DEPARTMENT OF LIFE SCIENCES

FACULTY OF SCIENCES AND TECHNOLOGY OF
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Neurotensin effect on skin dendritic cells and fibroblasts: repercussions in wound healing and diabetic wound healing.

Efeito da neurotensina em células dendríticas e fibroblastos da pele: repercussões na cicatrização de feridas e feridas diabéticas.

Dissertation presented to the University of Coimbra to complement the necessary requisites to acquire the Master degree in Cell and Molecular Biology. The work was performed under the scientific orientation of the Professor Eugénia Carvalho (Center for Neurosciences and Cell Biology/University of Coimbra) and the Professor Maria Teresa Cruz (Faculty of Pharmacy/University of Coimbra) and under supervision of the Professor Margarida Castro (Faculty of Sciences and Technology /University of Coimbra)

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Sumário

A cicatrização de feridas é um processo complexo delineado pelas seguintes fases: homeostase/coagulação, inflamação, migração-proliferação e remodelação. As células da pele são fundamentais no decorrer deste processo, nomeadamente as células de *Langerhans* (LCs), os mastócitos (MCs), os macrófagos, os queratinócitos, as células endoteliais e os fibroblastos (FBs). Esta dissertação baseou-se no estudo das células LCs e FBs, através do uso da linha celular dendrítica proveniente da pele de fetos de ratinhos (FSDC) e da linha celular de FBs proveniente de recém-nascidos humanos (BJ), em condições normais e inflamatórias *in vitro* (estimulação através de um lipopolisacarídeo – LPS), num ambiente hiperglicémico (30 mM de glucose).

A activação de terminais nervosos sensoriais da pele é igualmente importante para a cicatrização, induzindo a expressão e libertação de neuropeptídeos na pele que se ligam a receptores acoplados a proteínas G das células da pele, modulando a activação de diferentes vias de sinalização. Na presente dissertação é estudado o efeito da neurotensina (NT) na activação das vias de sinalização Akt, cinases de proteínas activada por agentes mitogénicos (MAPK), nomeadamente JNK, p38 e ERK MAPKs, e na activação do factor de transcrição NF-κB. O efeito da NT na viabilidade e migração celular, na produção de óxido nítrico (NO), na síntese de citocinas/quimiocinas pro/anti-inflamatórias e na expressão de factores de crescimento, também foram avaliadas.

Os resultados evidenciaram que os efeitos da NT dependem da sua concentração, do tipo e quantidade de receptores a que se ligam e a célula em que induz efeito. Assim, enquanto 100 nM de NT induziu efeitos pro-inflamatórios, 10 nM de NT induziu efeitos anti-inflamatórios nas células FSDCs; nas células BJ, a NT induziu efeitos anti-inflamatórios de um modo dependente da dose utilizada. A prévia descrição dos efeitos da NT nas células, também abrange condições de inflamação *in vitro*, nas

quais se observou um aumento da síntese da NT nas células FSDCs, não observado nas células BJ, e um aumento da expressão dos receptores de NT nas células BJ, em oposição à sua diminuição nas FSDCs. Por conseguinte, estes resultados evidenciam o papel modelador da NT na inflamação, em que a NT pode ser produzida nas LCs e actuar de um modo parácrino em células da pele, como os FBs.

O tratamento das células FSDCs com 10 nM de NT diminuiu a activação das vias inflamatórias NF-κB e JNK, a produção de NO, a expressão das citocinas IL-6, TNF-α e IL-10 e o factor de crescimento vascular endotelial (VEGF), assim como activou a via de sobrevivência ERK e aumentou a síntese do factor de crescimento epidermal (EGF). Por outro lado, 100 nM de NT promoveram efeitos opostos. O tratamento das células BJ com 10 ou 100 nM de NT diminuiu a expressão das quimiocinas CCL4, CCL5, CCL11 e IL-8, assim como diminuiu a expressão das citocinas IL-1β e IL-6, de um modo dependente da dose utilizada. NT também induziu a expressão de EGF e promoveu a migração das células BJ.

Reduzidas doses de NT modulam negativamente a capacidade imune das células FSDC e BJ, contrastando com o efeito pro-inflamatório de outros neuropeptídeos, como SP e CGRP. O aumento da expressão de EGF, adicionado à diminuição da capacidade imune das células, é importante na inibição da fase inflamatória da cicatrização, e consequente progresso para as fases de migração-proliferação e remodelação. Pelo contrário, altas doses de NT podem promover efeitos patológicos nas células FSDC (estado pro-inflamatório), limitando a progressão da cicatrização. Estes resultados perspectivam o aparecimento de novas terapias para o tratamento da ferida diabética, caracterizada por neuropatia e pro-inflamação, podendo mesmo terminar em úlceras incuráveis nos pés diabéticos (DFUs), com consequente amputação.

