

## **Skin homeostasis during inflammation: a role for nerve growth factor**

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**Summary.** The skin is a neuroendocrine immune organ in which many different molecules operate in autocrine-paracrine manner to guarantee tissue homeostasis in physiological and pathophysiological conditions. In this paper we examined NGF and p75 receptor expression in the skin, during CFA induced inflammation, in a time-course study. We also examined cutaneous innervation and proliferation, by means of immunohistochemistry and quantitative image analysis, RT-PCR and Western blot. Spontaneous and evoked pain-behavior was also measured in experimental rats. The main results can be summarized as follows: 1). a peripheral sensory neuropathy develops in this condition, as indicated by thermal hyperalgesia, thus leading to a sensory denervation of the hind-paw skin as indicated by disappearance of CGRP and PGP9.5-IR fibers; 2). NGF and p75 expression (mRNA and protein) increases in the skin (keratinocytes) in the acute phase of CFA inflammation; 3). at this stage, a higher proliferative activity is observed in the skin, as defined by the expression of cell cycle-associated protein Ki67; 4). in the long-lasting chronic phase there is a further up-regulation of NFG and p75 expression in the skin; 5). *trkA* mRNA expression inversely correlates with p75 and NGF mRNA expression. These results suggest that CFA chronic inflammation evolves from inflammation to a small fibers sensory neuropathy and NGF seems to play a role in both events.

**Key words:** Skin, NGF, CGRP, p75, CFA

### **Introduction**

A growing body of evidence indicates that the bidirectional communication between the different histological components of the skin, e.g. epidermal and

dermal cells, sensory and sympathetic nerve endings, vessels and inflammatory cells, plays a key role in the physiology of the skin, in the pathophysiology of peripheral inflammation and in somatic pain generation (Zimmerman, 2001; DeLeo and Yezierski, 2001; Smith and Liu, 2002; Marchand et al., 2005; McMahan et al., 2005). The activation of sensory nerve endings leads to vasodilation and inflammatory cells recruitment into peripheral tissues (neurogenic inflammation) (Schmelz and Petersen, 2001; Richardson and Vasko, 2002). Moreover, the cellular release of cytokines and neuroactive substances that modify excitability of peripheral nerve endings is involved in neuropathic pain generation and maintenance (Richardson and Vasko, 2002; Marchand et al., 2005; Moalem and Tracey, 2005). Thus, a number of chemical mediators present in the skin influence physiological (embryogenesis, vasoconstriction, vasodilatation, body temperature, barrier function, secretion, growth, differentiation, cell nutrition, nerve growth) and pathophysiological (inflammation, immune defense, apoptosis, proliferation, wound healing) functions within the skin, which acts as a true neuroimmune endocrine organ (Roosterman et al., 2006).

In this complex context, selected molecular players could interact with more than one type of cell, thus also becoming preferential targets for therapeutic intervention. Nerve growth factor (NGF) seems to be a good candidate for this possibility. NGF was first defined as an important survival factor in the development and proliferation of neurons of the nervous system, including sensory neurons of the dorsal root ganglia (DRG) (Levi-Montalcini and Angeletti, 1968). More recently, NGF has been identified as having a role in mature tissue and organs (Aloe and Calzà, 2004) as it is also a significant mediator in inflammation and skin physiology (McMahan, 1996; Roosterman et al., 2006), and the low- (p75) and high- (*trkA*) affinity NGF receptors have been postulated as also being involved in inflammation modulation (Allen and Dawbarn, 2006).

In order to highlight NGF's role in skin

pathophysiology, we investigated NGF and p75 expression in the skin during different phases of paw inflammation induced by complete Freund's adjuvant (CFA) injection in the rat. CFA injection, which is a widely used model for peripheral inflammation, results in a severe cutaneous paw inflammation with skin thinning, polyarthritis and the final alteration of joint structure (Calzà et al., 2000; Holmdahl et al., 2001).

## Materials and methods

### Animals

Male, pathogen free, 2 month-old, Sprague Dawley rats (Charles River, Varese, Italy) were used. The animals were housed under standard light/dark conditions (light on 7.00, off 19.00) in polypropylene cages. Paw inflammation was induced by a single intradermal injection (150 µl) of heat-killed *Mycobacterium butyricum* (DIFCO) suspended in complete Freund's adjuvant on the upper side of the tail under brief fentanyl/midazolam anesthesia (performed at the breeding center, Charles River, Saint Aubin Les Elfreuf, France). Paw inflammation was evaluated by clinical examination of the animals by two investigators blind to the animals' condition and by paw oedema measurement. A numerical score was attributed to oedema and reddening observed in the anterior and posterior paws and scrotum (0=absence; 1=low; 2=moderate; 3=severe) (Calzà et al., 1998, 2000). Animal evaluation started 3 days after the adjuvant injection. A total score (sum of the score attributed to the different areas for the symptoms evaluated) was then assigned to the animals during each observation. Only animals with signs of inflammation (score >1 for at least two body segments) 5 days after CFA-injection were included in the study. Animals developing skin lesions were excluded from the study. The volume for both hind paws in CFA-injected animals, such as control animals, was measured in triplicate using a water displacement plethysmometer (UGO BASILE, Italy) on day 3, 12 and 17 after CFA-injection. No further measurements were possible due to severe pain and joint stiffness. Data were plotted as volume (ml) of each paw at each time point.

