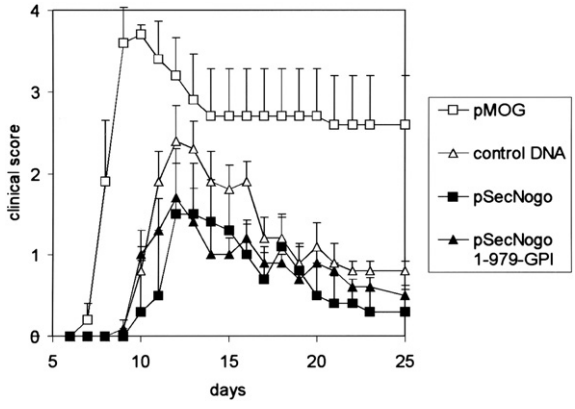

Fig. 4. Vaccination with Nogo-A DNA does not affect the course of EAE. Clinical course of EAE induced by immunization with PLP$_{139-154}$ 4 weeks after vaccination with Nogo-A DNA, MOG-DNA or control DNA. Whereas vaccination with pMOG results in severe exacerbation of EAE, vaccination with either pSecNogo or pSecNogo$_{1-979}$-GPI did not enhance EAE compared to vaccination with control DNA in either the acute or chronic phase of disease (day 12: pMOG, $p<0.05$, pSecNogo$_{1-979}$-GPI, pSecNogo, NS; day 20: pMOG, $p<0.01$, pSecNogo$_{1-979}$-GPI, pSecNogo-A, NS). Data are representative of 2 independent experiments (total number of mice: pMOG, $n=5$; pSecNogo$_{1-979}$-GPI, $n=10$; pSecNogo-A, $n=11$; control DNA, $n=16$). Error bars represent the standard error of the mean.

The therapeutic potential of Nogo-A neutralization following spinal cord injury has been a major focus of research in recent years. However, Nogo-A may also play a role in autoimmune demyelinating diseases such as multiple sclerosis. An increase of Nogo-A expression by oligodendrocytes was found in chronic demyelinating plaques (Satoh et al., 2005) and soluble Nogo-A is found in the CSF of patients with multiple sclerosis but not in patients with other neurologic diseases (Jurewicz et al., 2007). Furthermore, both serum and CSF antibodies to Nogo-A are found in patients with multiple sclerosis and acute neurological disorders (Reindl et al., 2003). In this study, we observed no signs of either neurologic or systemic disease in any of the animals for up to six weeks after DNA vaccination, demonstrating that the immune response induced by DNA vaccination is not pathogenic in healthy recipients. Furthermore, we assessed the pathogenic potential of DNA vaccination during the clinical course of experimental autoimmune encephalomyelitis. The tissue damage associated with this disease leads to opening of the blood-brain barrier, disruption of the myelin structure and may lead to exposure of hidden epitopes to the extracellular space. Even in this highly proinflammatory context, no increase in experimental autoimmune encephalomyelitis score was observed. This is in contrast to results obtained by vaccination with the myelin antigen MOG. While a MOG-specific response was not pathogenic in the absence of an encephalitogenic challenge, MOG-DNA vaccination exacerbated demyelination, inflammation and disease severity of experimental autoimmune encephalomyelitis (Bourquin et al., 2000).

An important factor for the pathogenicity of MOG-DNA vaccination is the presence of MOG-specific antibodies that have been shown to induce demyelination (Bourquin et al., 2000; Litzenburger et al., 2000). In the present study, despite induction of a Nogo-A-specific antibody response, no pathogenicity was seen following Nogo-DNA vaccination even in the context of an ongoing inflammatory response of the CNS. This may be explained by the inaccessibility of Nogo-A to extracellular antibodies. Most of Nogo-A is localized to the endoplasmic reticulum, but a small percentage of Nogo-A is detectable on the oligodendrocyte surface (GrandPre et al., 2000; Oertle et al., 2003; Voeltz et al., 2006). We therefore suspect that the absolute density of Nogo-A exposed at the surface of myelin, while sufficient to inhibit axonal outgrowth, is too low to obtain effective cross-linking and complement activation in vivo and thus to generate a pathogenic response.

The accompanying T-cell response may play a protective role following DNA vaccination. Vaccination with DNA encoding myelin antigens can result in partial suppression of the inflammatory T-cell response, thus protecting from experimental autoimmune encephalomyelitis (Lobell et al., 2003; Lobell et al., 1998; Ruiz et al., 1999). Different mechanisms were identified, including elicitation of a Th1 response (Lobell et al., 2003) and modulation of costimulation (Ruiz et al., 1999). In the present study, a mixed Th1/Th2 response was observed following DNA vaccination. It is conceivable that in this case the absence of a strongly polarized Th1 response may prevent encephalogenicity. DNA vaccination can also induce regulatory T cells that protect from autoimmunity in an animal model of uveitis (Silver et al., 2007). In the present study, regulatory T cells in the blood were however not increased (data not shown). T cells induced by vaccination with Nogo-A peptides following spinal cord injury can also be neuroprotective, as demonstrated by passive transfer of Nogo-A-specific T cells (Hauben et al., 2000; Hauben et al., 2001). In addition, vaccination with the Nogo peptide 623–640 decreased demyelination in experimental autoimmune encephalomyelitis induced by MOG peptide (Karnezis et al., 2004). This effect was probably due to a shift in the phenotype of encephalitogenic MOG-

specific T cells from Th1 to Th2. Interestingly, the effect of T-cell responses to Nogo-66 is variable: responses against some epitopes of Nogo-66 are associated with suppression of ongoing experimental autoimmune encephalomyelitis, whereas other epitopes can be encephalitogenic (Fontoura and Steinman, 2006). It is thus possible that Nogo-A vaccination may in some cases induce a neuroprotective T-cell response that counterbalances any deleterious effects that may be mediated by the humoral arm of the immune system.

While the role of Nogo-A in restricting axonal regeneration is well documented, Nogo-A is one of a number of myelin-associated inhibitors of axonal growth. Thus, DNA vaccination might be used to immunize against a combination of epitopes from different inhibitory proteins. In each case, a similar evaluation of the pathogenicity of specific antibodies is however imperative, as illustrated by the demyelinating potential of antibodies specific for the myelin-associated glycoprotein (MAG), another neurite outgrowth inhibitory protein (Domeniconi et al., 2002; Hays et al., 1987; Mukhopadhyay et al., 1994). Furthermore, DNA vaccination might be combined with additional nucleic acid patterns, such as DNA or RNA oligonucleotides, that function as adjuvants (Bourquin et al., 2007; Heckelsmiller et al., 2002).

In summary, we demonstrate that DNA vaccination provides a safe and efficient method to induce an autoantibody response to Nogo-A even during an ongoing inflammatory process within the CNS. These findings provide novel and important information for the use and safety of DNA vaccination for inducing Nogo-A-specific antibodies to enhance spinal cord repair.

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