Palavras-Chave: Úlcera do pé diabético, neuropeptídeos, neurotensina, cicatrização de feridas, células da pele, inflamação e vias de sinalização.

Abstract

Wound healing (WH) is a complex process which involves homeostasis/coagulation, inflammation, migration-proliferation and remodeling. Skin cells like Langerhans cells (LCs), mast cells (MCs), macrophages, keratinocytes, endothelial cells and fibroblasts (FBs) are fundamental to all stages of WH. In the present work, LCs and FBs were studied using a fetal skin dendritic cell line (FSDC) and a cell line obtained from normal newborn human foreskin fibroblasts (BJ), in homeostatic and *in vitro* inflammatory conditions, stimulated by lipopolysaccharide (LPS), and under a hyperglycemic (30 mM of glucose) environment.

The activation of sensory nerve endings of the skin is also important in WH, inducing the expression and release of neuropeptides into the skin that bind to G-protein coupled receptors in skin cells and modulate the activation of different signaling pathways. This work shows the effects of the neuropeptide neurotensin (NT) in the activation of the intracellular signaling pathways of Akt, Mitogen-activated protein kinases (MAPK) (JNK, p38 and ERK) and the transcription factor NF-κB. Moreover, the effect of NT on cell viability and migration, nitric oxide (NO) production, synthesis of pro- and anti-inflammatory cytokines/chemokines and expression of growth factors was also evaluated.

The results showed that NT effects were dependent on NT concentration, on the type and amount of receptors that bind to NT and, consequently, on the host cells. Accordingly, while NT induced a pro (100 nM) or anti-inflammatory (10 nM) effect in FSDCs, in BJ cells, NT only induced a dose-response anti-inflammatory effect. NT also promoted its effects in *in vitro* inflammatory conditions. In addition, NT synthesis was upregulated in FSDCs and barely synthesized in BJ cells, as well as, NT receptors were downregulated in FSDCs and upregulated in BJ cells, in inflammatory conditions.

These results enlighten that NT is an essential mediator of inflammation, produced by LCs to act in a paracrine way in surrounding cells, like FBs.

NT (10 nM) treatment downregulated the inflammatory pathways of NF-κB and JNK, the production of NO, the expression of the acute-phase proteins IL-6 and TNF-α, the anti-inflammatory cytokine IL-10 and the vascular endothelial growth factor (VEGF), while the survival pathway ERK and the epidermal growth factor (EGF) were upregulated in FSDCs. However, NT (100 nM) upregulated NO production and the expression of acute-phase proteins, while downregulated the expression of EGF.

BJ cells treated with NT (10 or 100 nM) also downregulated the expression of pro-inflammatory chemokines, namely CCL4, CCL5, CCL11 and IL-8, as well as, the expression of pro-inflammatory cytokines, like IL-1β and IL-6, in a dose-dependent way. NT also induced the expression of EGF and promoted the migration of BJ cells.

Low doses of NT negatively modulated the immune capacity of FSDCs and BJ cells, in contrast to other neuropeptides effects, like SP and CGRP that induce a pro-inflammatory status. In addition to this negative immunomodulation, the expression of EGF increases, important in the downregulation of the inflammatory phase of WH and promotion of the migration-proliferation and remodeling phases of WH. In contrast, high doses of NT may promote pathological effects in FSDCs (pro-inflammatory state), limiting the promotion of WH. These results may give new perspectives in the design of new therapies for diabetic wound healing, a disease characterized by neuropathy and a pro-inflammatory environment that can culminate into incurable diabetic foot ulcerations (DFUs) or even foot amputation.

Keywords: Diabetic foot ulcers, neuropeptides, neurotensin, wound healing, skin cells, inflammation and signaling pathways.

