ARTHRITIS & RHEUMATISM
Vol. 63, No. 11, November 2011, pp 3243–3252
DOI 10.1002/art.30564

Nerve Growth Factor

A Key Local Regulator in the Pathogenesis of Inflammatory Arthritis

Siba P. Raychaudhuri,[1] Smriti K. Raychaudhuri,[1] Kondala R. Atkuri,[2] Leonard A. Herzenberg,[2] and Leonore A. Herzenberg[2]

Objective. **The effect of nerve growth factor (NGF) and its receptor (NGFR) in inflammatory diseases is a novel research field. The purpose of this study was to investigate the role of NGF/NGFR in human T cell subpopulations and fibroblast-like synovial cells (FLS) and examine its pathophysiologic significance in psoriatic arthritis (PsA) and rheumatoid arthritis (RA).**

Methods. **Expression of NGF/NGFR was examined in synovial fluid (SF), FLS, peripheral blood (PB)–derived T cells, and SF-derived T cells from patients with PsA, RA, and osteoarthritis (OA). NGF levels were determined by enzyme-linked immunosorbent assay. NGF-induced T cell/FLS proliferation was examined by MTT assay. Low-affinity (p75)/high-affinity (TrkA) NGFR expression was determined by high-dimensional fluorescence-activated cell sorting. A monochlorobimane assay was used to determine the effect of NGF on T cell survival.**

Results. **Levels of NGF were higher in SF samples from PsA and RA patients as compared to SF samples from OA patients. NGF-induced FLS proliferation was more marked in PsA and RA patients. TrkA was up-regulated on activated SF T cells from PsA (mean ± SD 22 ± 6.2%) and RA (8 ± 1.3%) patients, whereas in SF samples from OA patients, TrkA+CD3+ T cells were not detectable. NGF induced the proliferation of PB T cells, induced the phosphorylation of Akt in activated T cells, and consistent with known pAkt activity, inhibited tumor necrosis factor α–induced cell death in these T cells.**

Conclusion. **Based on our findings, we propose a model in which NGF secreted by FLS into PsA and RA synovium promotes the survival of activated autoreactive T cells as well as FLS proliferation. Thus, NGF has the potential to sustain the chronic inflammatory cascades of arthritis of autoimmune origin.**

Nerve growth factor (NGF) is produced by cells in the nervous system (1,2), by lymphocytes in the immune system (3–7), by fibroblasts located in peripheral organs (8), and by cell lines derived from synovial fibroblasts (9). This protein is recognized by 2 receptors (NGFRs): a high-affinity receptor (TrkA), which is central to the studies presented herein, and a low-affinity receptor (p75), which we have not found to be relevant to our studies. Neurotropic and other NGF activities in the nervous system have been well defined (1,2). In addition, several studies have implicated NGF in the development and function of the murine immune system (3–7). NGF and TrkA have been detected during early development (10) and, in adult mice, in the thymus and secondary lymphoid organs (11). In addition, TrkA/NGFR$^{-/-}$ mice have been shown to have structural abnormalities in the thymus and a clear decrease in thymocyte density in the organ (10). NGF has been shown to increase in vivo antibody responses to a T cell–dependent antigen (4) and to increase interleukin-2 (IL-2) receptor expression (5,6) and c-Fos transcription (6).

Several studies have shown that NGF synthesis is up-regulated in inflammation (2,7,9,12–16). Immune cells involved in innate and acquired immunity show basal expression of NGF, and its synthesis is enhanced

Supported by an Arthritis Foundation Investigator award to Dr. S. P. Raychaudhuri.

[1]Siba P. Raychaudhuri, MD, Smriti K. Raychaudhuri, MD: University of California School of Medicine, Davis, VA Medical Center, Sacramento, and Stanford University School of Medicine, Stanford, California; [2]Kondala R. Atkuri, PhD, Leonard A. Herzenberg, PhD, Leonore A. Herzenberg, PhD: Stanford University School of Medicine, Stanford, California.

Address correspondence to Siba P. Raychaudhuri, MD, VA Medical Center Sacramento/University of California, Davis, 10535 Hospital Way, Building 807, Mather, CA 95655. E-mail: sraychaudhuri@ucdavis.edu.

Submitted for publication November 14, 2010; accepted in revised form July 19, 2011.

after stimulation with specific antigens and cytokines (2,3–6). Cytokines such as IL-1β, tumor necrosis factor α (TNFα), and IL-6 can induce NGF production in fibroblasts, endothelial cells, keratinocytes, and glial cells (8,9,12,17–19). These non-neuronal cells that produce NGF also express TrkA, which upon binding to its ligand, activates intracellular pathways and nuclear factors in an autocrine manner, similar to processes recognized in neuronal cells.

A contributing role of NGF/NGFR has been suggested in various inflammatory diseases, such as inflammatory airway disease, arthritis, and psoriasis (2,7,9,12–16). NGF can be expected to increase pain in the arthritic joint via several mechanisms, for example, by promoting the proliferation of terminal nerves (12), the up-regulation of substance P (20,21), and the degranulation of mast cells (22). Further, it promotes the in-migration and degranulation of neutrophils (22,23), which raises elastase levels and, hence, increases the destruction of normal joint tissue. NGF signals the invasion of nerve fibers into the sites of inflammation, which places NGF at the crossroads between the neurologic and immune-mediated mechanisms that maintain and exacerbate inflammatory disease.

The current state of knowledge about the interactions of T lymphocytes with NGF and its receptor system is mostly limited to mouse studies. In the present study, we investigated the pathophysiologic significance of the NGF/NGFR system in human T lymphocyte subpopulations and fibroblast-like synovial cells (FLS), as well as its contributing role in the inflammatory and proliferative cascades of psoriatic arthritis (PsA) and rheumatoid arthritis (RA).

MATERIALS AND METHODS

Reagents. Human NGFβ was purchased from Calbiochem. Recombinant human TNFα was purchased from R&D Systems. Polyclonal anti-human NGF antibody, anti-TrkA antibody, and isotype controls were procured from Santa Cruz Biotechnology. Anti-p75 and peripheral blood (PB) subset antibodies were procured from BD PharMingen. Live/Dead markers were obtained from Molecular Probes/Invitrogen.

Study population. This study was approved by the Institutional Review Boards of Stanford University and the Veterans Affairs Medical Center at Sacramento. Specimens were collected from patients with active PsA, osteoarthritis (OA), and RA as well as from healthy control subjects. The control group was matched for sex and age with the patient groups. OA of the knee (24) and RA (25) were diagnosed according to the clinical, laboratory, and radiographic criteria of the American College of Rheumatology. PsA was diagnosed according to the Classification of Psoriatic Arthritis (CASPAR) Study Group criteria (26).

