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A Virus-Like Particle-Based Anti-Nerve Growth Factor Vaccine Reduces Inflammatory Hyperalgesia: Potential Long-Term Therapy for Chronic Pain

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Chronic pain resulting from inflammatory and neuropathic disorders causes considerable economic and social burden. For a substantial proportion of patients, conventional drug treatments do not provide adequate pain relief. Consequently, novel approaches to pain management, involving alternative targets and new therapeutic modalities compatible with chronic use, are being sought. Nerve growth factor (NGF) is a major mediator of chronic pain. Clinical testing of NGF antagonists is ongoing, and clinical proof of concept has been established with a neutralizing mAb. Active immunization, with the goal of inducing therapeutically effective neutralizing autoreactive Abs, is recognized as a potential treatment option for chronic diseases. We have sought to determine if such a strategy could be applied to chronic pain by targeting NGF with a virus-like particle (VLP)-based vaccine. A vaccine comprising recombinant murine NGF conjugated to VLPs from the bacteriophage Qβ (NGFQβ) was produced. Immunization of mice with NGFQβ induced anti-NGF–specific IgG Abs capable of neutralizing NGF. Titers could be sustained over 1 y by periodic immunization but declined in the absence of boosting. Vaccination with NGFQβ substantially reduced hyperalgesia in collagen-induced arthritis or postinjection of zymosan A, two models of inflammatory pain. Long-term NGFQβ immunization did not change sensory or sympathetic innervation patterns or induce cholinergic deficits in the forebrain, nor did it interfere with blood-brain barrier integrity. Thus, autovaccination targeting NGF using a VLP-based approach may represent a novel modality for the treatment of chronic pain. ***The Journal of Immunology*****, 2011, 186: 000–000.**

Chronic pain is a highly debilitating condition with multiple etiologies and pathophysiologies. It is a serious health problem that affects ~20% of European and United States adult populations (1–4). Current therapies provide adequate relief to <30% of those suffering chronic pain (2, 3, 5). The two most widely used classes of analgesic drugs, nonsteroidal anti-inflammatory drugs and opioids, are limited in their efficacy and tolerability. Furthermore, their long-term administration is accompanied by serious side effects (1). Therefore, it is generally acknowledged that additional approaches to managing chronic pain are needed.

Chronic pain may be nociceptive, neuropathic, or a combination of both. Neuropathic pain is a result of damage or improper functioning of the nervous system due to disease or trauma (6). Nociceptive pain arises from the stimulation of specialized sensory nerve fibers, so-called nociceptors. The excitability of these nociceptors can be enhanced by endogenous mediators such as bradykinin, histamine, 5-hydroxytryptamine, neuropeptides, PGs (PGEs), protons, potassium, ATP, proinflammatory cytokines, and NGF released during tissue injury, metabolic stress, and inflammation (7).

NGF is a member of the neurotrophin family expressed in the CNS during embryonic development. It acts as a trophic factor supporting the expansion and survival of peptidergic sensory and sympathetic neurones (8). In the adult, increases in NGF during inflammation stimulate the expression of proteins functionally important in pain perception. These include receptors and voltage-gated ion channels in nociceptors (9). Research conducted in the last two decades has established an important role for NGF in a number of persistent pain states, most notably those associated with inflammation (10–12).

NGF is produced by various peripheral cells including keratinocytes, epithelial cells, smooth muscle cells, and Schwann cells and by mast cells and macrophages during inflammation (1). It is synthesized as a precursor (pro-NGF) that is proteolytically cleaved by intracellular proprotein convertases. The mature form of NGF is secreted and interacts with two types of receptors that activate different sets of signaling pathways: the tropomyosin-receptor kinase (TrkA) and the pan-neurotrophin $p75^{NTR}$ receptor (1). NGF is thought to mediate hyperalgesia via its interaction

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Abbreviations used in this paper: AUC, area under the curve; BBB, blood-brain barrier; CGRP, calcitonin gene-related peptide; ChAT, choline acetyltransferase; CIA, collagen-induced arthritis; CX, cortex; DBB, diagonal bands of Broca; HC, hippocampus; LY, lysates; MS, medial septum; NGF, nerve growth factor; NGFQβ, nerve growth factor conjugated to virus-like particles from the bacteriophage Qβ; PBS-T, PBS + 0.5% Tween; RA, rheumatoid arthritis; RT, room temperature; SCG, superior cervical ganglion; smNGF, nerve growth factor purified from murine submaxillary glands; SN, supernatants; TrkA, tropomyosin-receptor kinase; TRPV1, transient receptor potential vanilloid receptor 1; VLP, virus-like particle.

with the high-affinity TrkA receptor (13, 14). Engagement of the TrkA receptor leads to phosphorylation of the transient receptor potential vanilloid receptor 1 (TRPV1), an ion channel with an important role in hyperalgesia (15). Phosphorylation of TRPV1 increases its basal activity and promotes trafficking and insertion of TRPV1 containing vesicles into the cell membrane. In the longer term, proteins including sodium channels, such as Nav1.8 and acid-sensing ion channel 3, brain-derived neurotrophic factor, calcitonin gene-related peptide (CGRP), and substance P and TRPV1 itself are induced (7). These proteins further facilitate activation and excitability of nociceptors and second-order neurons in the CNS. During tissue injury and inflammation, NGF also triggers the release of pain mediators from mast cells, such as bradykinin, 5-hydroxytryptamine, histamine, and PGE_2 and NGF itself. This results in a positive-feedback loop that reinforces the sensitization of nociceptors (16).

Injection of NGF results in the development of rapid and persistent pain and hyperalgesia (12, 17, 18). Elevated NGF levels have been detected in a number of chronic pain states in humans, including arthritis, cystitis, prostatitis, and chronic headaches (19–24). Recognition of NGF as a mediator of pain and its involvement in persistent pain in adults has stimulated the development of numerous biologics and pharmaceuticals that antagonize its activity (5, 11). Neutralization of NGF by specific Abs has been shown to decrease thermal and mechanical hyperalgesia in animal models of acute and chronic pain that include: cutaneous injection of CFA (17, 25), arthritis-associated pain (26), and bone cancer (27). A phase II clinical trial with the anti-NGF mAb tanezumab demonstrated suppression of osteoarthritic pain (28). An acceptable safety profile in >675 treated patients has seen clinical testing expand into numerous chronic pain indications and phase III trials (29).

Chronic pain in humans is generally defined as a condition that lasts for periods of 6 mo or more. Effective treatment would hence require continuous sequestration of NGF. Active vaccination to induce neutralizing anti-NGF Abs is an approach with the potential to achieve this goal. Induction of enduring neutralizing anti-NGF Abs titers could obviate the need for frequent administrations of expensive mAbs or pharmaceuticals. To test the feasibility of this concept, we generated a vaccine comprising mature NGF covalently conjugated to a virus-like particle (VLP) carrier derived from the bacteriophage Qβ. We have previously validated this approach both preclinically and clinically for other Ags (30–35).

In this study, we report preclinical efficacy and preliminary safety of the vaccine NGF conjugated to VLPs from the bacteriophage Qβ (NGFQβ). Induced Abs proved to neutralize NGF and suppress hyperalgesia in different rodent models of acute and chronic inflammatory pain.

Materials and Methods

Mice

DBA1 and C57BL/6 mice were purchased from Harlan Netherlands (Horst, The Netherlands). All mice were maintained under specific pathogen-free conditions and used for experimentation according to protocols approved by the Veterinary Office of the Kanton of Zurich (Zurich, Switzerland).

Cloning, expression, and purification of murine NGF

The nucleotide sequence encoding aa 19–241 of mouse pro-NGFβ and an additional 9 aa extension at the C terminus comprising a hexahistidine-tag and GGC sequence was ligated into the eukaryotic expression vector pCB28 in frame with the signal sequence of the κ L chain of γ Ig-derived of the pSECTag2/Hygro A,B,C vector (Invitrogen, Carlsbad, CA). The resulting plasmid, NGFpCB28, encodes for the signal sequence followed by the pro-NGFβ sequence, a His_6 tag, and a linker containing two glycines and a cysteine at the C terminus. HEK 293T cells were transfected with NGFpCB28, and supernatants containing processed mature His_6-tagged NGF were harvested and NGF purified by Ni^{2+} affinity purification.

NGF sandwich ELISA

Mouse anti-mouse NGF mAb (Chemicon International, Temecula, CA) was diluted in carbonate buffer (0.1 M $NaHCO_3$ [pH 9.6]) to a concentration of 1 μg/ml and coated overnight at 4°C on microtiter wells. After blocking with 2% BSA in PBS + 0.5% Tween (PBS-T), plates were incubated for 2 h at room temperature (RT) with increasing concentrations of rNGF or NGF purified ex vivo from submaxillary glands (AbD Serotec, Düsseldorf, Germany) in PBS-T + 2% BSA. Then, plates were washed six times with PBS-T and incubated with a 1:500 dilution of a sheep anti-NGF polyclonal Ab (AbD Serotec) for 1 h at RT. After another six washing steps with PBS-T, a 1:1000 dilution of HRP-labeled rabbit anti-sheep Ab (Chemicon International) was applied for 1 h at RT. After six final washes, the enzymatic reaction was started by adding 100 μl substrate solution (0.4 mg/ml 1,2 phenylene diamine dihydrochloride [Sigma-Aldrich, St. Louis, MO] and 0.01% H_2O_2 in 66 mM NaH_2PO_4 and 35 mM citric acid [pH 5]) to all wells. The color reaction was stopped with 5% H_2SO_4 and absorbance measured at 450 nm using an ELISA reader (Bio-Rad, Hercules, CA).