Immunohistochemical and molecular biology experiments were carried out in groups of animals sacrificed 21, 52 and 88 days after CFA injection (Calzà et al., 1998, 2000; Giuliani et al., 2004). Five (immunohistochemical) and 4 (molecular biology) animals were included in each time-point group.

Experiments were conducted in accordance with current European Union and Italian law, with Italian Ministry of Health authorization number 123/2004-B. All experiments were designed to minimize the number of animals used as well as their discomfort. All allgesiometric assays were conducted in accordance with the ethical guidelines set out by the International Association for the Study of Pain (IASP) (Zimmermann, 1983).

### Immunocytochemical studies

Indirect immunofluorescence technique was performed on cryostat sections as previously described (Calzà et al., 2000). Briefly, after paraformaldehyde fixation, skin biopsy from the plantar surface of both hind-paws were removed, post-fixed, rinsed, quickly frozen and cryostat sections (14 µm) immediately processed for the immunofluorescence procedure. Slides from all animals were run in the same assay. The following primary antisera have been used in the study: goat anti p75 (Santa Cruz C-20, Santa Cruz CA), rabbit anti NGF (Santa Cruz M-20), rabbit anti CGRP (Peninsula Lab, Bubendorf, Switzerland), rabbit anti PGP 9.5 (Biogenesis, Kingston, NH). Fluorescein isothiocyanate- (FITC), cyanine- (Cy2) and rhodamine red-x succinimidyl- (RRX) conjugated secondary antisera were used, and slices were mounted in glycerol and PBS (3:1, v/v) containing 0.1% 1,4-phenylene-diamine to minimize fluorescence decay.

Images were collected using a Nikon Eclipse fluorescence microscope equipped with a Nikon DXM1200F camera (x125 final magnification) and quantification of skin immunocytochemical staining was carried out using Image Pro Plus software (Media Cybernetics, Silver Spring, MD). Five animals/group/time were used in each analysis. All samples were collected using the same exposure time and analyzed at the same time by a blind observer. For skin analysis, 5 sections/animal were used. Once all images had been collected, they were subjected to a normalization procedure in order to equalise background levels across sections. The entire epidermis included in the field (area of 100000 square microns) was then evaluated by measuring the total length of immunoreactive fibres, the epidermis area and thickness. Due to variation in epidermis thickness in the different CFA phases, the innervation index was calculated as follows: fibre length/epidermis area x epidermis thickness.

### RNA isolation and reverse transcription

Messenger RNAs (mRNA isolation kit, Roche Molecular Biochemicals) obtained from skin were subjected to DNase treatment (1U/ml, Roche Molecular Biochemicals), 10mM DTT (GIBCO BRL) and 4 U/ml RNase inhibitor (PROMEGA), at 37°C for 30 min. First strand cDNAs were obtained using 200U of the M-Moloney murine leukaemia virus (MuLV) reverse transcriptase enzyme (GIBCO/ BRL), 1x First Strand Buffer, 0.5 mM of each d(NTP)s (Roche Molecular Biochemicals) and 50 mM of the p(dN)6 random primers (Roche Molecular Biochemicals), incubation at 37°C for 50 min and 70°C for 15 min.

### Semiquantitative traditional PCR

Before setting the amplification conditions for each pair of primers used, linear regression curves assaying

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different amounts of retrotranscribed mRNA (cDNA) (Fig. 3) and different numbers of cycles of amplification (data not shown) were performed. Both of these parameters (cDNA amount and number of cycles of amplification) were chosen in the linear range. The oligonucleotides used as specific primers were: NGF, forward 5'-CCAAGGACGCAGCTTTCTAT-3', reverse 5'-CTCCGGTGAGTCTGTTGAA-3' (Scott et al., 1983) (402 bp); p75, forward 5'-GTCGTGGGCCTTGTGGCC-3', reverse 5'-CTGTGAGTTCACACTGGGG-3' (Troy et al., 2002) (497bp); *GADPH* (glyceraldehyde phosphate dehydrogenase), forward 5'-TCCATGACAACCTTGGCATCGTGG-5', reverse 5'-GTTGCTGTTGAAGTCACAGGAGAC-3' (Tso et al., 1985) (376bp). Amplifications were performed in a mixed reaction medium consisting of 670 mM Tris-HCl pH 8.8, 160 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.1% Tween-20, 2 mM MgCl<sub>2</sub>, 0.2 mM dNTPs, 0.05 U/ml of Taq DNA polymerase (EuroClone) and the corresponding oligonucleotides (forward and reverse) at a final concentration of 0.4 mM. Amplification parameters were: for NGF, 95°C 30 sec, 60°C 1 min 30 sec, 72°C 1 min 30 sec, 40 cycles; for p75, 95°C 30 sec, 54°C 1 min, 72°C 1 min, 40 cycles; for GAPDH, 95°C 30 sec, 60°C 30 sec, 72°C 30 sec, 30 cycles. The analysis of GAPDH mRNA was used as a control for cDNA quantities used as templates for PCR assays. PCR products were electrophoresed on 2% agarose gels, stained with ethidium bromide (SIGMA) and visualized under UV light. Specificity of amplifications was confirmed by the appearance of a single band of the expected size. The bands obtained were analysed using the AIS Imaging System software (Ontario, Canada). Results are shown as mean ± SEM of two different experiments and at least three different animals per group.