Patients underwent a complete physical examination, evaluation of their psoriasis and arthritis severity, appropriate blood tests, and radiologic studies. Synovial fluid (SF) and synovial tissues were collected from PsA, RA, and OA patients with active arthritis of one or both knees. The inclusion criteria we used for RA and PsA were designed to limit participants to only those who had active disease; thus, active RA and active PsA were defined by the presence of at least 3 swollen and 3 tender joints. In addition, for PsA, the presence of plaque psoriasis with a qualifying lesion at least 2 cm in diameter was required. The percentage of body surface area affected by psoriatic skin involvement was also determined. The number of swollen joints, the number of tender joints, the patient's assessment of pain, the erythrocyte sedimentation rate, and the C-reactive protein level were determined in all patients. We tried to enroll patients who had not taken DMARDs (methotrexate, leflunomide, cyclosporine, hydroxychloroquine, sulfasalazine, azathioprine) or biologic agents within the last 3 months.

The types of specimens, numbers of subjects, types of disease, and use of DMARDs for specific experiments are described under the appropriate sections. The specific assays to which patient samples were subjected are described under the appropriate sections.

Surface expression of NGFRs TrkA and p75. *Expression on T lymphocytes from peripheral blood.* Ficoll-Hypaque density-gradient centrifugation was used to isolate peripheral blood mononuclear cells (PBMCs) from blood samples obtained from normal subjects (n = 10) and patients with PsA (n = 10), RA (n = 10), or OA (n = 10). PBMCs (2×10^6/ml) were activated by 72 hours of culture in wells coated with CD3 (clone UCHT-1)/CD28 (clone CD28.2) (5 μg of each per ml; BD PharMingen). Cells were harvested, washed with phosphate buffered saline (PBS) containing 2% fetal bovine serum (FBS), and stained with Live/Dead marker (Molecular Probes) and surface markers of T cells (CD3, CD4, and CD8; BD PharMingen). Then, cells were permeabilized with Cytofix/Cytoperm, washed twice with wash buffer, resuspended in wash buffer containing 2% FBS, and kept on ice. Cells were then incubated with either phycoerythrin (PE)–conjugated p75 antibody (BD PharMingen) or PE-conjugated TrkA antibody (Santa Cruz Biotechnology). Controls were unstained cells and cells stained with irrelevant primary mouse or rabbit sera. Unstained cells were used to determine autofluorescence.

Cells were analyzed in a FACSAria (Becton Dickinson). At least 500,000 events were collected in a FACSDiva electronic system. Data were analyzed using FlowJo software (Tree Star). Expression of p75 and TrkA were determined in gated live cells only. SH-SY5 cells (ATCC) were used as positive controls for expression of p75 and TrkA.

Expression on T lymphocytes from SF. SF samples were collected from PsA (n = 9), RA (n = 12), and OA (n = 10) patients, all of whom had active arthritis. Patients were taking nonsteroidal antiinflammatory drugs (NSAIDs) or acetaminophen. Five patients with RA were taking hydroxychloroquine, 3 patients with RA were taking methotrexate (<10 mg/week), and 1 patient with new-onset PsA was taking prednisone (10 mg/day). Patients were not receiving biologic agents. For simultaneous analysis of functional and phenotype characteristics of the T cells at the single-cell level, we performed multicolor high-dimensional (Hi-D) fluorescence-activated cell sorter (FACS) analysis (12-color, 13-parameter). The SF

mononuclear cells (SFMCs) were stained with Live/Dead stain and antibody cocktail (CD3, CD4, CD8, CD45RA, CD11a, HLA–DR, CD14, and CD19) as described above.

T cell proliferation assays. CD3+ T cells from the PBMCs obtained from normal subjects (n = 10) and PsA (n = 10), RA (n = 12), and OA (n = 10) patients were purified to 96% purity by negative selection with magnetic beads (Dynabeads Untouched human T cells; Invitrogen). One million CD3+ T cells/ml were cultured in 24-well plates with NGF in RPMI-1640 containing 25 m*M* HEPES (Gibco-BRL) supplemented with 2 m*M* L-glutamine, 100 units/ml of penicillin, and 100 μg/ml of streptomycin (Gibco-BRL) at 37°C in a humidified incubator containing 5% CO_2. Cells were incubated under the following conditions: with medium alone, with NGF alone (10, 100, 200, and 500 ng/ml), with K252a alone (100 n*M*; Axxora), with NGF plus K252a (100 n*M*), with NGF-neutralizing antibody alone (100 ng/ml; BD PharMingen), or with NGF plus NGF-neutralizing antibody (100 ng/ml). Cells