NGF receptor binding assay

The rat TrkA receptor (R&D Systems, Minneapolis, MN) was diluted in carbonate buffer (0.1 M $NaHCO_3$ [pH 9.6]) to a concentration of 1 μg/ml and coated overnight at 4°C onto microtiter wells. After blocking, plates were incubated with increasing concentrations of recombinant or ex vivo-purified NGF as described above. Receptor-bound NGF was detected with a sheep anti-NGF polyclonal Ab as described above.

To test the capacity of induced Abs to inhibit the interaction of NGF with its receptor TrkA sera of mice immunized with Qβ or NGFQβ were collected and total IgG fractions isolated from sera by protein G (Amersham Biosciences, Piscataway, NJ) purification. NGF was biotinylated at its free sulfhydryl groups with Maleimide-PEG11-Biotin (Pierce, Rockford, IL) and biotinylated NGF preincubated for 1 h at 37°C with increasing concentrations of purified Abs. The biotinylated NGF was then given to the plate coated with TrkA receptor and incubated for 2 h at RT. After six washes with PBS-T, a 1:1000 dilution of HRP-labeled streptavidin (Jackson ImmunoResearch Laboratories, West Grove, PA) was added for 1 h at RT. After six final washing steps, the enzymatic reaction was started as described above.

To test the capacity of TrkA binding to NGF covalently attached to Qβ particles, 1 μg/ml mouse anti-Qβ mAbs (Cytos Biotechnology, Schlieren, Germany) were coated onto ELISA plates as described. After binding of different concentrations of VLPs to the immobilized Ab, exposed NGF was detected with 1 μg/ml rat TrkA containing a human IgG Fc fusion. Bound receptor was detected with a 1:1000 dilution of HRP-labeled goat anti-human IgG (Jackson ImmunoResearch Laboratories).

NGF bioactivity assay

NGF bioactivity was assessed by measuring proliferation of the erythroblastoma cell line TF-1 (American Type Culture Collection, Manassas, VA) in response to increasing concentrations of NGF. Briefly, 10^4 TF-1 cells were seeded in 100 μl DMEM medium (supplemented with 10% FCS, 10 mM HEPES, 1% penicillin/streptomycin, and 1% glutamax) per well of a 96-well flat-bottom plate. Increasing concentrations of rNGF or ex vivo-purified NGF was added to the cells. After 40 h, cells were labeled with BrdU labeling reagent (Roche Diagnostic Systems, Somerville, NJ), which is incorporated into proliferating cells. Twenty-four hours later, cells were fixed and subsequently incubated with a peroxidase-conjugated anti-BrdU mAb (Roche Diagnostic Systems). After extensive washing, 100 μl tetrabenzyl-benzidine substrate solution was given to each well. The color reaction was stopped with 5% H_2SO_4 and absorbance measured at 450 nm. To test the in vitro neutralizing activity of Abs raised by immunization with NGFQβ, sera of mice immunized with Qβ or NGFQβ were collected and total IgG purified as described above. The capacity of purified total IgGs to neutralize the bioactivity of NGF was tested by incubating a constant concentration of 1 ng/ml NGF purified from submaxilliary glands with increasing concentrations of purified total IgGs for 1 h at RT. The NGF was then added to the cells, and cell proliferation was quantified by BrdU incorporation.

Production of NGFQβ conjugate vaccine

VLPs of the bacteriophage Qβ were reacted for 30 min with a 5-fold molar excess of the heterobifunctional cross-linker succinimyl-6-(β-maleimidopropionamido)hexanoate (Pierce) at RT. Reaction products were dialyzed against two changes of coupling buffer (20 mM MES, 300 mM

NaCl, 10% glycerol [pH 6]) for 2 h at RT. Qβ-VLPs derivatized in such a way were then used for coupling to the target protein. Precoupling, purified NGF was reduced for 1 h at RT with a 5-M excess of tri(2-carboxyethyl) phosphine hydrochloride (Pierce). Reduced NGF was incubated with derivatized Qβ for 4 h at 4˚C. To estimate the coupling efficiency, conjugated NGF-VLPs were analyzed by anti-His tag Immunoblot using anti-penta His-specific Ab (Qiagen, Valencia, CA).

Immunogenicity tests

Male DBA/1 mice were immunized s.c. with 50 μg Qβ VLPs coupled to NGF three times in the absence of any further adjuvant. As negative controls, mice were immunized with Qβ-VLPs alone. After each immunization, blood was taken. Serum was prepared by spinning the blood samples in serum tubes (Microtainer, BD Biosciences, San Jose, CA) at 10,000 × *g* for 10 min. Detection of NGF-specific Abs in serum samples was done by ELISA using tag- and linker-free NGF purified from submaxillary glands of male mice for coating. Briefly, NGF was diluted to a concentration of 2.5 μg/ml in carbonate buffer (0.1 M $NaHCO_3$ [pH 9.6]) and coated overnight at 4˚C on microtiter wells. After blocking with 2% BSA in PBS-T, plates were incubated for 2 h at RT with serum samples diluted in PBS-T + 2% BSA. Then plates were washed six times with PBS-T and bound Ab detected with a 1:1000 dilution of a peroxidase-conjugated goat anti mouse IgG-Fc Ab (Jackson ImmunoResearch Laboratories). Titers are expressed as those serum dilutions that lead to half-maximal OD450 (OD50).

Zymosan A-induced inflammatory pain model

The ability of the NGFQβ vaccine to reduce inflammatory hypersensitivity in response to thermal and mechanical stimulation caused by the injection of the yeast extract zymosan A (Sigma-Aldrich) into the plantar side of hind paws of C57BL/6 mice was evaluated in the following way.

C57BL/6 mice were immunized three times with 50 μg NGFQβ or Qβ alone. Seven days after the last immunization inflammatory pain was induced by injection of 20 μl 3 mg/ml solution of zymosan A in 0.9% NaCl into the plantar side of the left hind paw. Hypersensitivity in response to thermal and mechanical stimulation, which arises shortly postonset of the zymosan A-induced inflammation, was determined in the following way: at each time point, thermal hypersensitivity of the plantar side of both hind paws was determined in response to thermal and mechanical stimulation. For determination of thermal sensitivity, the latency of paw withdrawal poststimulation with a heat source of defined intensity (infrared beam) was measured. Latency was determined with an electronically controlled instrument (Plantar Test, Ugo Basile, Collegeville, PA). The intensity of the heat source was adjusted so that it yielded a latency of ~12–16 s in naive mice. For each hind paw and time point, six to eight measurements were taken and mean values calculated. To assess mechanical sensitivity, we determined the force that was needed to elicit a paw withdrawal in response to stimulation with dynamic von Frey Filaments (IITC Life Science, Woodland Hills, CA). All behavioral assessments were done in a blind fashion, meaning that the experimenter did not know the treatment status of the mice under investigation.

Collagen-induced arthritis model

The ability of the NGFQβ vaccine to reduce cachexia and pain in autoimmune arthritis was evaluated in a mouse model of rheumatoid arthritis (RA), so-called collagen-induced arthritis (CIA). In this model, RA was induced by intradermal injection of collagen type II (MD Biosciences, St. Paul, MN) in CFA followed by an intradermal injection of collagen type II in IFA 21 d later. The inflammation progresses steadily and culminates in ankylosis and permanent joint destruction accompanied by weight loss.

Male DBA/1 mice were immunized three times with 50 μg NGFQβ; as a negative control mice were immunized only with Qβ. Seven days after the last immunization, RA was induced. The inflammatory process was monitored over 7–9 wk, and clinical scores were assigned to each limb according to the following definitions: 0, normal; 1, mild erythema and/or swelling of digits/paw; 2, erythema and swelling extending over whole paw/joint; and 3, strong swelling and deformation of paw/joint with ankylosis. Hypersensitivity in response to thermal stimulation was determined as described above. All behavioral assessments were done in a blind fashion, meaning that the experimenter did not know the treatment status of the mice under investigation.

Immunohistochemistry

Immunostainings were performed in adult female C57BL/6 mice either immunized with NGFQβ or Qβ alone. Brains, spinal cords, superior cervical ganglia (SCG), and hind paws were prepared from mice deeply anesthetized with nembutal (50 mg/kg, i.p.) and intracardially perfused with 4% paraformaldehyde. All tissues were postfixed in 4% paraformaldehyde, and spinal cords, ganglia, and brains were subsequently cryoprotected in 30% sucrose overnight, whereas hind paws were incubated for 4 d in 10% EDTA for decalcification as described (36).