List of abbreviations

AMPs – Antimicrobial peptides

ANS – Autonomous Nervous System

APCs – Antigen-presenting cells

BCA – Bicinchoninic acid assay

bFGF – basic Fibroblast growth factor

BM – Bone Marrow

BSA – Bovine serum albumin

cAMP – cyclic Adenosine monophosphate

CAMs – Cell Adhesion Molecules

cGMP – cyclic Guanosine monophosphate

CGRP – Calcitonin gene-related peptide

CNS – Central Nervous System

COX – Cycloxigenase

CRF – Corticotropin-releasing factor

CRH – Corticotropin-releasing hormone

CTGF – Connective tissue growth factor

DAG – Diacylglycerol

DCs – Dendritic Cells

DEPC – Diethyl pyrocarbonate

DFUs – Diabetic foot ulcers

DMEM – Dulbecco's Modified Eagle Medium

DRG – Dorsal root ganglia

DUSPs – Dual specificity phosphatases

DWH – Diabetic wound healing

ECF – Enhanced chemifluorescence

ECM – Extracellular Matrix

EGF – Epidermal growth factor

ERK – Extracellular signal-regulated kinases

FBs – Fibroblasts

FGF – Fibroblast growth factor

FSDCs – Fetal skin dendritic cell line

FSDCs – Fetal skin dendritic cells

G-CSF – Granulocyte-Colony Stimulating Factor

GF – Growth factor

GM-CSF – Granulocyte Macrophage-Colony Stimulating Factor

GPCRs – G-protein coupled receptor

HGF – Hepatocyte growth factor

HPRT1 – Hypoxanthine phosphoribosyltransferase 1

ICAMs – Intercellular adhesion molecules

IFN – Interferon

IκB-α – inhibitor of NF-κB

IL – Interleukin

IMDM – Iscove's Modified Dulbecco's Media

iNOS – inducible Nitric oxide synthase

IP_3 – Inositol 1,4,5-triphosphate

IRF – Interferon Regulatory factor

JNK – c-Jun N-terminal kinases

KCs – Keratinocytes

LCs – Langerhans cells

LPS – Lipopolysaccharide

MAPK – Mitogen-activated protein kinases

MCs – Mast cells

M-CSF – Macrophage colony-stimulating factor

MIP – Macrophage inflammatory protein

MKPs – MAPK phosphatases

MMPs – Metalloproteinases

MSH – Melanocyte-stimulating hormone

MTT – 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

MΦs – Macrophages

NEP – Neutral endopeptidase

NF-κB – Nuclear factor immunoglobulin κ light chain of activated B cells

NGF – Nerve growth factor

NK – Neurokinin Receptors

NKs – Natural killer cells

NO – Nitric oxide

NPY – Neuropeptide Y

NT – Neurotensin

NTR – Neurotensin receptor

PACAP – Pituitary adenylate cyclase activating polypeptide

PAF – Platelet-activating factor

PARs – Proteinase-activated receptors

PBS – Phosphate buffered saline

PDGF – Platelet-derived growth factor

PDK-1 – Phosphoinositide-dependent protein kinase

PI3-K – Phosphoinositide 3-kinase

PIP_2 – Phosphatidylinositol-4,5-bisphosphate

PIP_3 – Phosphatidylinositol(3,4,5)-trisphosphate

PKA – Protein kinase A

PKB – Protein kinase B (=Akt)

PKC – Protein Kinase C

PLC – Phospholipase C

PMN – Polimorphonuclear cells (neutrophils + eosinophils + basophils)

PNS – Peripheral Nervous System

POMC – Pro-opiomelanocortin

PRRs – Pattern recognition receptors

ROIs – Reactive Oxygen Intermediates

SC – Stratum corneum

SN – Secretoneurin

SNS – Sensorial Nervous System

SP – Substance P

STT – Somatostatin

TBS-T – Tris-buffered saline containing Tween

TEWL – Transepidermal water loss

TGF – Transforming growth factor

TGN – Trans Golgi Compartment

Th – T helper cells

TLRs – Toll-like receptors

TNF – Tumor necrosis factor

TRPV1 – Transient receptor potential vanilloid 1

VCAM – Vascular cell adhesion molecule

VEGF – Vascular endothelial growth factor

VIP – Vasointestinal Polypeptide

VSMC – Vascular smooth muscle cell

WB – Western Blot

WH – Wound healing

Chapter I. Introduction

1. Anatomy and histology of the skin

The skin has a complex anatomy consisting of diverse cells and other components. Blood vessels are responsible for the supply of nutrients to the cell. Hair follicles help to regulate the skin temperature, absorb harmful radiation, prevent external agents to enter the skin and, in addition to sweat glands, promote a large surface area for sweat evaporation. Sweat glands can be divided in eccrine and apocrine glands. Ducts of eccrine glands pass through the dermis and epidermis and reach directly into the skin, producing sweat (water and salts) responsible for the regulation of body temperature and for the release of toxins from the body. Apocrine glands are formed from the same structure as the hair follicle and sebaceous glands and are responsible for the production of individual sexual odor. Sebaceous glands produce an oily secretion, called sebum that is secreted through the sebaceous duct into the hair follicle. These glands consist of lactic and fatty acids that are responsible to maintain the acidic pH (between 3 and 5) of the skin, which inhibits the growth of most microorganisms. Sensory and anatomic fibers make the connection between the Central Nervous System (CNS) and the Peripheral Nervous System (PNS), including the skin.