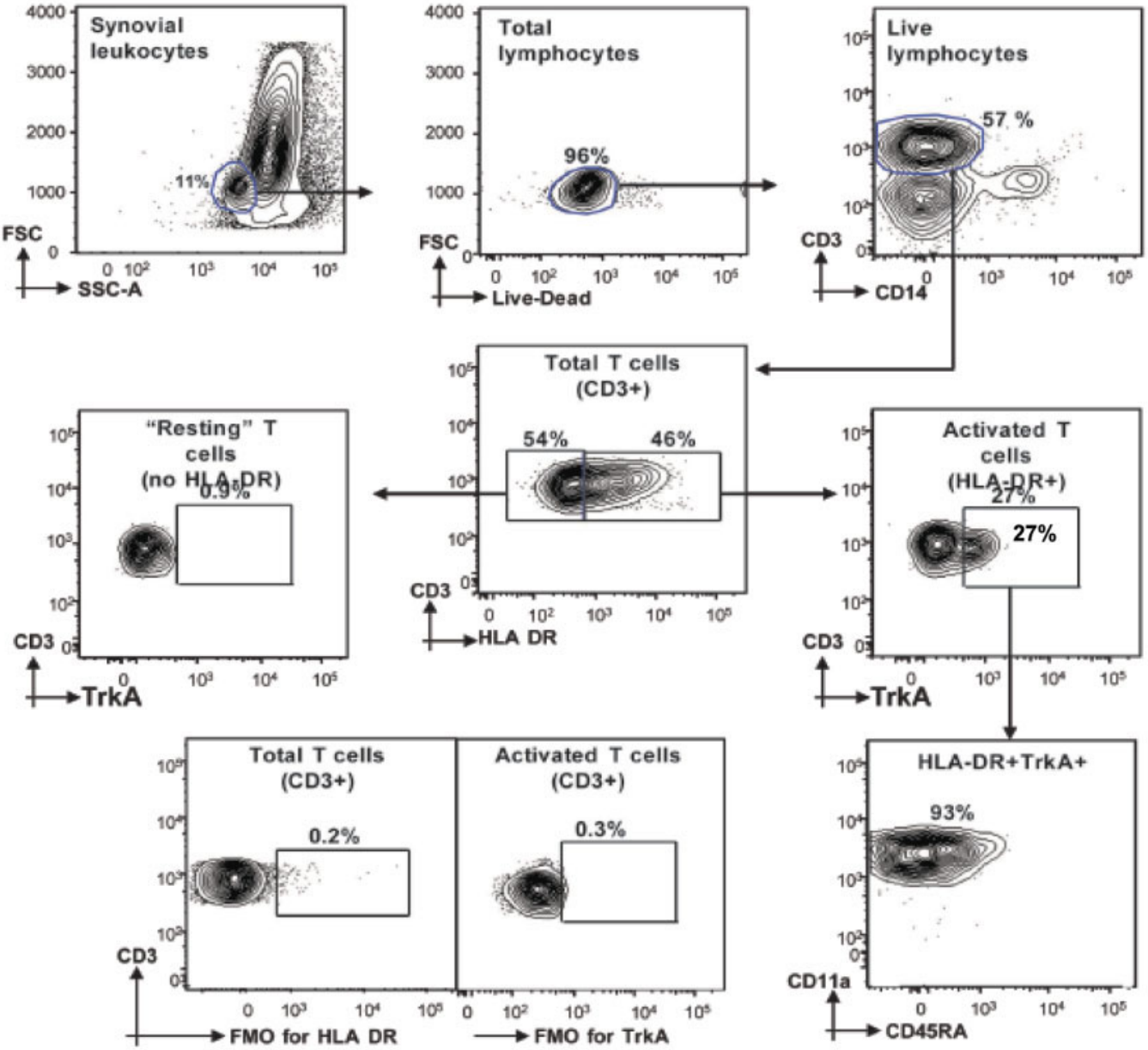

Figure 1. TrkA+ T cells in the synovial fluid (SF) of patients with psoriatic arthritis (PsA). Cells derived from SF samples obtained from a PsA patient were stained and gated as indicated. TrkA+ T cells were present in the SF samples and displayed a memory T cell phenotype. Numbers shown in each panel are the percentage of positive cells. FMO = fluorescence minus one (stain 1). Color figure can be viewed in the online issue, which is available at http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)1529-0131.

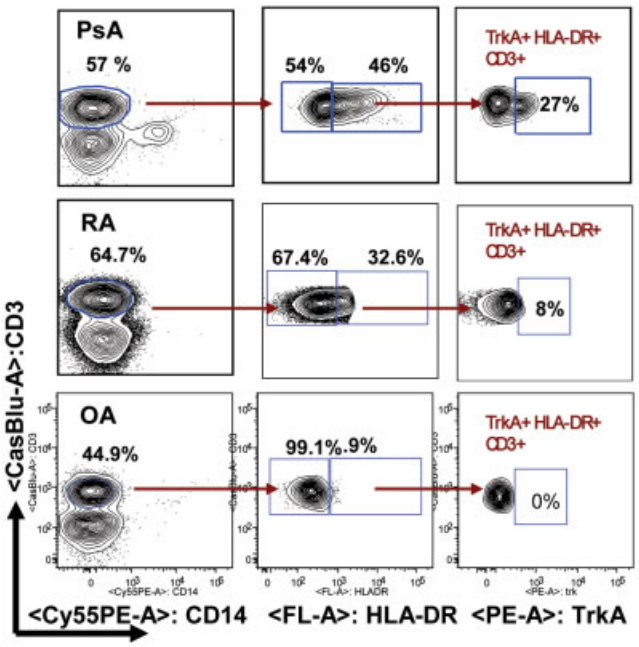

Figure 2. Frequencies of TrkA+ activated T cells in the synovial fluid (SF) of patients with psoriatic arthritis (PsA), rheumatoid arthritis (RA), and osteoarthritis (OA). Cells were stained and gated as indicated. TrkA+ activated T cells were elevated in PsA and RA SF, but not in OA SF. The mean ± SD percentages of TrkA+DR+CD3+ cells in the 3 patient groups were as follows: 22 ± 6.2% in PsA patients (n = 9; $P < 0.01$ versus RA patients, by Student's *t*-test), 8.5 ± 1.3% in RA patients (n = 12), and none in OA patients (n = 10). Numbers shown in each panel are the percentage of positive cells. CasBlu = Cascade Blue; FL = fluorescein isothiocyanate; PE = phycoerythrin. Color figure can be viewed in the online issue, which is available at http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)1529-0131.

were cultured for 4, 6, and 8 days. We used MTT assay, hexosaminidase assay, and cell counting methods to measure T cell proliferation. Each test was run in quadruplicate and was performed 5 times. Results are expressed as the mean ± SD. Analysis of variance (ANOVA) with post hoc testing was used for comparison of the means.

Effect of NGF on T cell survival and apoptosis. PBMCs from normal subjects (n = 10) were activated by 72 hours of culture in 24-well plates coated with CD3/CD28 (5 μg of each per ml). NGF (10, 100, or 1,000 ng/ml) was added after 48 hours of incubation. To induce apoptosis, cells were then treated with 20 ng/ml of TNFα for the last 30 minutes to 2 hours of incubation. Experiments were performed with activated and unactivated T lymphocytes, with and without TNFα or NGF. Cells were stained with monochlorobimane (live cell marker), surface markers of T cells (CD3, CD4, and CD8), and allophycocyanin-conjugated annexin V (apoptosis marker).

Western blot analysis of pAkt. CD3+ T cells purified by negative selection from normal subjects (n = 10) were cultured with and without NGF for 72 hours in a 24-well plate. Cells were lysed in 1× sodium dodecyl sulfate (SDS) loading buffer, and the lysates were centrifuged at 13,000*g* for 30 minutes at 4°C. Protein content in the supernatants was determined with the BCA protein assay system (Pierce). Protein (40 μg) in cell extracts was resolved by 10% SDS–polyacrylamide gel electrophoresis, transferred to PVDF membranes, probed with antibodies, and the blot was developed using an enhanced chemiluminescence substrate system (Amersham Biosciences). Antibodies specific for pAkt and α-tubulin (Cell Signaling Technologies) were used.

Quantitative enzyme-linked immunosorbent assay (ELISA) of NGF in SF samples from OA, RA, and PsA patients. SF was aspirated from the knee joints of patients with OA (n = 30), RA (n = 30), and PsA (n = 20). All patients had active arthritis. The patients were taking NSAIDs or acetaminophen. Five patients with RA were taking hydroxychloroquine, 3 patients each with PsA and RA were taking methotrexate (<10 mg/week). Patients were not receiving biologic agents or any other immunomodulatory drugs at least for 3 months prior to this study. SF samples were centrifuged at 2,000 revolutions per minute for 10 minutes to remove cellular debris and were stored at –20°C in aliquots until the time of analysis.

NGF concentrations were measured by ELISA using an NGF E_{max} immunoassay system (Promega). Total NGF was obtained by acid treatment of the SF supernatant according to the Promega protocol. For comparison, paired serum samples from the patients as well as serum from 10 healthy subjects were used as controls. Sensitivity for NGF detection with this kit is 15 pg/ml.