CGRP-positive structures were analyzed as described (37) in 40-μm-thick transverse hind paw sections taken at the level of metatarsal bones and in 14-μm-thick coronal lumbar spinal cord sections incubated overnight with a primary rabbit anti-CGRP Ab (AB15360; Chemicon International) in Tris-buffer at 4˚C, followed by a secondary Alexa 488 goat anti-rabbit Ab (A11008; Molecular Probes, Eugene, OR) for a 1 h RT incubation; 14-μm-thick ganglia sections and brains were stained to assess the state of the sympathetic cerebrovascular innervation. Ganglia were stained with primary rabbit anti-DBH (DiaSorin, Saluggia, Italy) and secondary Alexa 488 goat anti-rabbit Ab for quantification of the total surface area. Cerebral blood vessels were stained with Alexa 488-conjugated isolectin B4, which binds to endothelial cells, and their sympathetic innervation was visualized through counterstaining with primary rabbit anti-tyrosine hydroxylase (AB152; Chemicon International) and secondary Cy3 goat anti-rabbit Abs (78136; Jackson ImmunoResearch Laboratories).

Choline acetyltransferase (ChAT)-positive cholinergic neurons were quantified in medial septum and diagonal bands of Broca of the basal forebrain. Free-floating 40-μm-thick coronal brain sections were incubated with a primary rabbit anti-ChAT Ab (AB143; Chemicon International) and secondary biotin goat anti-rabbit Ab (111-065-003; Jackson ImmunoResearch Laboratories) after preincubation in avidin (1%)–biotin (1%) complex (VectaStain, Vector Laboratories, Burlingame, CA). Substrate diaminobenzidine was used for peroxidase-catalyzed visualization of immunoreactive cells.

A separate set of NGFQβ- or Qβ-vaccinated mice was used to test for a potential leakage of the blood-brain barrier (BBB). Extravasation of peripheral blood fibrinogen was used as marker of BBB impairment as described (38). Sections were prepared as described above for ChAT stainings; extravascular fibrinogen was stained with a rabbit anti-fibrinogen Ab (A0080; DakoCytomation, Carpinteria, CA) and detected as described in the ChAT staining. Mice with known BBB leakage caused by either kainate-induced temporal lobe epilepsy or by injection of 1.6 mol/l mannitol (200 μl) in the tail vein (39) were included as positive controls.

Quantification

CGRP-positive structures were quantified in the spinal dorsal horn and hind paw skin of five immunized and five control mice. Eight sections per mouse were analyzed using ImageJ software (National Institutes of Health, Bethesda, MD), and CGRP-positive structures were either quantified automatically (spinal cord) or counted manually (hind paw skin dermis; area counted was 120 μm below epidermis covering 240 μm per section in length). The total DBH-positive area of the ganglia was quantified using ImageJ software (National Institutes of Health). ChAT immunoreactive basal forebrain neurons of medial septum and diagonal bands of Broca were counted in an area of 2 mm^2 per section in four sections at 200 μm intervals as described (40) between Bregma 0.38–1.34 mm using ImageJ software (National Institutes of Health). All quantifications were performed on randomized sample images arranged by an observer blinded to the treatment of the mice. Statistical analysis was done with GraphPad Prism 4 (GraphPad, San Diego, CA).

Results

Production of an rNGF-VLP conjugate vaccine

Murine pro-NGF, containing a 9 aa extension at the C terminus comprising a hexahistidine-tag and GGC sequence, was expressed in HEK293T cells. The terminal cysteine was introduced into the construct to enable covalent conjugation of NGF to the surface of the VLP via the heterobifunctional chemical cross-linker succinimyl-6-(β-maleimidopropionamido)hexanoate. Immunoblot analysis of HEK cells transiently transfected with NGFpCB28 showed expression of the 29-kDa pro-NGF and a higher molecular mass glycosylated form (Fig. 1*A*). Analysis of cell-culture medium showed the presence of a 14.7-kDa band corresponding to mature NGF. A fainter 20-kDa band previously described by others for cell lines constitutively secreting NGF was also observed (41). Edman analysis of the secreted mature NGF showed the expected N-terminal sequence, which confirmed the mature protein was correctly processed (data not shown). NGF was purified from culture

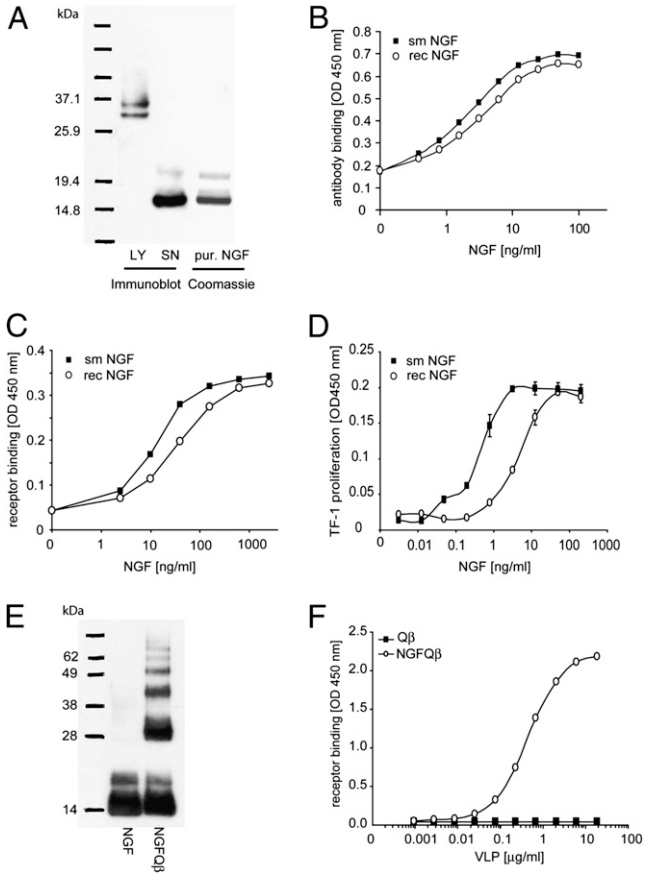

FIGURE 1. Production and functional analysis of the rNGFQβ vaccine. *A*, Production of rNGF. Murine NGF was expressed in HEK293T cells. Antipentahistidine immunoblot analysis of HEK293T lysates (*first lane*) and cell-culture supernatants (*second lane*) revealed expression of the NGF proprotein by HEK293T cells and secretion of the processed 14.7-kDa mature form of NGF into the cell-culture supernatant. Coomassie staining of NGF purified from supernatants by affinity chromatography confirmed homogeneity (*third lane*). *B*, Recognition of NGF by anti-NGF Abs. Recombinantly expressed NGF (white circles) was compared with ex vivo-purified NGF (black squares). Different dilutions of both proteins were applied to ELISA plates coated with anti-NGF mAb. Bound NGF was detected with polyclonal anti-NGF Abs. Averages of triplicates ± SEM. *C*, NGF receptor binding. The NGF-specific receptor TrkA was coated onto ELISA plates and descending concentrations of both proteins applied. Receptor bound NGF was detected with polyclonal NGF Abs as in *B*. Averages of triplicates ± SEM. *D*, Bioactivity of NGF. The NGF responsive cell line TF-1 was stimulated with descending concentrations of NGF and proliferation determined by BrdU incorporation during DNA synthesis. Averages of triplicates ± SEM. *E*, Analysis of NGFQβ vaccine. NGF was cross-linked to VLPs with a heterobifunctional cross-linker. An immunoblot of NGF (*left lane*) or the NGFQβ conjugate vaccine (*right lane*) analyzed with an anti-pentahistidine–specific Ab showed a dominant band appearing at 29 kDa corresponding to a Qβ monomer (14.2 kDa) conjugated to one molecule NGF (14.7 kDa). Higher molecular mass bands correspond to Qβ-oligomers linked to one NGF molecule. *F*, Binding of TrkA to NGFQβ. Ascending concentrations of Qβ (filled squares) or NGFQβ (open circles) were immobilized on an ELISA plate coated with anti-Qβ mAb. Bound vaccine was incubated with TrkA receptor containing a human Fc domain. Binding of receptor to NGF displayed on VLP was detected with peroxidase-conjugated goat anti-human Abs. Averages of triplicates ± SEM. LY, lysates; SN, supernatants.

supernatants to homogeneity by immobilized metal ion affinity chromatography (Fig. 1*A*).

The authenticity of the purified rNGF Ag was further investigated by measuring its bioactivity and its ability to bind conformation-dependent mAbs and the TrkA receptor. For these analyses, NGF purified from murine submaxillary glands (smNGF) was used as a standard comparator. ELISA (Fig. 1*B*) demonstrated that both rNGF and smNGF were equally well recognized by two neutralizing NGF Abs. Binding to TrkA was also shown to be similar for both recombinant and smNGF (Fig. 1*C*). rNGF-induced proliferation of TF-1 cells with 10-fold lower bioactivity than smNGF (Fig. 1*D*). The relatively minor differences in receptor binding and bioactivity may reflect some interference from the C-terminal linker sequence. From these studies, we concluded the rNGF to be of a quality suitable for vaccine production and immunization studies.