### Relative quantitative real-time PCR reactions

Real time PCR reactions were performed in the Mx3005P™ real-time PCR system (Stratagene, CA, USA) using SYBR-green I dye. Rat primers for trk-A and GAPDH real-time PCR were designed for SYBR-green real-time reactions by using the software Beacon Designer 4.0 (Premier Biosoft International, Palo Alto, CA, USA). The sequences of designed primers for trk-A amplification are: forward 5'-AAGCCGTGGAA CAGCATC-3', reverse 5'-TGCTACAGGGTTTCA TCCAG-3' (accession number MN\_021589); the sequences of primers for GAPDH amplification are: forward 5'-GGCAAGTTCAATGGCACAGTCAAG-3', reverse 5'- ACATACTCAGCACCAGCATCACC-3' (accession number M17701). The reactions were performed in a final volume of 25 ml consisting of 1x master mix (Brilliant SYBR-green QPCR master mix, Stratagene), 1.6 nM reference dye Rox (Stratagene) and 0.4 mM forward and reverse primers. Two step PCR reactions have been performed: 1. denaturation (95°C, 10 min; 2. annealing/extension (40 cycles of: 95°C for 15 sec, 60°C for 30 sec); At the end of PCR reaction the

melting curve of amplified products was performed following this temperature/time scheme: heating from 55°C to 95°C with a temperature increase of 0.5°C/sec. The relative mRNA level of trk-A gene was calculated in the base of the threshold cycles (CT) values obtained for each sample. The housekeeping gene GAPDH was used to normalize the amount of retro-transcribed mRNA used for PCR reactions.

Before processing the samples corresponding to the different groups of animals, the efficiencies of trk-A and GAPDH real-time PCR reactions were calculated by amplifying cDNA dilution series in the presence of trk-A or GAPDH primers in the conditions above described. Threshold cycle (CT) values for each pair of primers were used by MxPro QPCR software 3.0 (Stratagene, CA, USA) to calculate standard curves, slopes and efficiencies. As they were near 100% (99,1% for trk-A and 98% for GAPDH), in order to determine the relative amount of trk-A mRNA in each treated group of animals relative to the control group, we could apply the equation  $2^{-\Delta\Delta C_T}$  where

$$\Delta\Delta C_T = \text{DCT mean value of treated animals} - \Delta C_T \text{ mean value of control animals}$$

$$\Delta C_T \text{ treated animals} = C_T \text{ trk-A} - C_T \text{ GAPDH}$$

$$\Delta C_T \text{ control animals} = C_T \text{ trk-A} - C_T \text{ GAPDH}$$

The specificity of real-time PCR reactions was evidenced by the melting curve, obtaining a unique peak at the correspondent melting temperature (T<sub>m</sub>). Moreover, we wanted to analyse PCR products by electrophoresis on 2.5% agarose gel stained with ethidium bromide and visualized under UV light. A single band of the expected size was obtained for each pair of primers used (Fig. 3C).

### Ki67 Western blotting

Tissue homogenates from hind paws were prepared using a 10 mM Hepes, 1 mM DTT, pH 7.5 lysis buffer containing a protease inhibitor cocktail (Sigma, St. Louis, MO). Equal amounts of protein (5 µg) were separated in 15% SDS-polyacrylamide gels and electroblotted to nitrocellulose membranes. In order to block non specific protein binding sites, filters were incubated with blocking solution (Pierce) for 2 hours at room temperature and the primary antibody rabbit polyclonal anti-Ki67 1:2500 (Novo Castra) was then incubated overnight at 4°C. After washing for 1 hour with TTBS (TBS-0.05% Tween-20), filters were incubated with the secondary antibody anti-rabbit conjugated to horseradish peroxidase (Dako) 1:4000 for 30 min. at RT and washed again for another hour. The proteins were detected using an ECL chemiluminescent kit (Pierce). Densitometric analysis was performed using the AIS Imaging System.

### Statistical analysis

Descriptive statistics are reported as means±SEM. Data were analyzed with t-test and ANOVA followed by

multiple comparison tests. Duncan's test was used to compare experimental (CFA-injected, 21 and 79 days after CFA) and control groups in the ICC and molecular biology experiments; the Tukey multiple comparison test was used for plantar test experiments. Results were considered significant when the probability of them occurring due to chance alone was less than 5%.