MTT assay for FLS proliferation. Synovial tissues were obtained from patients with PsA (n = 4), OA (n = 4), and RA (n = 4) who had undergone knee surgery. OA patients were not receiving any immunosuppressive agents. Low-dose prednisone and hydroxychloroquine was continued in RA patients. Methotrexate was discontinued in RA and PsA patients 6–8 weeks before surgery. Tissues were processed and FLS were cultured according to our standardized protocols (9). Third-passage FLS (5,000 cells/200 μl of Dulbecco's modified Eagle's medium [complete medium]) in 96-well plates were cultured for 5 days under the following conditions: with medium alone, with NGFβ alone (100 ng/ml), with K252a alone (100 n*M*), with NGF plus K252a (100 n*M*), with NGF-neutralizing antibody alone (100 ng/ml), or with NGF plus NGF-neutralizing antibody (100 ng/ml).

A dose-response curve was performed with various doses of NGF (10, 100, 200, and 500 ng/ml), and we observed that 100 ng/ml of NGF is the optimal dose for FLS proliferation (9). The MTT assay was run according to our previously described protocol (9). Briefly, PBS-diluted MTT (5 mg/ml) was added to each well, and the plate was incubated for 4 hours. Acid isopropanol (0.04*N* HCl in isopropanol, 100 μl/well) was added. The plates were maintained at room temperature for 5 minutes, and then the absorbance at 570/690 nm was read with a microtiter plate reader.

Statistical analysis. Analyses of FACS data, calculations of absolute cell numbers, and comparisons of means/medians of each phenotype under different experimental conditions were performed using FlowJo software (Tree Star). Statistical analyses were performed with the JMP statistical software package (SAS Institute). Cell proliferation data were analyzed by ANOVA. A 2-sided *t*-test was used to compare the ELISA data, the flow cytometry data, and the mean levels or numbers of each outcome in the patient groups versus the control group. The nonparametric Mann-Whitney U test was used for non-normal data. *P* values less than 0.05 were considered statistically significant.

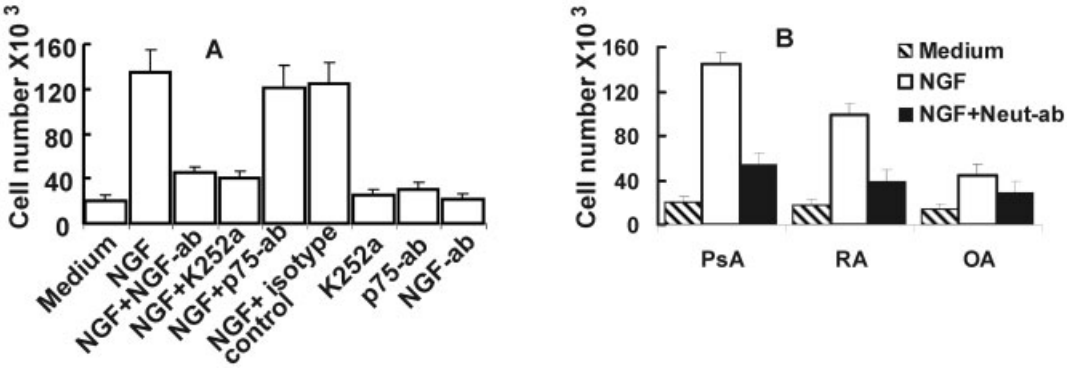

Figure 3. Fibroblast-like synoviocyte (FLS) proliferation as determined by MTT assay. Through its high-affinity receptor TrkA, nerve growth factor (NGF) induces marked proliferation of FLS derived from patients with psoriatic arthritis (PsA) and patients with rheumatoid arthritis (RA). **A,** Proliferation of FLS induced by NGF is mediated through its high-affinity receptor TrkA. Third-passage FLS derived from PsA patients (5,000 cells/200 μl of Dulbecco's modified Eagle's medium [DMEM; complete medium]) were cultured for 5 days in 96-well plates with medium alone, NGFβ alone (100 ng/ml), NGFβ plus NGF-neutralizing antibody (NGF-ab), NGFβ plus K252a, NGFβ plus p75 antibody (p75-ab), NGF plus isotype control, or K252a, p75 antibody, or NGF antibody alone. **B,** NGF demonstrates marked mitogenic effects on FLS derived from PsA and RA patients as compared to those from osteoarthritis (OA) patients. The effect of NGFβ (100 ng/ml) on the proliferation of FLS derived from PsA (n = 4), OA (n = 4), and RA (n = 4) synovial tissue samples was measured. Third-passage FLS (5,000 cells/200 μl of DMEM complete medium) were cultured for 5 days in 96-well plates. Values are the mean ± SD of quadruplicate cultures from 5 independent experiments.