NGF was rendered highly repetitive by chemical conjugation to the surface of the VLP Qβ. Immunoblot analysis of the coupling reaction revealed bands with molecular weights corresponding to NGF with one or more Qβ coat protein monomers (Fig. 1*E*). It was estimated by densiometric analysis there were ~60 NGF molecules per VLP. Further analysis of the vaccine demonstrated that NGF coupled to the VLP retained its ability to bind the TrkA receptor (Fig. 1*F*). These results show that NGF maintained its conformational integrity and accessibility to the receptor binding site even when displayed on the surface of the VLP.

Induction of NGF-neutralizing Abs by NGFQβ

Groups of mice were immunized s.c. with NGFQβ without the addition of adjuvant. By day 10, after only one immunization, NGF-specific IgG Abs were induced in all mice. Titers could be boosted by subsequent immunizations. Twenty days after the third immunization (day 40), the peak titer measured was 1:50,000. Then, immunization was discontinued and from day 40 onwards, the level of NGF-specific Abs declined (Fig. 2*A*).

To investigate whether the Abs induced by NGFQβ were neutralizing, an ELISA-based receptor-inhibition assay was performed. As shown in Fig. 2*B*, protein G-purified IgG from mice immunized with NGFQβ inhibited binding of NGF to TrkA, whereas IgG from mice immunized with Qβ alone did not. The neutralizing effect of the Abs was also demonstrated in a cell proliferation assay. Abs purified from NGFQβ-immunized mice inhibited proliferation of NGF-responsive TF-1 cells, whereas IgG from control mice failed to do so (Fig. 2*C*). From these results, we concluded that NGFQβ is able to overcome immune tolerance to NGF and induce NGF-specific Abs. Moreover, these Abs were able to efficiently neutralize the action of NGF in vitro.

Suppression of zymosan A-induced inflammatory pain

To investigate the ability of immunization against NGF to suppress pain in vivo, we tested NGFQβ in a rodent model of inflammatory hyperalgesia. Zymosan A injected into the plantar side of the hind paws of rodents causes local inflammation that fully develops within 6–8 h then slowly subsides over a period of 1 wk. Inflammation causes mice to exhibit increased sensitivity to thermal stimulation of the inflamed paw (thermal hyperalgesia) and reduced tolerance to punctuate mechanical stimulation (mechanical sensitization).

Groups of mice were immunized three times with either Qβ or NGFQβ. Measurement of NGF-specific Abs 1 wk after the last immunization showed titers of ~1:30,000. At this time point, inflammation was induced in the left hind paws by injection of zymosan A. Throughout the course of the experiment, the extent of inflammation was determined by measuring paw volume. Similar levels of inflammation were recorded for both control and test groups (data not shown). Sensitivity to mechanical and thermal stimulation was measured at various intervals after zymosan

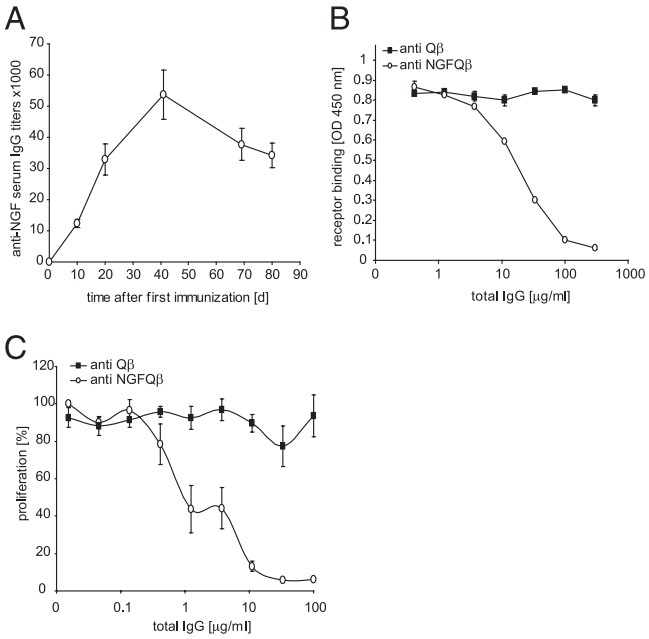

FIGURE 2. NGFQβ vaccination induces neutralizing Abs. *A*, Immunogenicity. Mice were immunized three times at days 0, 10, and 20 s.c. with 50 µg Qβ or NGFQβ and NGF-specific serum IgG titers were determined. Average titers ($n = 8$) ± SEM are shown. *B*, Induced Abs inhibit NGF receptor binding. IgGs were purified by protein G chromatography from sera of animals immunized with Qβ (filled squares) or NGFQβ (open circles). Total of 25 ng/ml biotinylated NGF was preincubated with increasing concentrations of purified Abs and applied to an ELISA plate coated with TrkA receptor. The extent of TrkA binding in the presence of IgGs was determined with peroxidase-conjugated streptavidin detecting receptor-bound NGF. Averages of triplicates ± SEM. *C*, Induced Abs neutralize the bioactivity of NGF. TF-1 cells were stimulated with 1 ng/ml of NGF, preincubated with increasing concentrations of IgGs purified from Qβ (filled squares) or NGFQβ (open circles) immunized animals. Shown is relative proliferation in percent of proliferation of cells stimulated with 1 ng/ml NGF without Abs. Averages of triplicates ± SEM.

A injection with dynamic von Frey aesthesiometer and a Hargreaves plantar test apparatus, respectively.

Within 7 h, during the acute phase of inflammation, the mechanical force tolerated by the inflamed paws rapidly declined from ~4 g to 1 g (Fig. 3*A*). During this time, there was only a minor difference in tolerance to stimulation between control and test groups. However, from 24 h onwards, a difference in tolerance to the applied mechanical force was measured. NGFQβ-immunized animals withstood significantly higher applied force than animals immunized with Qβ (area under the curve [AUC] 7–156 h; $p < 0.0001$). This effect was observed until day 7, but was no longer evident by day 14, at which time the zymosan A-induced inflammation had receded. Indeed, the results of three independent experiments showed statistically significant reductions in mechanical allodynia of up to 60% between days 1 and 7 (Fig. 3*B*).

As shown in Fig. 3*C*, reduced hyperalgesia to thermal stimulation was also observed for NGFQβ-immunized mice. The reduction in sensitivity occurred with similar kinetics to those observed for mechanical stimulation (AUC 7–96 h; $p < 0.05$). Again, assessment of independent experiments showed an average reduction in thermal hyperalgesia of the inflamed foot of up to 60% in the days following onset of inflammation (Fig. 3*D*).

Suppression of inflammatory pain in CIA

A second inflammatory pain model, arthritic hyperalgesia arising during collagen-induced arthritis, was used to further test the analgesic effects of NGFQβ. Groups of mice were immunized three times with either Qβ or NGFQβ. Once NGF-specific Ab titers had reached levels of ~25,000, arthritis was induced by sequential injections of collagen in CFA followed by IFA. Animals were monitored for the development of arthritis and clinical scores assigned to each hind and fore paw. Sensitivity to thermal stimulation was measured at various time intervals for a period of 60 d in hind paws.

Fig. 4*A* shows that average clinical scores and disease progression in hind paws were similar for both test and control groups. Disease incidence was also comparable (data not shown). However, a marked difference in sensitivity to thermal stimulation was noted (Fig. 4*B*). After onset of arthritis, a progressive decrease in latency from ~12 s (day 31) to 7 s (day 74) was measured in arthritic hind paws of animals from the control group. In contrast, during the same period, the average latency in arthritic paws of NGFQβ-immunized animals remained essentially unchanged (AUC 31–74 d; $p < 0.01$). To further examine the dependency of hyperalgesia on disease progression and the potential effect of NGFQβ vaccination, all latency values recorded for individual hind paws throughout the course of the experiment were grouped according to the clinical arthritis score of the corresponding paw at each time point (Fig. 4*C*). In the CIA model, it is common for one or more limbs not to develop inflammation in a proportion of animals. The paws from both control and NGFQβ immunized animals that failed to develop clinical signs of CIA showed no statistically significant difference in response to thermal stimulation. For paws of control animals, an increase in clinical score was associated with heightened sensitivity to thermal stimulus. Paws with clinical scores ranging from 0.5–1.0, 1.5–2.0, and 2.5–3.0 showed decreases in latency of 20%, 35%, and 45%, respectively. For NGFQβ-immunized animals, an increase in thermal hyperalgesia was only observed for paws with the highest clinical scores (2.5–3.0). The decrease in latency of 21% was similar to that observed in the paws of control animals, with scores ranging from 0.5–1.0.