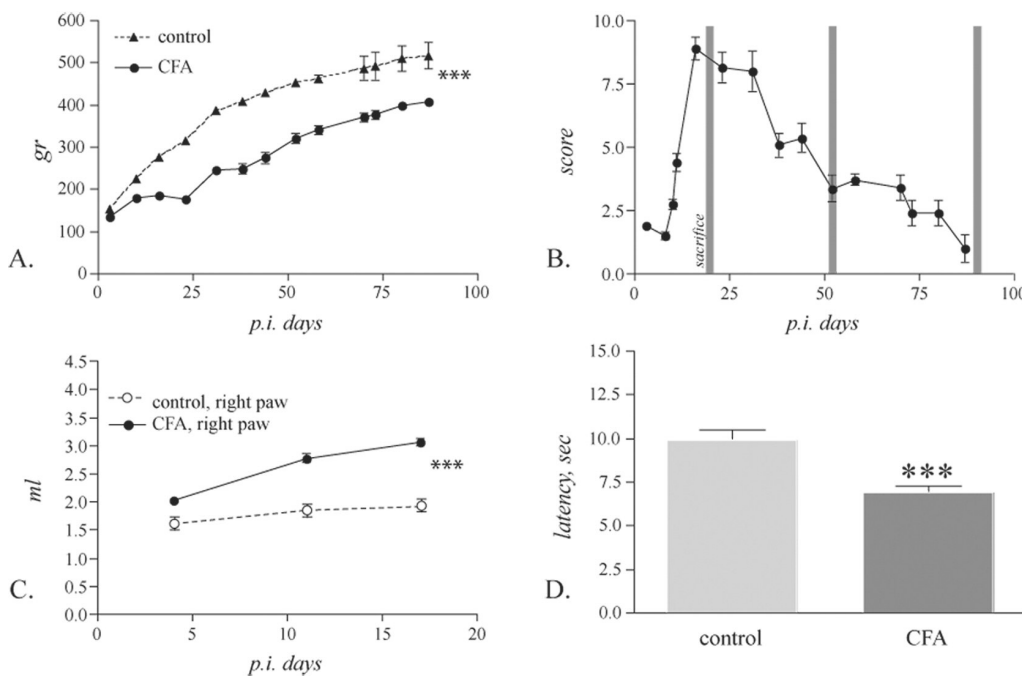
## Results

CFA injection at the base of the tail results in a severe cutaneous paw inflammation with skin thinning, polyarthritis and the final alteration of joint structure (Calzà et al., 2000). The course of the experimental pathology is illustrated in Fig. 1. The animals' distress was already evident from the body weight curve (A), which indicates a cessation of growth between 5 and 30 days, with corresponding increase in inflammation score (B), paw oedema (C) and thermal hyperalgesia (D). Paw oedema in one paw is evident already 5 days after injection, and at 17 days both hind paws in injected animals had a higher volume than control animals. There was a progressive increase in inflammation (Fig. 1B), which peaked 14-16 days after CFA injection and then remained elevated until day 27, after which it slowly decreased.

Hind-paw skin taken from animals without ulcerations was used for both morphological and biomolecular studies. Cutaneous innervation was evaluated by means of immunostaining for the protein gene product 9.5 (PGP9.5), which has been indicated as a sensitive marker for nerve terminals (Johnson et al., 1994) and for calcitonin gene-related peptide (CGRP),

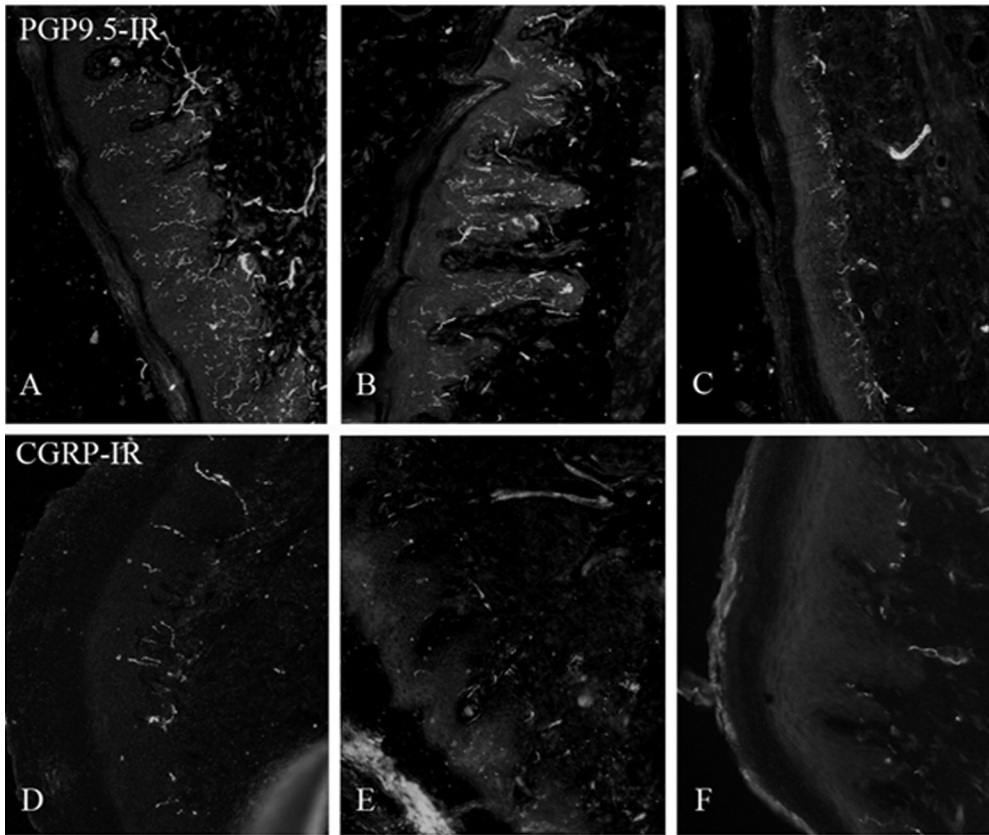
which is a marker for sensory innervation (Oaklander and Siegel, 2005). Our observations of PGP9.5 and CGRP-IR fibers in the normal rat footpad skin are consistent with those of previous studies (Ma and Bisby, 2000). PGP9.5-IR fibres are observed in the epidermis and dermis, and thinner fibers extend from the dermoepidermal junction into the epidermis (Fig. 2A). CGRP-IR fibers are also distributed in the superficial and deeper dermis, and some fibers penetrate into the epidermis, also reaching the superficial layers (Fig. 2D). No nerve fibers were recognized when the first antibody step was omitted. Quantitative analysis of skin innervation was performed in the epidermis in the acute phase (Fig. 2B,E) and at two different times: 55 and 88 days after CFA injection (Fig. 2C,F); results have been normalized according to the epidermis thickness and illustrated by the graphs in Fig. 2. In the epidermis, PGP9.5-IR innervation significantly increases in CFA animals at day 21, when irregular, thin fibres are observed in the most superficial layers, and nerve trunks are found at the dermoepidermal junction. Conversely, at 88 days there is a significant reduction of PGP9.5-IR fibres, and also of CGRP-IR somatosensory innervation compared to control animals.