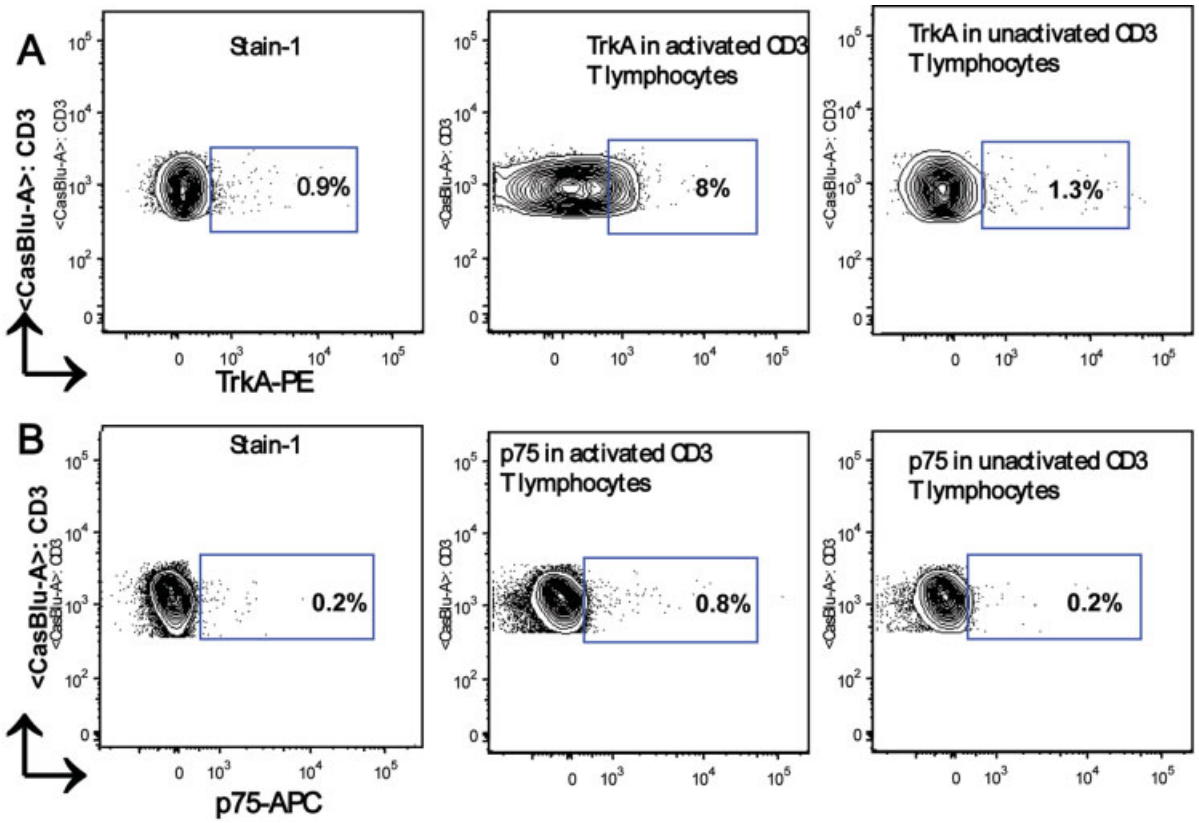

Figure 4. Expression of the high-affinity nerve growth factor (NGF) TrkA on CD3/CD28-stimulated peripheral blood T lymphocytes. T lymphocytes from peripheral blood mononuclear cells (1×10^6/ml) obtained from normal subjects (n = 10) were activated by 72 hours of culture in wells coated with CD3/CD28 (5 μg of each per ml). Cells were stained with Live/Dead markers, monochlorobimane stain (live cell marker), surface marker of T cells (CD3), and NGF receptor (TrkA or p75). **A,** A distinct population of gated, live, activated CD3+ T lymphocytes expressed TrkA. **B,** These activated CD3+ T cells did not express p75. Stain-1 represents isotype controls. Numbers shown in each panel are the percentage of positive cells. CasBlu = Cascade Blue; PE = phycoerythrin; APC = allophycocyanin. Color figure can be viewed in the online issue, which is available at http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)1529-0131.

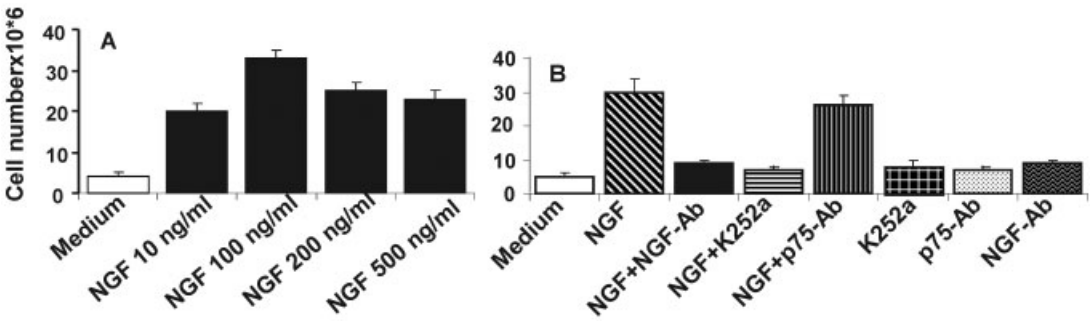

Figure 5. T cell proliferation as determined by MTT assay. Nerve growth factor (NGF) stimulates peripheral blood T cells via its receptor. T cells were purified by negative selection with magnetic beads and were then cultured for 6 days. **A,** Proliferation of untreated T cells or T cells treated with increasing concentrations of NGF. **B,** Proliferation of untreated T cells or T cells treated with NGF alone (100 ng/ml), NGF plus NGF-neutralizing antibody (NGF-ab), NGF plus K252a, NGF plus p75 antibody (p75-ab), or K252a, p75 antibody, or NGF antibody alone. Values are the mean ± SD of quadruplicate cultures and are representative of 5 experiments.

RESULTS

Up-regulation of TrkA on effector memory T lymphocytes from the SF of PsA and RA patients. Freshly isolated SFMCs from the joints of patients with PsA contained a subset of T cells that expressed the high-affinity NGF receptor (TrkA) as well as HLA–DR and memory T cell markers, as determined by Hi-D FACS (Figure 1). We observed that in the SF of PsA patients, a significant percentage of the activated HLA–DR+CD3+ T cells expressed TrkA (mean ± SD 22 ± 6.2%), whereas the unactivated (HLA–DR–) T cells had minimal TrkA expression (Figure 1). The low-affinity NGF receptor (p75) was not increased in these cells.

SF samples from RA patients had a comparatively lower, but still significant, percentage of TrkA+ cells (8 ± 1.3%) in the activated CD3+ T cell subset. In contrast, OA synovial fluid did not show any detectable TrkA+CD3+ T cells (Figure 2). In PBMCs from RA, PsA, and OA patients, expression of p75 and TrkA was unremarkable in unactivated T cells. We did not find any correlation between TrkA expression in SF T cells and the treatment regimen or disease severity.

Elevated NGF levels in the SF of PsA and RA patients and marked proliferation of FLS induced by NGF via TrkA. We recently reported that FLS derived from PsA and RA joints spontaneously produced higher levels of NGF and that the percentages of TrkA+ FLS were higher compared to those in FLS from OA patients and patients with meniscal tear (9). Consistent with those findings, we observed in the present study NGF levels of 395.5 ± 85.2 pg/ml, 230 ± 35 pg/ml, and 56 ± 6 pg/ml (mean ± SD) in SF samples from RA (n = 30), PsA (n = 20), and OA (n = 30) patients, respectively, as determined by ELISA. We did not notice any correlation of NGF levels in SF with the treatment regimen or disease severity.

To determine the functional significance of this high level of NGF, we used an MTT assay to investigate the effects of NGF on the proliferation of FLS (Figure 3A). The specificity of NGF-induced FLS proliferation was determined by the addition of an excess of NGF-neutralizing monoclonal antibody, which caused a significant reduction in NGF-induced proliferation (Figure 3A). The addition of isotype-matched control antibody did not affect proliferation. To examine the role of p75 and TrkA, we used anti-p75 monoclonal antibody and K252a, a natural alkaloid that blocks NGF-induced phosphorylation of TrkA. There was marked inhibition of NGF-induced FLS proliferation by both anti–NGF-neutralizing antibody and K252a ($P < 0.01$) (Figure 3A), suggesting that NGF-induced proliferation is mediated through TrkA.

Using the same dose of NGFβ (100 ng/ml), we noticed significantly increased proliferation of FLS derived from PsA and RA patients as compared to FLS from OA patients (Figure 3B). In our previous report (9), we noted that TrkA is expressed in a higher percentage of FLS derived from PsA and RA patients as compared to FLS derived from OA patients. Thus, the NGF-induced increase in proliferation of FLS derived from PsA and RA patients as shown in Figure 3B is likely from the increased expression of TrkA in these cells.