Suppression of weight loss in CIA

NGF has also been implicated as a mediator of weight loss (26). Progressive unintentional weight loss, also known as cachexia, can be a clinical consequence of a chronic systemic inflammation, such as RA (42). Indeed, inhibition of NGF by anti-NGF mAbs has been reported to decrease rheumatoid cachexia in rats (26). Hence, we investigated the ability of immunization with NGFQβ to influence body weight in the CIA model. Animals were immunized and CIA induced as described above. Fig. 5*A* shows Qβ- and NGFQβ-immunized mice developed arthritis of a similar magnitude and over a similar time course. However, a statistically significant difference in average body weight between control and NGFQβ-treated mice was observed postonset of disease (Fig. 5*B*). Control mice failed to gain weight, whereas those treated with NGFQβ continued to gain weight during the course of the disease. This result suggests, as observed for anti-NGF mAb-treated rats, mice immunized with NGFQβ were similarly protected from cachexia.

Kinetics of Ab response and long-term effects of vaccination

An important safety requirement for therapeutic vaccines targeting endogenous molecules is that the Ab response they induce be reversible. To establish this, mice were immunized three times with NGFQβ over a period of 4 wk and the subsequent anti-NGF Ab response measured for ~11 mo (Fig. 6*A*). Over the course of the experiment, anti-NGF titers declined in all animals to ~10% of peak levels measured immediately postimmunization. The decline

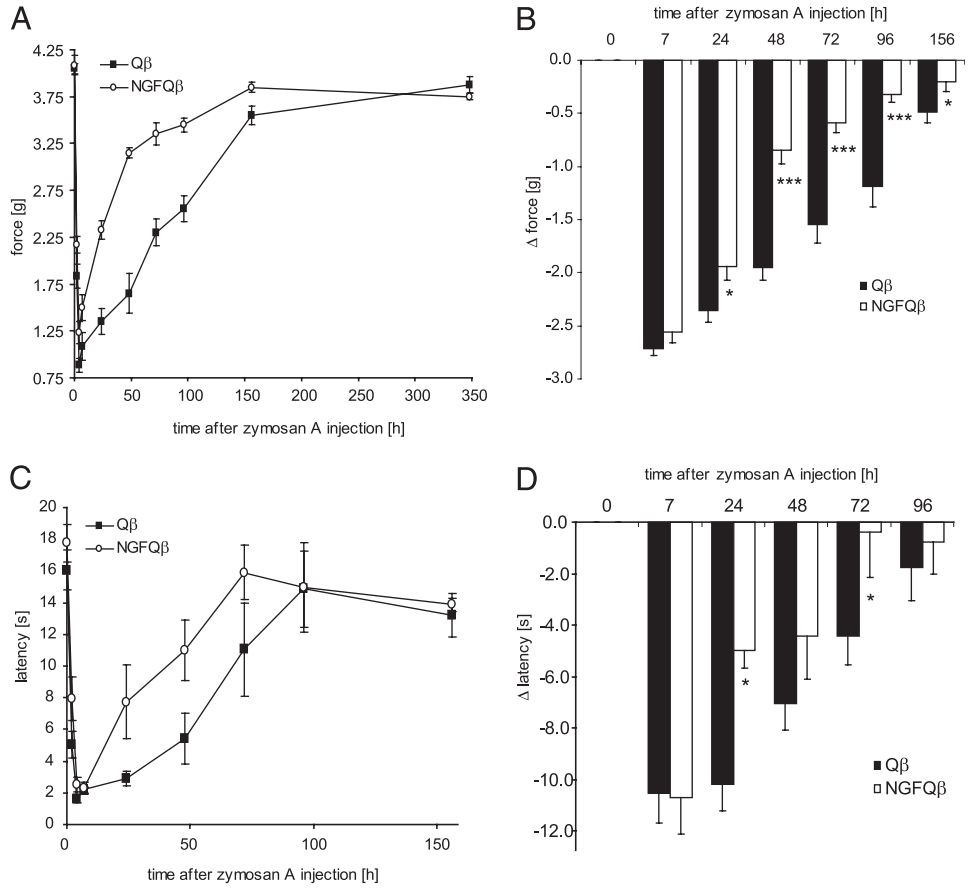

FIGURE 3. NGFQβ vaccination suppresses hyperalgesia in a zymosan A model of inflammatory pain. C57BL/6 mice ($n = 6$) were immunized with 50 μg Qβ or NGFQβ at days 0, 14, and 28. Seven days after the last immunization, inflammatory pain was induced by injection of 60 μg zymosan A in 20 μl saline into the left hind paw. *A*, Mechanical sensitization. At different intervals postinjection of zymosan A, tolerance toward mechanical stimulation was determined by applying a punctual stimulus with a von Frey filament. The tolerated force on the plantar side of the left hind paw is shown as mean ± SEM for mice immunized with Qβ (filled squares) or NGFQβ (open circles) (AUC 7–156 h). $p < 0.0001$. *B*, Average results of three independent experiments show the difference in tolerated mechanical tolerance at different time points versus baseline for mice immunized with Qβ (black bars) and NGFQβ (white bars). Average values ± SEM are shown. *C*, Thermal hyperalgesia. At different intervals postinjection of zymosan A, tolerance toward thermal stimulation was determined by applying heat to the plantar side of the foot with a electronically controlled heat source (plantar test). Latency before paw withdrawal is shown ± SEM for mice immunized with Qβ (filled squares) or NGFQβ (open circles) (AUC 7–96 h). $p < 0.05$. *D*, Average results of three independent experiments show the latency difference versus baseline at different time points for mice immunized with Qβ (black bars) and NGFQβ (white bars). Average values ± SEM are shown. Student *t* test: $*p < 0.05$, $***p < 0.001$.

in Ab titer was at first rapid with a $t_{1/2}$ of ~35 d, then reached a slower phase with a $t_{1/2}$ of ~160 d.

Although not expected for immunological reasons, we examined whether it was possible for endogenous NGF to boost the anti-NGF Ab response induced by vaccination with NGFQβ. Anti-NGF Abs were measured in NGFQβ-immunized mice postinjection of either buffer, NGFQβ, rNGF, or CFA, the latter being a potent inducer of inflammation that is known to induce NGF production in vivo (17, 25). Fig. 6*B* shows anti-NGF Abs could be boosted by administration of NGFQβ, but not by administration of rNGF or CFA.

The effect of maintaining high levels of neutralizing Abs over an extended period of time on the normal perception of mechanical pressure and thermal stimulus was investigated. Mice were immunized three times biweekly with Qβ or NGFQβ followed by further immunizations every 4–6 wk. In this fashion, anti-NGF Ab titers in the range of 20,000–40,000 were stably maintained for ~1 y (Fig. 6*C*). At day 286, the peripheral baseline response of animals to thermal and mechanical stimulation was assessed. Fig. 6*D* shows there was no significant difference in pain-related behavior after mechanical or thermal stimulation for control and test animals. Importantly, during this long-term study, there were no deaths and no adverse clinical signs noted. Moreover, there was no statistically significant difference in body weight and weight gain between both control and NGFQβ-immunized mice (data not shown).

Influence of NGFQβ vaccination on sensory neuron innervation

Previous reports on mice expressing anti-NGF Abs throughout embryonic development and adulthood from transgenes have raised concerns that long-term sequestration of NGF could lead to a loss of CGRP-positive sensory fibers and interfere with the integrity of the BBB (43, 44). The latter could allow penetration of anti-NGF Abs into the CNS, which could potentially result in a loss of cholinergic forebrain neurons. To address this, we compared CGRP-positive sensory nerve-fiber innervations of the spinal cord and skin of long-term NGFQβ-immunized mice with control animals. In addition, we analyzed BBB integrity, sympathetic neurons of the SCG and cholinergic neurons in the basal forebrain.

Mice were immunized every 2–4 wk for a period of ~5 mo with NGFQβ or Qβ VLP alone and anti-NGF titers measured. Four weeks after the last immunization, mice were sacrificed and analyzed. There was no statistically significant difference in the number of CGRP-positive nerve fibers in the dorsal horn outer