We then analyzed NGF, p75 and TrkA mRNAs expression in the skin according to the same time-schedule (Fig. 3). In the first phase (21 days after CFA injection), there is an increase in NGF mRNA expression (Fig. 3B), which is followed by protein expression in cells in the dermis but also in the epidermis, where NGF is found in the cytoplasm of single cells or of small cell clusters. There is then a drop in NGF mRNA expression, which is strongly re-

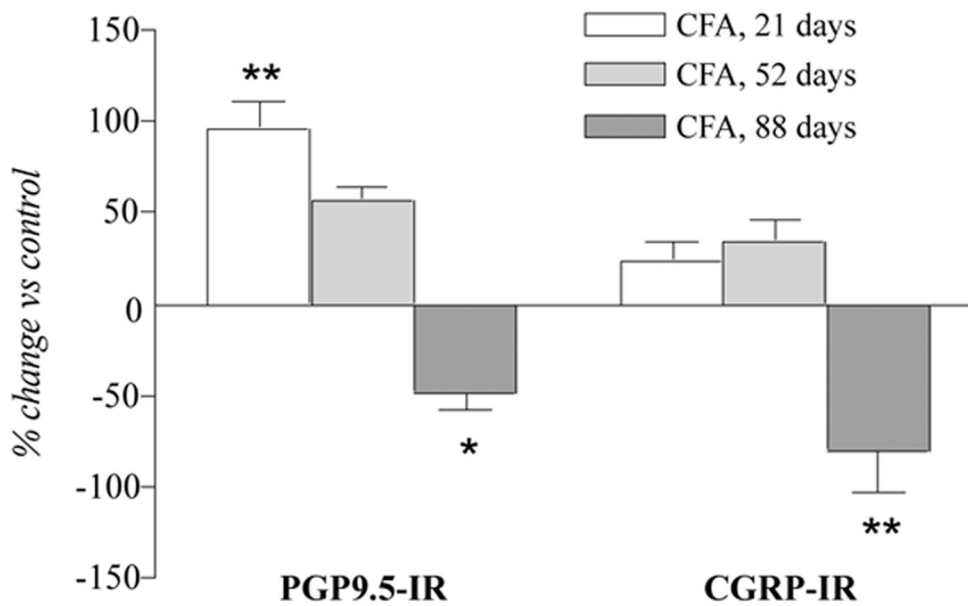


**Fig. 1.** Behavioral characterization of animals used in this study. **A.** Body weight curve of animals included in the study illustrates distress of CFA animals; statistical analysis ANOVA and post-hoc, \*\*\* $p < 0.001$ ; **B.** inflammation score, as evaluated throughout the experiment (see text for further explanation). Grey vertical lines indicate the day on which animals were sacrificed; **C.** paw oedema in CFA animals, as measured 4, 12 and 17 days after CFA injection vs control animals; statistical analysis ANOVA and post-hoc, \*\*\* $p < 0.001$ ; **D.** latency at thermal stimuli, as measured in freely moving animals 17 days after CFA injection, indicates severe hyperalgesia in CFA animals; statistical analysis: Student's t test,  $p < 0.001$ .