We further confirmed the pathophysiologic significance of NGF by testing the effects of SF from PsA patients on FLS proliferation and examining whether proliferation induced by SF could be blocked by anti-NGF antibody. By performing a dose-response curve with various dilutions of SF, we found that a 1:100

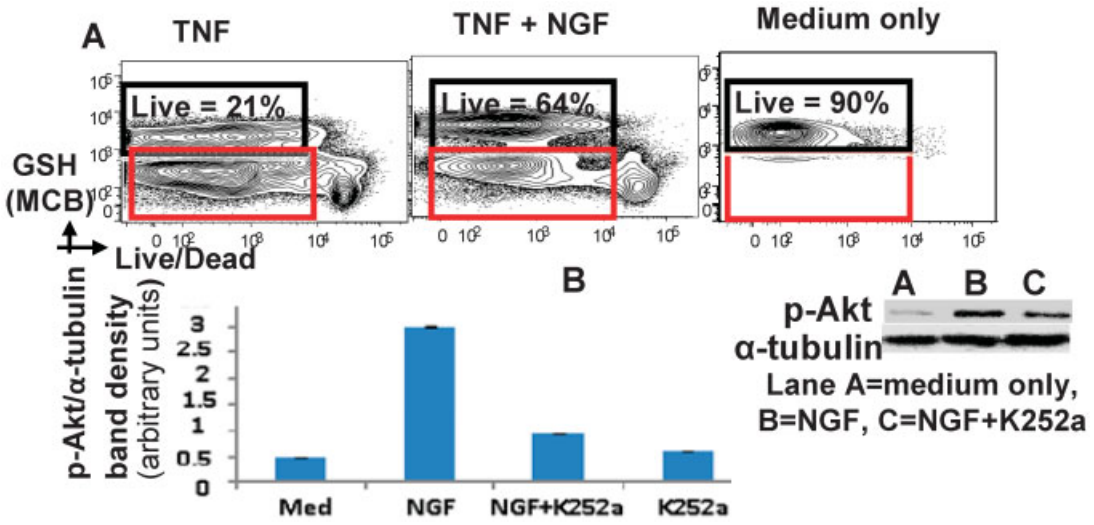

Figure 6. A, Nerve growth factor (NGF) inhibition of tumor necrosis factor α (TNFα)–induced cell death. Peripheral blood mononuclear cells (PBMCs) were cultured for 2 hours with TNFα (20 ng/ml) in the presence or absence of NGF (100 ng/ml), washed, and stained to evaluate cell death. Cells were stained with monochlorobimane (MCB) to detect intracellular glutathione (GSH), the levels of which distinguish live cells from dead cells, and with the Invitrogen Live/Dead reagent to identify dead cells. Results are representative of 10 experiments. **B,** Increased expression of pAkt in CD3+ T lymphocytes by NGF. PBMCs were cultured for 24 hours with plate-bound antibodies to CD3/CD28, and NGF with or without K252a was added for the last 2 hours. Cell lysates (20 μg of protein) were immunoblotted with antibodies to pAkt and α-tubulin (positive control). The blot is representative of data obtained at 2 hours. Densitometric estimates of the bands were obtained with ImageJ software. Results for pAkt were normalized to α-tubulin. K252a significantly decreased pAkt expression ($P < 0.01$ by one-way analysis of variance followed by Dunnett's post hoc test). Values are the mean ± SD of 10 experiments.

dilution of SF was the optimal dose for FLS proliferation. SF from patients was used in triplicate for this experiment. NGF-neutralizing antibody used at 100 ng/ml inhibited SF-induced proliferation of FLS from PsA patients ($P < 0.05$). More specifically, NGF-neutralizing antibody (100 ng/ml) in the presence of FLS from PsA patients 1, 2, and 3 inhibited proliferation by a mean ± SD of 30 ± 5%, 35 ± 5%, and 38 ± 7%, respectively.

Marked expression of TrkA in activated T cells. CD3/CD28 stimulation of PBMCs from healthy donors is commonly used to generate activated T cells. Following this protocol, we observed that a subset of CD3+TrkA+ cells appeared within 3 days when PBMCs from healthy donors were stimulated with antibodies to CD3 and CD28 and analyzed by Hi-D FACS. As in the in vivo studies, where the activated cells were obtained from PsA joints, the low-affinity NGF receptor (p75) was not detectably increased on CD3/CD28-stimulated T cells (Figure 4). The mean ± SD frequency of TrkA+ stimulated PB T cells (CD3+) was 4 ± 0.3% in healthy subjects, 6 ± 2.8% in PsA patients, 5 ± 1.8% in RA patients, and 3.9 ± 0.5% in OA patients. Ten PBMC samples were studied in each group. In unstimulated T cells, the mean ± SD frequency of CD3+TrkA+ cells was 1.8 ± 0.5%.

NGF-induced proliferation of PB T cells from healthy subjects. T cell proliferation was performed using CD3+ T cells derived from PBMCs of healthy donors with increasing concentrations of NGF (10, 100, 200, and 500 ng/ml in 2% serum-containing medium). Maximum proliferation was observed at an NGF concentration of 100 ng/ml (Figure 5A). This proliferation was clearly induced by NGF for 3 reasons. First, the addition of an excess of an anti-NGF monoclonal antibody with neutralizing activity significantly decreased this proliferation (Figure 5B), whereas the addition of an isotype-matched control antibody was ineffective (data not shown). Second, the addition of a TrkA inhibitor (the natural alkaloid K252a, which selectively inhibits the NGF-induced phosphorylation of TrkA) blocked NGF-induced proliferation (Figure 5B). Since the addition of the alkaloid in the absence of NGF did not affect cell survival or cell numbers in the culture, we conclude that the inhibition was due to specific inhibition of the NGF/TrkA interaction. Third, consistent with the absence of detectable amounts of the low-affinity NGF receptor p75 on activated cells (Figure 5B), mono-

clonal antibodies that inhibit NGF binding to p75 did not inhibit the NGF-induced proliferation of PBMCs (Figure 5B).

The data shown in Figure 5 were determined by MTT assays. We have noticed a similar degree of NGF-induced proliferation of T cells using hexosaminidase assay and cell counting methods. From these findings, we conclude that the low level of TrkA on unstimulated PB T cells (Figure 4) is sufficient to enable TrkA to trigger PB T cells to proliferate under the culture conditions we used. Since, as we have shown, TrkA expression is increased on activated T cells, this suggests that production of NGF could also trigger or support T cell proliferation under conditions in which T cells are activated in vivo.

NGF protection of TNFα-induced T cell death and evidence of NGF-induced phosphorylation of Akt in T cells. TNFα, which is well known to induce apoptosis in T cells (27), is present at high levels in the SF of PsA patients. Nevertheless, substantial numbers of viable T cells can be readily recovered and cultured from SF, which suggests that the T cells are protected against TNFα-induced apoptosis. This protection, we suggest, is at least partly due to NGF. Indeed, it may be explained by induction of the phosphorylation of Akt, a well-studied serine/threonine protein kinase whose expression is associated with cell survival and protection against apoptosis. NGF has been shown to induce pAkt in neural cells (28) and to be an important negative regulator of TNFα-induced apoptosis in these cells.