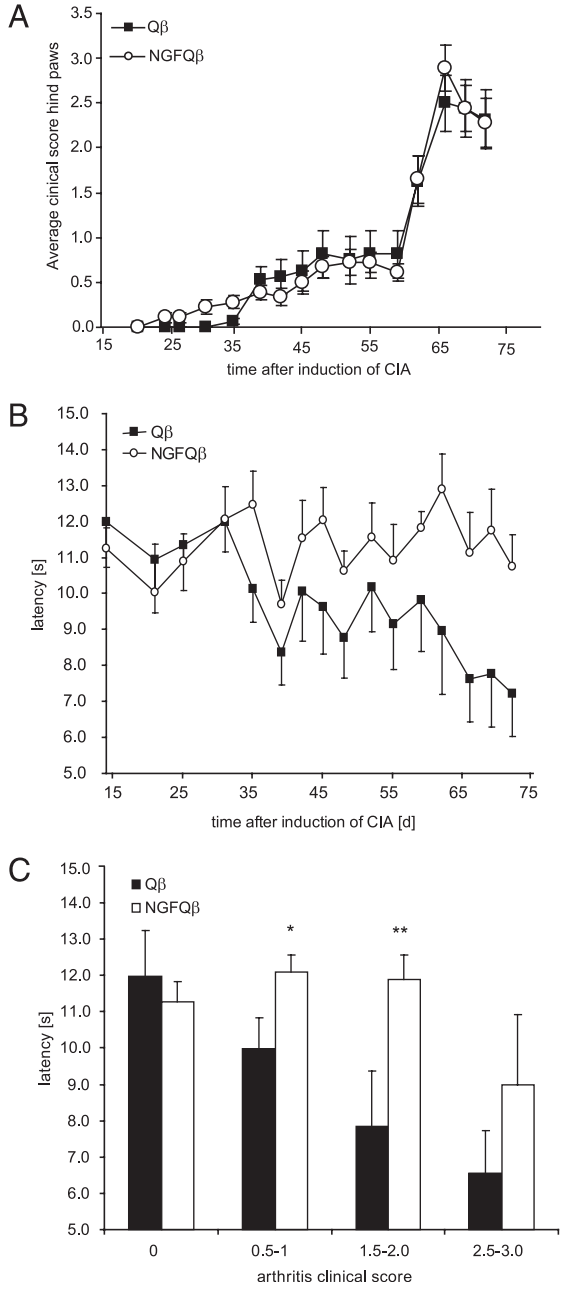

FIGURE 4. NGFQβ vaccination suppresses hyperalgesia in CIA. *A*, CIA clinical scores. Groups of mice were immunized with 50 μg Qβ (black squares) or NGFQβ (white circles) on days 0, 14, and 28. Disease was induced by injection of collagen in CFA followed by collagen in IFA 21 d later. Starting 20 d postinduction of disease, mice were monitored for clinical signs of arthritis, and scores from 0–3 were assigned to each limb according to the degree of reddening and swelling. Shown are mean values of cumulative clinical scores of hind paws per mouse with SEM. *B*, Thermal hyperalgesia time course. Starting 14 d after first induction of CIA, thermal hyperalgesia was assessed. Shown are averages in latency ± SEM (AUC postonset of disease at day 31). $p = 0.0036$. *C*, Thermal hyperalgesia versus clinical score. For mice of the same experiment as shown in *B*, latency is depicted as a function of arthritis scores. $*p < 0.05$, $**p < 0.01$.

lamina II, the main termination area of peptidergic sensory fibers in the spinal cord (Fig. 7*A*, 7*B*). We counted 330 ± 17 (mean ± SEM; $n = 5$ mice) CGRP-positive structures per dorsal horn section in NGFQβ-immunized mice compared with 348 ± 19 ($n = 5$) in control mice ($p = 0.61$, unpaired *t* test) (Fig. 7*E*). Anti-NGF titers in immunized mice varied between 14,500 and 275,000. There was no significant correlation between the number of CGRP-positive structures and anti-NGF titers ($r = -0.074$; $p = 0.906$, ANOVA). CGRP-positive nerve fibers in hind paw skin were similarly unaffected by immunization (Fig. 7*C*, 7*D*). There were 6.18 ± 0.61 CGRP-positive dermis fibers in NGFQβ-immunized mice compared with 5.8 ± 0.76 in control mice ($p = 0.71$). Again, there was no significant correlation of anti-NGF titers with the number of CGRP-positive fibers ($r = -0.58$, $p = 0.301$).

Influence of NGFQβ vaccination on BBB and basal forebrain cholinergic neurons

The intact BBB does not allow high molecular mass blood components, such as fibrinogen, or IgG to pass into the brain. Therefore, the integrity of the BBB was assessed by staining the brains of NGFQβ-immunized and control (Qβ-immunized) mice for fibrinogen (Fig. 8*A–C*) and mouse IgG (not shown). Neither fibrinogen nor mouse IgG could be detected in brains of either NGFQβ- or Qβ-immunized mice ($n = 4$ per group). In line with these results, brains of immunized mice did not show elevated CD68 immunoreactivity confirming the absence of elevated microglial activation, an early marker of BBB dysfunction (45). To validate our experiments, we included diseased mice with a known defect in the BBB and analyzed their brains. We investigated six mice with kainate-induced temporal lobe epilepsy (Fig. 8*C*) and two mice injected with 1.6 mol/l mannitol (200 μl) into the tail vein to permeabilize the BBB (39) (data not shown). As expected, all these mice showed intense intracerebral fibrinogen labeling throughout the cortex and hippocampus.

We further compared the sizes of superior cervical ganglia, which harbor the somata of the sympathetic neurons innervating the cerebral blood vessels to maintain BBB integrity, and verified that cerebral blood vessels retained their innervation by sympathetic nerve fibers. Both groups of mice showed similar sizes of SCG (Supplemental Fig. 1). The intactness of the sympathetic innervation of the cerebral blood vessels by the sympathetic nerve fibers was confirmed by double labeling of cerebral blood vessel endothelial cells with IB4 and sympathetic nerve endings with tyrosine hydroxylase. Cerebral blood vessels of both groups of mice showed comparable patterns of innervations by tyrosine hydroxylase-positive fibers (Supplemental Fig. 2).

NGF is an important survival factor for basal forebrain cholinergic neurons during development; thus, anti-NGF induced by vaccination with NGFQβ could affect the survival of these cells. We analyzed the numbers of cholinergic neurons in two areas of the basal forebrain, the medial septum, and the diagonal bands of Broca (Fig. 8*D*, 8*E*). Cholinergic neurons were identified by the presence of ChAT by neurostereological quantification in NGFQβ-immunized and control mice ($n = 5$ each). We counted on average 155 ± 15 ChAT-positive neurons per 2 mm^2 and section in control mice versus 144 ± 14 in NGFQβ-immunized mice ($p = 0.61$, unpaired *t* test; $n = 5$ each) (Fig. 8*F*). There was no correlation of the number of ChAT-positive neurons with anti-NGF titers ($r = -0.12$; $p = 0.848$).

Discussion

One of the most challenging problems in modern medicine is the treatment of patients with chronic pain. Chronic pain is said to be the most costly health problem in the United States. In Europe, it accounts for nearly 500 million working days lost each year and costs the European economy ~35 billion Euro annually (3). Almost one in five people in Europe and the United States suffer from chronic pain with an average duration of 7 y. Twenty percent of chronic pain patients suffer for >20 y. In two thirds of these subjects, pain is inadequately controlled with current drugs (3).

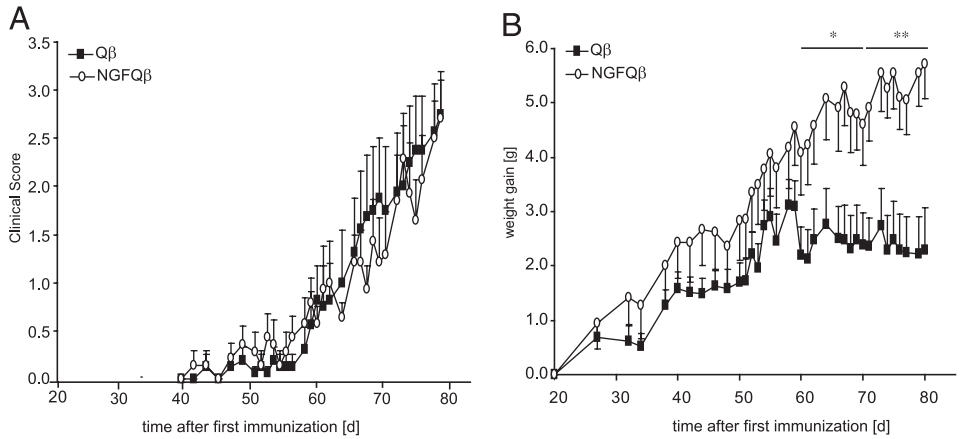

FIGURE 5. NGFQβ vaccination suppresses cachexia in CIA. *A*, CIA clinical scores. Groups of mice were immunized with 50 μg Qβ (black squares) or NGFQβ (white circles) on days 0, 10, and 20. Disease was induced by injection of collagen in CFA on day 27 followed by collagen in IFA 21 d later. Mice were monitored daily for clinical signs of arthritis, and scores from 0–3 were assigned to each limb according to the degree of reddening and swelling. Shown are mean values of cumulative clinical scores of all paws per mouse with SEM. *B*, Body weight in CIA. Average body weight of both groups of mice was assessed. Weight differences in comparison with body weight on the day of last immunization were calculated. For each day, weight gain of the NGFQβ-immunized group versus control group was analyzed by student *t* test. Significance is expressed as $*p < 0.05$, $**p < 0.001$.

Hence, the development of innovative analgesic drugs with novel mechanisms of action, and compatible with long-term use, represents one of the major goals for the pharmaceutical industry.