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IR/area x thickness



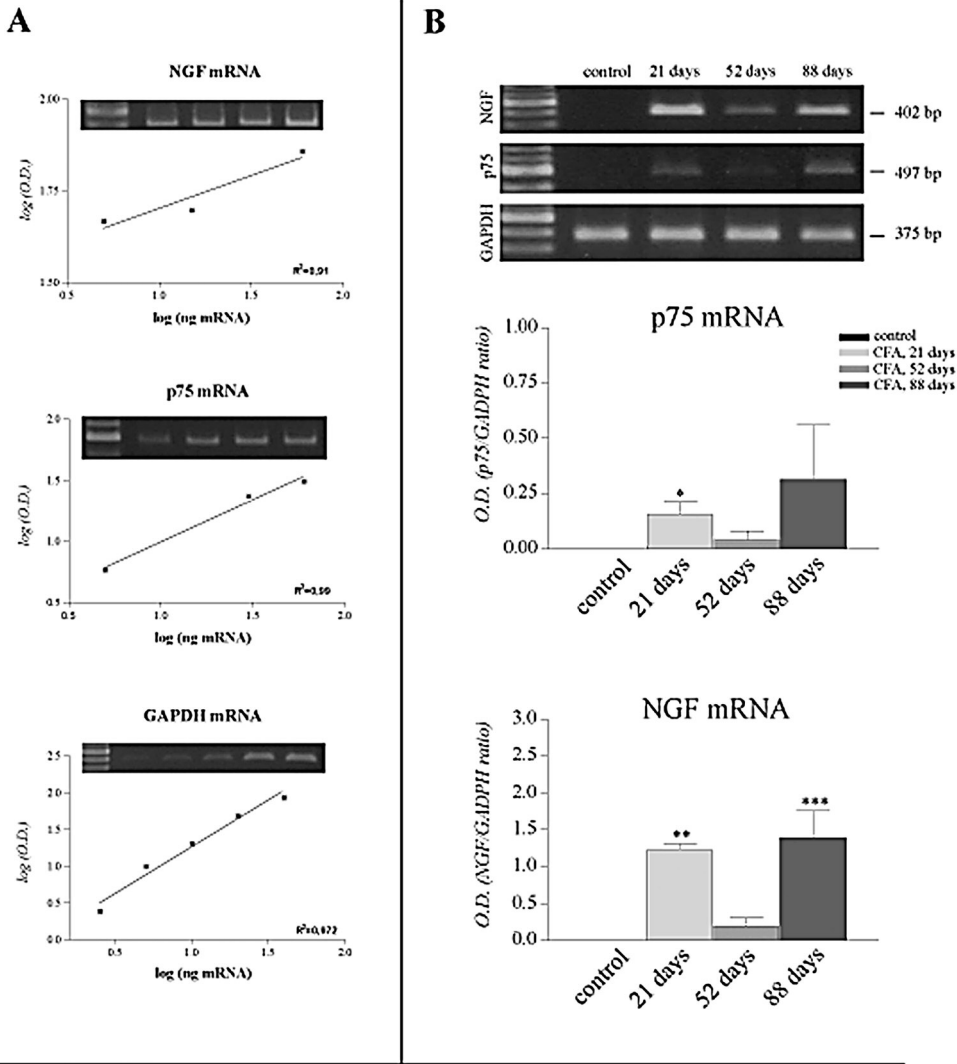
**Fig. 2.** Skin innervation in control and experimental animals. A-C: micrographs show PGP9.5-IR innervation of the plantar surface of hind-paw in control (A), CFA acute (B) and CFA chronic, 88 days (C); D-E micrographs show CGRP-IR innervation of the plantar surface of hind-paw in control (D), CFA acute (E) and CFA chronic, 88 days (F); the graph illustrates quantification epidermis sensory innervation, normalized according to epidermis thickness (see text for further details) and expressed as a percentage change compared to control animals (mean+SEM). Five animals were included in each group. Statistical analysis: one-way ANOVA and post-hoc test, \* $p < 0.05$ , \*\* $p < 0.01$ . Bar: 100  $\mu\text{m}$ .

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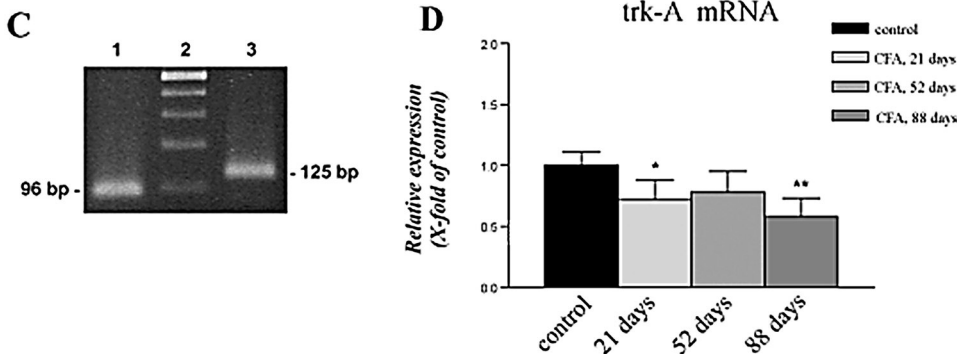
expressed in the last phase (88 days after CFA injection). A similar expression profile is shown by p75 mRNA (Fig. 3B), which is expressed by infiltrating cells in the dermis (not shown). Conversely, we found *trk-A* mRNA expression to be significantly down-regulated 21 and 88

days after CFA injection (28% and 42%, respectively, Table 1), being inversely regulated with respect to NGF and p75 mRNAs.

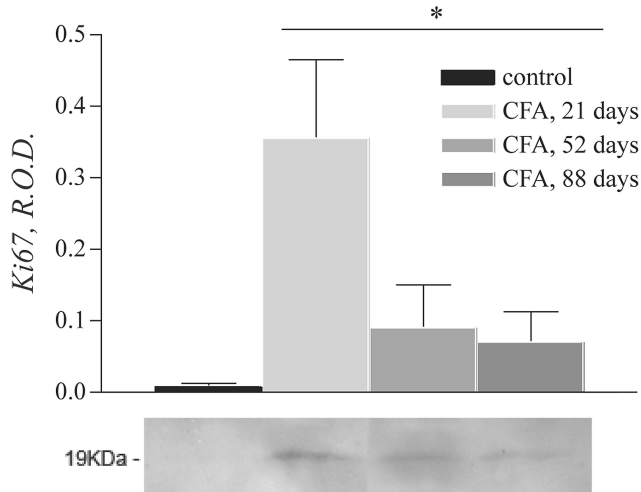
In view of the role of innervation in proliferative rate control of keratinocytes, in order to have an index of