To test this hypothesis, we cultured PBMCs from healthy donors with plate-bound antibodies to CD3/CD28 for 24 hours. During the last 2 hours of culture, we added TNFα, TNFα plus NGF, or nothing (Figure 6A). As predicted, NGF dramatically decreased TNFα-induced cell death in these cultures. Compared to the $22 \pm 2.5\%$ (mean $\pm$ SD) of live cells in TNFα-treated cultures, there were $60 \pm 4.5\%$ live cells in cultures treated with TNFα plus NGF ($P < 0.001$). Also as predicted, NGF (in the absence of TNFα) clearly up-regulated pAkt levels in PBMCs (purified magnetically using Dynabeads) (Figure 6B).

DISCUSSION

In the present study, we observed that SF samples from patients with inflammatory arthritis were enriched in NGF, that NGF induced marked proliferation of FLS cultured from the synovium of PsA and RA patients, and that TrkA was up-regulated on freshly isolated activated T cells from the joints of PsA and RA patients. With regard to our studies with PBMC-derived activated T cells, we observed that NGF induced T cell proliferation via TrkA and that T cell activation with CD3/CD28 up-regulated TrkA expression. Further, we noticed that NGF induced the phosphorylation of Akt (pAkt) in activated T cells and decreased the sensitivity of these cells to apoptosis induction. Thus, we propose that dysregulated production of NGF has the potential to proliferate and prolong the lifespan of activated T cells and that it also induces the proliferation of FLS and, hence, perpetuates the disease process.

Dean et al (29) were the first to report that NGF acts on lymphocytes. Those authors showed that NGF increased the blastogenic response of mouse spleen cells. There are conflicting data with regard to TrkA expression on lymphocytes. Ehrhard et al (3) demonstrated that in mitogen-activated murine CD4+ T cell clones, TrkA is expressed only on activated cells and that the TrkA in these clones is active, in that NGF signals the up-regulation of c-Fos expression (6). In contrast, Lambiase et al (30) reported that in human CD4+ T cell clones, both stimulated and unstimulated cells express TrkA receptors. This difference may reflect the species origin of the clones or something intrinsic to the way they were isolated.

The present study extends and clarifies these observations. We defined the mechanisms through which NGF may act on human T cells and introduced evidence supporting a model in which NGF plays a key role in the pathogenesis of autoimmune arthritis. We had previously shown that FLS cultured from PsA or RA synovial tissues produce significantly more NGF than do those from healthy individuals (9). Here, we demonstrated that SF from PsA and RA patients is enriched in NGF, which substantiates the notion that synovial fibroblasts are a prime source of NGF. Further, we observed that CD3/CD28 activation up-regulates TrkA expression on human PB T lymphocytes, which is consistent with the data from murine T cells reported by Erhard et al (3,6). We also observed that NGF is mitogenic for PB T lymphocytes and that this function is mediated via TrkA; that is, it is inhibited by the natural alkaloid K252a, which selectively inhibits NGF-induced Trk protooncogene tyrosine phosphorylation (31) (Figure 2).

We also showed that in addition to its mitogenic activity, NGF could increase T cell numbers by blocking T cell apoptosis induced by TNFα, which is known to deplete activated T cells in in vitro cultures (27). Furthermore, we showed that as in neural cells (32), this apoptosis blocking effect operates via phosphorylation of Akt, a key apoptosis regulator (33,34). Previous

studies have shown that binding of NGF to the ligand-binding domain of TrkA leads to TrkA autophosphorylation and activation of various signaling cascades. Activated phosphatidylinositol 3-kinase induces the activation of Akt through 3′-phosphorylated phosphatidylinositol as well as phosphoinositide-dependent kinase, which in turn, phosphorylates and activates Akt. This leads to the phosphorylation of CREB and IKK, which stimulates the transcription of prosurvival factors; pAkt also phosphorylates Bad, Forkhead, and caspase 9 and, thus, inhibits the proapoptotic pathway. Since we have directly demonstrated that NGF induces the phosphorylation of Akt in CD3+ T lymphocytes (Figure 4), we conclude that the underlying mechanism through which NGF promotes the survival of T lymphocyte populations is via induction of Akt phosphorylation.

Our data suggest that these mechanisms are likely to be operative in autoimmune diseases. Here we have shown that SF samples from PsA and RA patients have high levels of NGF. In addition, we have shown that in patients with inflammatory arthritis, a significant number of activated T cells (CD3+DR+) in the SF are TrkA+ (Figure 2) and that >90% of these TrkA+ cells are activated memory T cells (CD45RO+CD45RA–CD11a+). It is therefore expected that NGF would selectively promote the survival of activated memory T cells, the key pathologic cells in both PsA and RA.

Earlier reports suggested that NGF may influence the proliferative and inflammatory cascades in disease directly by regulating local pathologic events, such as proliferation of target tissues, promotion of angiogenesis, and induction of inflammatory responses, and/or they suggested that NGF might act indirectly by modulating the synthesis of neuropeptides, which in turn, induce the inflammatory reaction (2,7–9,12,17–19,35). In the series of experiments described herein, we found that NGF is produced at the site of inflammation and that it acts via its high-affinity receptor (TrkA) on the synovial cells (FLS) and memory T cells, the two key pathologic cells in the inflammatory/proliferative cascades of autoimmune rheumatic diseases. We further observed that NGF can promote the survival of these T cells and, hence, can prolong inflammation, which provides another paradigm for the perpetuation of a T cell–mediated inflammatory process such as that in PsA and RA.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. S. P. Raychaudhuri had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. S. P. Raychaudhuri, S. K. Raychaudhuri, Atkuri, Leonard Herzenberg, Leonore Herzenberg.

Acquisition of data. S. P. Raychaudhuri, S. K. Raychaudhuri, Atkuri, Leonard Herzenberg, Leonore Herzenberg.

Analysis and interpretation of data. S. P. Raychaudhuri, S. K. Raychaudhuri, Atkuri, Leonard Herzenberg, Leonore Herzenberg.