Toward this end, an anti-NGF vaccine comprising recombinant, mature NGF covalently coupled to the surface of VLPs derived from the bacteriophage Qβ was produced. Such conjugate VLP-

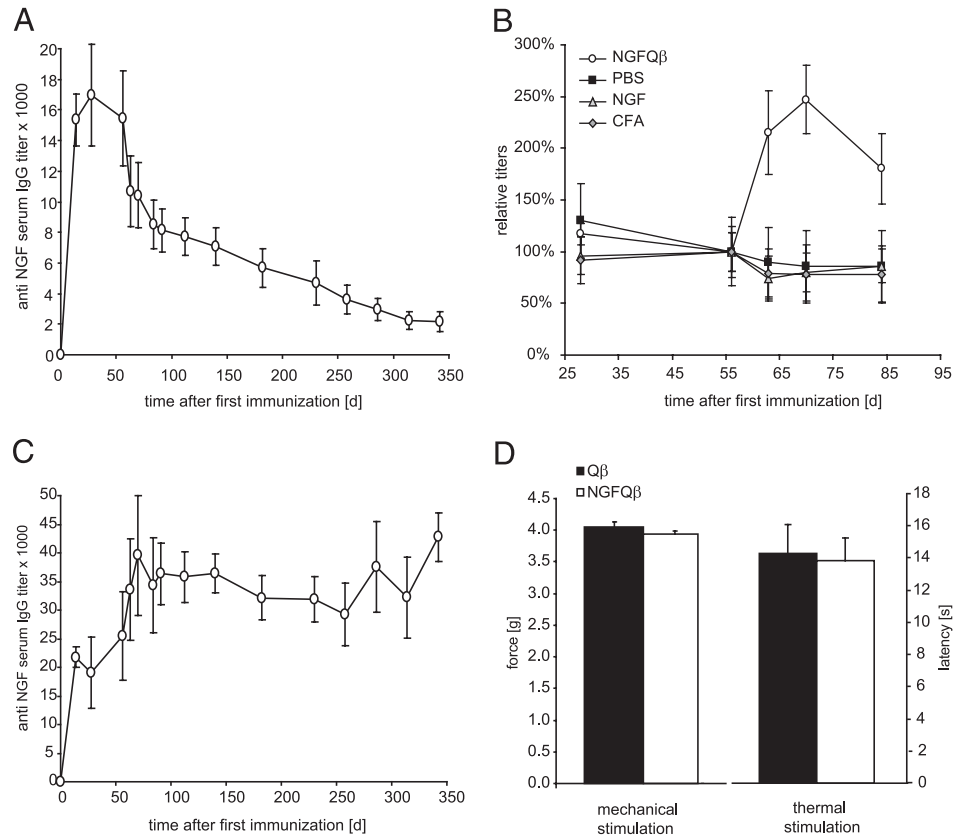

FIGURE 6. Long-term effects of NGFQβ vaccination. *A*, Titer reversibility. Female C57BL/6 mice ($n = 8$) were immunized with 50 μg NGFQβ s.c. at days 0, 14, and 28, then immunizations were discontinued. NGF-specific serum IgG titers were measured by ELISA over a period of ~1 y. Average titers (OD50 at 450 nm) ± SEM are shown. *B*, Endogenous boost. Female BL/6 mice were immunized as in *A*. At day 56, mice were immunized s.c. with either NGFQβ (50 μg in 200 μl buffer; open circles), PBS (200 μl; filled squares), NGF (50 μg in 200 μl buffer; gray triangles), or CFA (200 μl; gray diamonds). NGF-specific serum titers were then determined again at days 63, 70, and 84. Shown are titers ± SEM in percent relative to titers at day 56 for each group. *C*, Continuous immunization against NGF. Female BL/6 mice were immunized s.c. with 50 μg Qβ or NGFQβ at days 0, 14, and 28. In contrast to experiment A, immunizations were continued and mice injected with either Qβ or NGFQβ every 4–6 wk for a period of ~1 y. Titers were determined at the indicated time points. Shown are average titers (OD50 at 450 nm) ± SEM. *D*, Baseline sensitivity. For mice immunized as described in *C*, baseline sensitivity to mechanical and thermal stimulation was determined at day 286 after first immunization with Qβ (black bars) or NGFQβ (white bars). Shown is the average of left and right hind paws ± SEM.

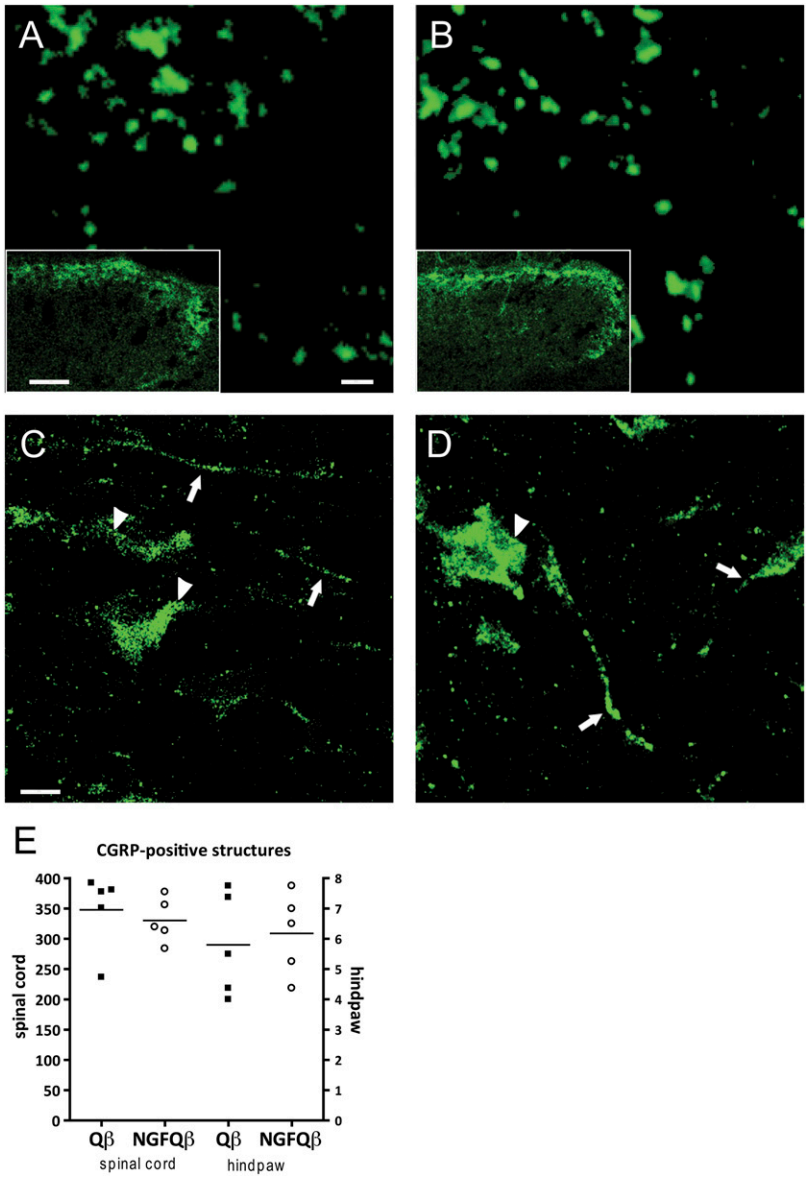

FIGURE 7. Innervation of lumbar spinal dorsal horn and hind paw skin by CGRP-positive sensory nerve fibers. CGRP-positive terminals in the lumbar spinal cord dorsal horn of Qβ- (*A*) and NGFQβ-immunized mice (*B*). Scale bar, 1 μm. *Insets* are lower magnification (scale bar, 50 μm). Transverse skin sections obtained from the metatarsal level of the hind paw dermis of Qβ-immunized control mice (*C*) and of NGFQβ-immunized mice (*D*). Scale bar, 5 μm. Arrows indicate CGRP-positive fibers. CGRP-positive cell bodies (arrowheads) are probably keratinocytes, cells in sweat glands, or hair follicles. *E*, Quantitative analyses. *Left side* shows number of CGRP-positive structures per coronal section in the dorsal horn of one side of the spinal cord averaged from eight sections per mouse. *Right side* shows average number of CGRP-positive fibers per transverse skin section. Each symbol represents a separate mouse. Horizontal lines indicate mean values.

based vaccines incorporate many of the immunological properties of viruses and serve as excellent immunogens that are able to overcome B cell tolerance to self-Ags without the requirement for strong adjuvants (46). Using this approach to vaccine design, we have previously demonstrated that levels of neutralizing Ab responses against self-proteins can be induced that are high enough to show therapeutic effectiveness in mice (31–35). We have also demonstrated that efficacy established in animal models can be translated into efficacy in humans (47, 48). An important safety feature and advantage of VLP-based vaccines is that autoantibodies can be induced without the addition of powerful adjuvants. The use of such adjuvants brings with it the risk of breaking T cell tolerance and potentially inducing autoreactive pathogenic T cells and/or irreversible Ab responses (49).

In the current study, we observed that immunization with NGFQβ, in the absence of added adjuvant, induced high titers of NGF-specific Abs in all treated animals. Ab levels were increased by additional administration of the vaccine and could be maintained at high levels for at least 1 y by periodic boosting. In the absence of boosting, titres waned by 90% within a year. The kinetics of the Ab response are similar to those reported previously (35). Importantly, neither endogenously produced NGF nor rNGF administered s.c. had an influence on the Ab response. The inability of NGF alone to boost the Ab response in the absence of linked T cell help provided by the VLP indicates NGF-specific T cells were not induced, another important safety feature for this type of vaccine.