**Fig. 3.** p75 and NGF mRNA expression in the skin of all experimental groups. **A:** standard curves for the semiquantitative RT-PCR assay for NGF, p75 and the internal standard GAPDH; **B:** the graphs represent the expression of NGF and p75 mRNA levels in the skin of the different groups, as studied by semiquantitative traditional PCR. Agarose gels in the upper part of panel B illustrate one representative PCR experiment with PCR products. Statistical analysis: one-way ANOVA and Dunnett's post-hoc test, \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ . **C:** shows an agarose gel with the real-time PCR products of *trk-A* (line 1) and GAPDH (line 3) amplifications. A DNA marker of 100 bp ladder has been used to estimate the size of the obtained PCR products (line 2). In graph D the relative expression of *trk-A* mRNA in treated groups of animals compared with control group has been represented. Four animals were included in each group. Student t test was used for statistical analysis, \* $p < 0.05$ , \*\* $p < 0.01$ .



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**Fig. 4.** Ki67 protein expression in the skin of all experimental groups, illustrating increased expression in the experimental animals. The blot represents a typical experiment. Four animals were included in each group. Statistical analysis: one-way ANOVA and post-hoc test, \* $p < 0.05$

cellular activity in the skin during CFA chronic inflammation, we measured the expression of the nuclear proliferation-associated antigen Ki67 (Fig. 4). We found a three-fold increase in the protein expression during the acute phase, whereas it was reduced to a one-fold increase in the chronic phases.

### Discussion

The experimental model used in this study is characterized by a severe cutaneous and deep (joint) inflammation involving paw soft tissues and joint structures, which evolves over time. The first phase, corresponding to day 21 after CFA injection, is characterized by severe joint oedema, spontaneous pain and thermal hyperalgesia; the two last phases, corresponding to days 52 and 88 after CFA injection, are identified by decreased inflammation scores and resolution of acute inflammation (Calzà et al., 2000). Here we demonstrated a complex regulation of NFG and p75 receptor expression in the skin during CFA inflammation, characterized by a biphasic expression profile. NGF and p75 mRNAs levels increase in acute inflammation, when NGF-IR cells are observed in the basal and spinal layers of the epidermis and also in a large number of infiltrating cells in the dermis, and in chronic, long-lasting phase, when epidermis sensory innervation disappears. The first increase could be related to the NGF role in inflammation, whereas the increase observed in the long-lasting phase corresponding to sensory neuropathy, could reflect a neurotrophic attempt by NGF. In the acute phase, a higher proliferative activity, as defined by the expression of cell cycle-associated protein Ki67, is also observed in the skin. In the long-term, epidermis sensory innervation

**Table 1.** Trk-A mRNA relative expression in treated groups of animals compared with control group.

GROUPS	$\Delta C_T$	$\Delta\Delta C_T$	Relative Expression to Control (range)
Control	5.73±0.11	0±0.11	1 (0.93-1.08)
21 days	6.21±0.16 *	0.72±0.16	0,72 (0.64-0.8)
52 days	6.08±0.17	0.78±0.17	0,78 (0.69-0.88)
88 days	6.5±0.15 **	0.58±0.15	0.58 (0.53-0.58)

$\Delta C_T$ :  $C_T$  trk-A average -  $C_T$  GAPDH average;  $\Delta\Delta C_T$ :  $\Delta C_T$  mean value of treated animals -  $\Delta C_T$  mean value of control animals; The S.E.M. (standard error mean) of the difference is calculated from the S.E.M. of the trk-A and GAPDH values. For the calculation of  $\Delta\Delta C_T$  a reference group has to be selected in order to subtract this constant value from the rest of the groups. As an arbitrary constant value is subtracted, the S.E.M. of  $\Delta\Delta C_T$  is the same as the S.E.M. of  $\Delta C_T$  (User Bulletin #2, ABI PRISM 7700 Sequence Detection System, 11 December 1997, updated 10/2001). Relative expression is calculated by the equation  $2^{-\Delta\Delta C_T}$  and the range for each group (numbers in parenthesis) has been calculated by  $2^{\Delta\Delta C_T + SEM}$  and  $2^{\Delta\Delta C_T - SEM}$ . Student t test was used for statistical analysis, \* $p < 0.05$ , \*\* $p < 0.01$ .

disappearance suggests that a small-fibre pathology is present in this chronic inflammatory model. Skin sensory denervation could cause neuropathic pain (Lauria, 2005), as described in postherpetic neuroalgia (Wasner et al., 2005), diabetic painful neuropathy (Shun et al., 2004), Sjogren syndrome (Chai et al., 2005) and also rheumatoid arthritis (Pozza et al., 2000).