REFERENCES

1. Levi-Montalcini R. The nerve growth factor 35 years later. Science 1987;237:1154–62.
2. Bonini S, Rasi G, Bracci-Laudiero ML, Procoli A, Aloe L. Nerve growth factor: neurotrophin or cytokine? Int Arch Allergy Immunol 2003;131:80–4.
3. Ehrhard PB, Erb P, Graumann U, Otten U. Expression of nerve growth factor and nerve growth factor receptor tyrosine kinase Trk in activated CD4-positive T-cell clones. Proc Natl Acad Sci U S A 1993;90:10984–8.
4. Manning PT, Russell JH, Simmons B, Johnson EM. Protection from guanethidine-induced neuronal destruction by nerve growth factor: effect of NGF on immune function. Brain Res 1985;340:61–9.
5. Thorpe LW, Werrbach-Perez K, Perez-Polo JR. Effects of nerve growth factor on the expression of interleukin-2 receptors on cultured human lymphocytes. Ann N Y Acad Sci 1987;496:310–1.
6. Ehrhard PB, Erb P, Graumann U, Schmutz B, Otten U. Expression of functional trk tyrosine kinase receptors after T cell activation. J Immunol 1994;152:2705–9.
7. Aloe L. Nerve growth factor and neuroimmune responses: basic and clinical observations. Arch Physiol Biochem 2001;109:354–6.
8. Hattor IA, Iwasaki S, Murase K, Tsujimoto M, Sato M, Hayashi K, et al. Tumor necrosis factor is markedly synergistic with interleukin-1 and interferon γ in stimulating the production of nerve growth factor in fibroblasts. FEBS Lett 1994;340:177–80.
9. Raychaudhuri SP, Raychaudhuri SK. The regulatory role of nerve growth factor and its receptor system in fibroblast-like synovial cells. Scand J Rheumatol 2009;38:207–15.
10. Perez-Pinera P, Garcia-Suarez O, Prieto JG, Germana A, Ciriaco E, del Valle ME, et al. Thymocyte depletion affects neurotrophin receptor expression in thymic stromal cells. J Anat 2006;208:231–8.
11. Ciriaco E, Dall'Aglio C, Hannestad J, Huerta JJ, Laura R, Germana G, et al. Localization of Trk neurotrophin receptor-like proteins in avian primary lymphoid organs (thymus and bursa of Fabricius). J Neuroimmunol 1996;69:73–83.
12. Raychaudhuri SP, Jiang WY, Raychaudhuri SK. Revisiting the Koebner phenomenon: role of NGF and its receptor system in the pathogenesis of psoriasis. Am J Pathol 2008;172:961–71.
13. Barthel C, Yeremenko N, Jacobs R, Schmidt RE, Bernateck M, Zeidler H, et al. Nerve growth factor and receptor expression in rheumatoid arthritis and spondyloarthritis. Arthritis Res Ther 2009;11:R82.
14. Forsgren S. New data favouring that neurotrophins are of importance in arthritis. Arthritis Res Ther 2009;11:122.
15. Seidel MF, Herguijuela M, Forkert R, Otten U. Nerve growth factor in rheumatic diseases. Semin Arthritis Rheum 2010;40:109–26.
16. Grimsholm O, Guo Y, Ny T, Forsgren S. Expression patterns of neurotrophins and neurotrophin receptors in articular chondrocytes and inflammatory infiltrates in knee joint arthritis. Cells Tissues Organs 2008;188:299–309.
17. Heese K, Hock C, Otten U. Inflammatory signals induce neurotrophin expression in human microglial cells. J Neurochem 1998;70:699–707.

18. Horigome K, Pryor JC, Bullock ED, Johnson EM. Mediator release from mast cells by nerve growth factor: neurotrophin specificity and receptor mediation. J Biol Chem 1993;268:14881–7.
19. Pincelli C, Sevignani C, Manfredini R, Grande A, Fantini F, Bracci-Laudiero L, et al. Expression and function of nerve growth factor and nerve growth factor receptor on cultured keratinocytes. J Invest Dermatol 1994;103:13–8.
20. Lindsay RM, Shooter EM, Radeke MJ, Misko TP, Dechant G, Thoenen H, et al. Nerve growth factor regulates expression of the nerve growth factor receptor gene in adult sensory neurons. Eur J Neurosci 1990;2:389–96.
21. Yang XD, Liu Z, Liu HX, Wang LH, Ma CH, Li ZZ. Regulatory effect of nerve growth factor on release of substance P in cultured dorsal root ganglion neurons of rat. Neurosci Bull 2007;23:215–20.
22. Kawamoto K, Aoki J, Tanaka A, Itakura A, Hosono H, Arai H, et al. Nerve growth factor activates mast cells through the collaborative interaction with lysophosphatidylserine expressed on the membrane surface of activated platelets. J Immunol 2002;168:6412–9.
23. Bischoff SC, Dahinden CA. Effect of nerve growth factor on the release of inflammatory mediators by mature human basophils. Blood 1992;79:2662–9.
24. Altman R, Asch E, Bloch D, Bole G, Borenstein D, Brandt K, et al. Development of criteria for the classification and reporting of osteoarthritis: classification of osteoarthritis of the knee. Arthritis Rheum 1986;29:1039–49.
25. Arnett FC, Edworthy SM, Bloch DA, McShane DJ, Fries JF, Cooper NS, et al. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. Arthritis Rheum 1988;31:315–24.
26. Taylor W, Gladman D, Helliwell P, Marchesoni A, Mease P, Mielants H, and the CASPAR Study Group. Classification criteria for psoriatic arthritis: development of new criteria from a large international study. Arthritis Rheum 2006;54:2665–73.
27. Aggarwal S, Gollapudi S, Gupta S. Increased TNF-α-induced apoptosis in lymphocytes from aged humans: changes in TNF-α receptor expression and activation of caspases. J Immunol 1999; 162:2154–61.
28. Chao MV. Neurotrophins and their receptors: a convergence point for many signalling pathways. Nat Rev Neurosci 2003;4:299–309.
29. Dean DH, Hiramoto RN, Ghanta VK. Modulation of immune response: a possible role for murine salivary epidermal and nerve growth factors. J Periodontol 1987;58:498–500.
30. Lambiase A, Bracci-Laudiero L, Bonini S, Bonini S, Storace G, D'Elios MM, et al. Human $CD4^+$ T cell clones produce and release nerve growth factor and express high-affinity nerve growth factor receptors. J Allergy Clin Immunol 1997;100:408–14.
31. Berg MM, Sternberg DW, Parada LF, Chao MV. K-252a inhibits nerve growth factor-induced trk proto-oncogene tyrosine phosphorylation and kinase activity. J Biol Chem 1992;267:13–6.
32. Culmsee C, Gerling N, Lehmann M, Nikolova-Karakashian M, Prehn JH, Mattson MP, et al. Nerve growth factor survival signaling in cultured hippocampal neurons is mediated through TrkA and requires the common neurotrophin receptor P75. Neuroscience 2002;115:1089–108.
33. Bortul R, Tazzari PL, Cappellini A, Tabellini G, Billi AM, Bareggi R, et al. Constitutively active Akt1 protects HL60 leukemia cells from TRAIL-induced apoptosis through a mechanism involving NF-κB activation and $cFLIP_L$ up-regulation. Leukemia 2003;17: 379–89.
34. Crowder RJ, Freeman RS. Phosphatidylinositol 3-kinase and Akt protein kinase are necessary and sufficient for the survival of nerve growth factor-dependent sympathetic neurons. J Neurosci 1998; 18:2933–43.
35. Raychaudhuri SK, Raychaudhuri SP, Weltman H, Farber EM. Effect of nerve growth factor on endothelial cell biology: proliferation and adherence molecule expression on human dermal microvascular endothelial cells. Arch Dermatol Res 2001;293: 291–5.