An in vitro assessment of the quality of the Abs induced by immunization with NGFQβ demonstrated they blocked the binding of NGF to its cognate receptor TrkA in a solid-phase assay and neutralized NGF in a cellular proliferation assay. Importantly, the neutralizing ability of the Abs was also observed in vivo, where it was shown that immunization had an analgesic effect in two models of inflammatory pain.

Immunization of mice with NGFQβ reduced inflammatory hyperalgesia induced by s.c. injection of zymosan A, a model previously used by others to evaluate analgesic drugs (50). Statistically significant reductions in sensitivity to mechanical and thermal stimuli were observed during the 7-d course of the inflammation. The magnitude of the reduction is comparable to that reported with an anti-TrkA receptor mAb tested in a formalin-evoked pain licking responses in mice (51). It was noted that in

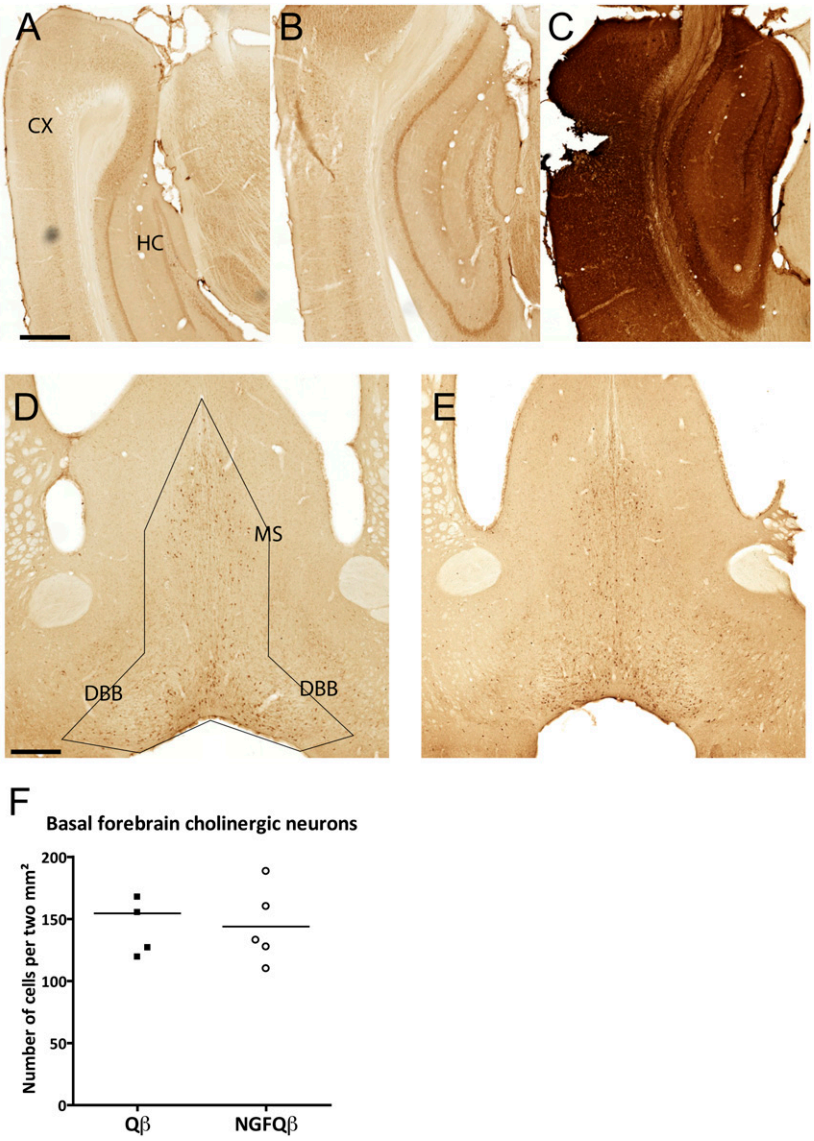

FIGURE 8. BBB and cholinergic forebrain neuron integrity. *A–C*, Immunoperoxidase staining of fibrinogen in cortex (CX) and hippocampus (HC). Scale bar, 500 μm. Neither Qβ-immunized control mice (*A*) nor NGFQβ-immunized (*B*) mice show any fibrinogen-immunoreactivity in the cortex or the hippocampus, whereas strong fibrinogen immunoreactivity was found in both structures in mice with a compromised BBB due to temporal lobe epilepsy (*C*). Cholinergic forebrain neurons labeled with anti-ChAT Abs in the medial septum and the diagonal bands of Broca (DBB) of Qβ-immunized control mice (*D*) and of NGFQβ-immunized mice (*E*). Scale bar, 500 μm. *F*, Quantification of cholinergic neurons in the basal forebrain is indicated in *D*. Each symbol represents a separate mouse. Horizontal lines indicate mean values. MS, medial septum.

the first 7 h after zymosan A injection, neutralization of NGF had little influence on pain perception. This result is perhaps not surprising because it has been reported that NGF mRNA only becomes markedly increased 2 h postonset of inflammation (23). In the very early stages of inflammation, sensitization to mechanical and thermal stimulation could predominately be due to release of pain mediators other than NGF.

The ability of NGFQβ to alleviate pain for an extended period of time during chronic inflammation was assessed in mice subjected to CIA. This is a recognized model of chronic inflammatory pain in which progressive inflammation and hyperalgesia develop in affected limbs for at least 6 wk. Three months postimmunization with NGFQβ, the average sensitivity to thermal stimulation in arthritic mice had gone back to the levels recorded before disease induction. In contrast, nonvaccinated animals developed hyperalgesia; sensitivity to thermal stimulation was increased by ~45%. Only for NGFQβ-immunized animals with the most severe inflammation (scores of 2.5–3) was sensitivity increased, but the hyperalgesia was ~50% reduced relative to nonvaccinated animals. Similarly, effective suppression of autoimmune arthritic pain in rats has been reported with anti-NGF mAbs (26).

Importantly, in both models, we demonstrated that neutralization of NGF did not affect responses in healthy mice when their paws were exposed to pressure or heat. Rather, only mechanical sensitization or thermal hyperalgesia associated with inflammation was affected. Furthermore, the presence of persistently high neutralizing NGF-specific Abs in healthy animals repeatedly vaccinated with NGFQβ for a period of 10 mo did not alter baseline sensitivity toward mechanical and thermal stimulation. This is in accordance with our results from the CGRP-immunoreactivity study clearly indicating that sensory innervation in hind paw skin and lumbar spinal dorsal horn is preserved post NGF immunization.

Studies (43, 44) on mice expressing monoclonal anti-NGF Abs from transgenes have raised concerns about the safety of long-term anti-NGF treatment. These studies reported disruption of the BBB after loss of sympathetic superior cervical ganglion neurons, which are required to maintain BBB integrity. They also showed a subsequent loss of cholinergic basal forebrain neurons. In the NGFQβ-immunized mice studied in this paper, we did not obtain evidence for a compromised BBB. Furthermore, sympathetic innervation of cerebral blood vessels and the size of the SCGs also did not differ between the two groups of mice. An absence of fibrinogen deposits in the brains of NGFQβ-immunized mice demonstrated the BBB was not adversely affected. The number of cholinergic neurons in the basal forebrain was also unaffected. An obvious difference with our system and that used in the previous

transgenic approach is that anti-NGF mAbs expressed as transgenes are present throughout embryonic development may thus interfere with the developmental functions of NGF.

Our results are in accord with preclinical studies investigating anti-NGF mAbs for the treatment of pain (26, 27, 52). In rodents and primates, no denervation or changes in sensory or sympathetic innervation density or structural toxicities in the nervous system have been reported (28). Moreover, the presence of natural anti-NGF autoantibodies in adult humans is not associated with neurologic deficits (53–58). Nevertheless, the effects of long-term neutralization of NGF must to be carefully considered. Clinical studies conducted with tanezumab will help establish the safety profile for long-term neutralization of NGF and may thus provide a framework to guide the development of an anti-NGF vaccine.

Vaccination represents a new approach to the relatively novel passive immunization therapies currently being developed for the treatment of chronic pain. Whether or not vaccination targeting NGF could be a safe and effective contribution to pain management remains to be determined and would require further preclinical testing and extensive clinical development. Nevertheless, the potential advantages of such an approach may warrant such investment. One advantage of autovaccination could be favorable pharmacodynamics. The longevity of vaccine-induced neutralizing anti-NGF Abs we have observed in mice could result in long-lived therapeutic benefit by alleviating pain for several months. Indeed, immunization once or twice per year may be sufficient to maintain Ab titers at levels high enough for sustained efficacy. Another significant advantage would be cost of goods for which a vaccine would provide clear cost savings over expensive mAb therapies. Thus, active autovaccination may have the potential to offer a treatment option for a debilitating condition that affects the quality of life of millions of people.

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Disclosures

T.A.R., P.B., M.F.B., and G.T.J. are employees of and hold stocks or stock options with Cytos Biotechnology. T.A.R, M.F.B., and G.T.J. are authors of a related patent application.

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