NGF is synthesized by a variety of cell types, including cutaneous epithelium (Davies et al., 1987) where proliferating keratinocytes are the main source (Yar et al., 1991; Albers et al., 1994; Pincelli et al., 1994). However, inflammatory cells as well, such as mast cells, lymphocytes and eosinophils can release NGF (Raychaudhuri and Raychaudhuri, 2004) and NGF itself stimulates chemotaxis *in vitro* (Gee et al., 1983). NGF tissue level increases in various diseases and inflammatory states of the skin, such as atopic dermatitis (Dou et al., 2006) and the administration of NGF in laboratory animals has been shown to cause neutrophil accumulation at the site of *in vivo* injection (Boyle et al., 1985; Lewin et al., 1993; Bennet et al., 1998). NGF synthesis also correlates with keratinocyte growth *in vitro* (Di Marco et al., 1991) and *in vivo* (Constantinou et al., 1994; Pincelli et al., 1994) and participates in hair growth control (Botchkarev et al., 2004). Here we report that this correlation exists also in this *in vivo* model, as indicated by the high expression of the Ki67 cell cycle-associated protein in acute CFA-induced inflammation. This could reflect skin tropism maintenance during severe inflammatory states that also produce ulcers. It has actually been shown that skin wounding in neonatal rats produces a marked increase in NGF levels (Constantinou et al., 1994), and application of NGF to wound sites can lead to accelerated tissue repair and an enhanced rate of wound healing (Lawman et al., 1985; Matsuda et al., 1998; Aloe, 2004; Kawamoto and Matsuda, 2004). Notably, in the CFA chronic phase, which is also characterized by increased NGF synthesis,

the epidermis is thinner than in control animals (data not shown). One possible explanation is that the NGF effect on skin cell proliferation is mediated through nerve endings, which are lacking at this stage, since the keratinocyte mitotic rate is neurally controlled (Hsieh and Lin, 1999).

A large body of evidence supports the role of NGF in inflammatory hyperalgesia (Lewin and Mendell, 1993; McMahon, 1996; Woolf, 1996; Anand, 2004). NGF levels are elevated in injury, inflammation and pain states, skin- (Foster et al., 2002) and endothelia- (Foster et al., 2003) being the main sources; administration of NGF provokes pain and hyperalgesia whereas inhibition of NGF function reduces pain and hyperalgesia in animal models. Genetic disorders, including mutations in the genes that encode trkA and NGF, modify pain perception, so that novel classes of pain drugs based on antagonism of NGF are under development (Hefty et al., 2006; Myers et al., 2006). Also, in this study we found high NGF tissue levels associated with hyperalgesia, as defined by a decreased latency in thermal threshold tests.

The NGF increase observed in the skin 88 days after immunization could be related to sensory neuropathy. In CFA arthritis, small fibers disappear from the synovium (Mapp et al., 1990) and other joint structures, and here we indicate that cutaneous innervation also decreases. We used CGRP and PGP9.5 to study small fibres in the skin. Both markers recognized small unmyelinated (C) and thinly myelinated (Ad) fibres, and topical capsaicin studies have indicated that more than 90% of epidermis neurites are C and Ad fibres that transduce and transmit pain (Oaklander and Siegel, 2005). This indicates that a peripheral sensory neuropathy develops in CFA chronic arthritis. NGF plays a key role in the survival and properties of sympathetic and sensory neurons (Levi-Montalcini, 1987), 40% of which are sensitive to NGF during adulthood (Snider and McMahon, 1998), and peripheral denervation itself induces an increase in NGF expression by target tissues, including keratinocytes, and this sustains neuropathic pain (Li et al., 2003). Moreover, a restorative effect of NGF administration has been reported in diabetes-induced cutaneous axon loss in mice (Christianson et al., 2003), so that NGF present in the skin could prevent complete denervation. An increased expression of NGF is also found in atopic dermatitis lesions, which are characterized by an inflammatory cell infiltrate consisting of eosinophils, lymphocytes, macrophages and increased numbers of mast cell, indicating a pathophysiological participation of these cytokine and cells in this diseases (Gronenberg et al., 2005). In the animal model of the disease (NC/Nga mice) this correlates with increased epidermal nerve fibers density (Horiuchi et al., 2005) and administration of anti-NGF antibodies significantly inhibited development of epidermal hyper-innervation (Takano et al., 2005).

We also studied NGF low- and high-affinity receptor mRNAs expression in skin. p75 in the skin is synthesized by epidermal basal cells (Dou et al., 2006; Roosterman

et al., 2006), fibroblasts, keratinocytes, keratinocytes of the hair follicle root (Peters et al., 2006a) where it probably participates in hair cycle progression as growth terminators (Peters et al., 2006b). Parallel regulation that we found in NGF and p75 mRNA expression during CFA supports an autocrine-paracrine role of neurotrophins in regulating skin physiology and pathophysiology. TrkA is also expressed by basal keratinocytes, melanocytes and sweat glands (Adly et al., 2006), and trkA immunostaining could be observed in the Meissner's and Pacinian corpuscles and around small arteries in glabrous digital skin (Vega et al., 1994). A reciprocal regulation between NGF and TrkA proteins leading to parallel variations has been reported in several experimental and spontaneous pathologies including atopic dermatitis (Dou et al., 2006). Here we observed that the TrkA mRNA expression is down-regulated when NGF mRNA is up-regulated. This occurs in tissue samples which pool different cell types. Thus, these changes could be not related. Moreover, differential gene expression pattern of NGF, TrkA and p75 has been described during differentiation in rat bone marrow stromal cells (Yaghoobi and Mowla, 2006). Both high-(trkA) and low- (p75) affinity receptors seem to take part in mediating the listed NGF effects in the skin, but having different and still partially unknown roles (Hokfelt et al., 1994; Chen et al., 1999; Malik-Hall et al., 2005; Roosterman et al., 2006). Pharmacological experiments aimed to interfere with NGF and specific receptors are needed to clarify this complex scenario.

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