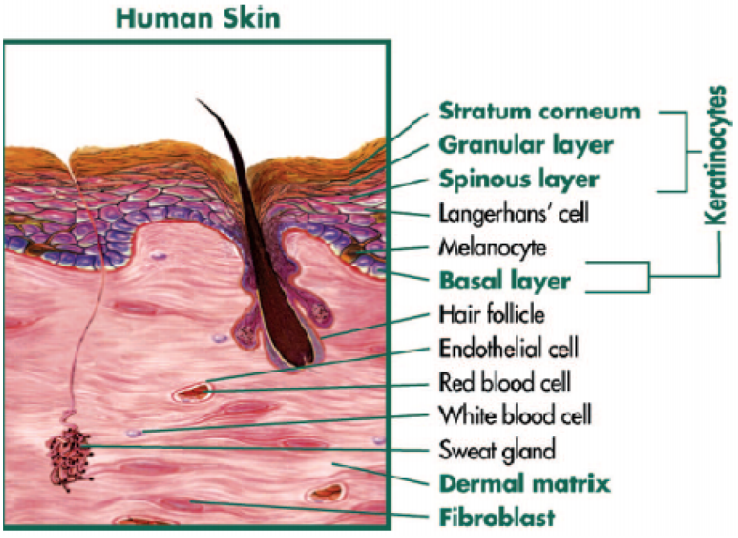


Figure 1 - Skin anatomy (Dinh T. & A. Veves, 2006).

The skin is divided in three main parts: epidermis, dermis and hypodermis (Figure 1). The epidermis is essentially occupied by keratinocytes (KCs) and Langerhans cells, the dendritic cells (DCs) of the epidermis. A lesser amount of melanocytes, LCs and Merkel cells are also part of the epidermis. Fibroblasts, macrophages (MΦs), mast cells and lymphocytes are dermis resident cells. Hypodermis is constituted mainly by adipocytes cells (Prost-Squarcioni C., 2006).

Due to an external agent or trauma, skin may activate inflammation. In this situation, monocytes, basophils, neutrophils, eosinophils, MCs, T and B lymphocytes and epidermal LCs become activated and may be recruited to the site of injury to mediate a proper inflammatory response. Indeed, these inflammatory cells play a major role in the skin, as they are more than anatomically required; they are a dynamic part of the skin.

2. Skin inflammation

Innate immunity provides the first and rapid line of defense against non-specific infections, such as pathogenic microorganisms or its components. The innate immune response attacks the pathogens and triggers non-specific cellular (by MΦs) and molecular response against them. Adaptive immunity is the second and slow response to external agents. It is a highly specific response that remains active in following injuries, by the immune system. The major agents of the adaptive immunity are lymphocytes and antibodies. Generally, most of the microorganisms encountered by a healthy individual are readily cleared within a few days by the defense mechanisms of the innate immune system before the activation of the adaptive immune system (Goldsby *et al*., 2003).

2.1. Innate immune system

Allergens, proteins, bacteria or UV radiation are substances that may penetrate the skin and initiate an inflammatory response (Wollenberg *et al*., 2001). Nonetheless, not only the exogenous agents can induce an inflammatory response. Endogenous factors may activate the immune response, such as pH, cytokines, kinins, histamines and proteases.

The human skin is the first barrier to environmental agents, preventing the entry of microbiologic flora and the damage of internal organs by physical agents. Innate immune system is responsible for this skin protection and acts through anatomic, physiologic, phagocytic and inflammatory barriers.

The skin anatomic barrier, besides hair follicles, sebaceous and sweat glands revised in the previous chapter, comprises a thick layer of lipids and proteins, nominated Stratum corneum (SC) that makes a barrier against the environmental ambient and prevents transepidermal water loss (TEWL) (Blank I.H., 1953; Winsor *et al*., 1944). Desquamation and secretion of mucus triggers the elimination of microorganisms which adhere to this layer. In addition to this anatomic barrier, a physiologic barrier composed of hydrolytic enzymes (Elias *et al*., 1991), antimicrobial peptides (AMPs) (Braff *et al*., 2005) and lipids (Sweeny *et al*., 1970), as well as, physiological skin's responses, such as pH, pattern recognition receptors (PRRs) like toll-like receptors (TLRs), fight against external factors (Goldsby *et al*., 2003).

2.1.1. Phagocytic activity and inflammation

If the previous referred barriers are not effective against pathogens, pathogens may start inflammation. Skin inflammation involves different mechanisms that can be divided in four events: vasodilation, increase in capillary permeability, influx of phagocytes and repair and regeneration of new tissue (Figure 2).

Vasodilation occurs through enlarged capillaries, which increase the temperature and induce tissue redness (erythema). The increased permeability of vessels facilitates the influx of fluid and cells from the capillaries into the tissue, provoking accumulation (exudate) and inducing tissue swelling (edema). The phagocytes enter into the tissue and kill the microbe, by releasing lytic enzymes. The digested material and the fluid of enzymes are nominated pus. After this initial process that culminates with inflammation, the tissue is repaired and regenerated.

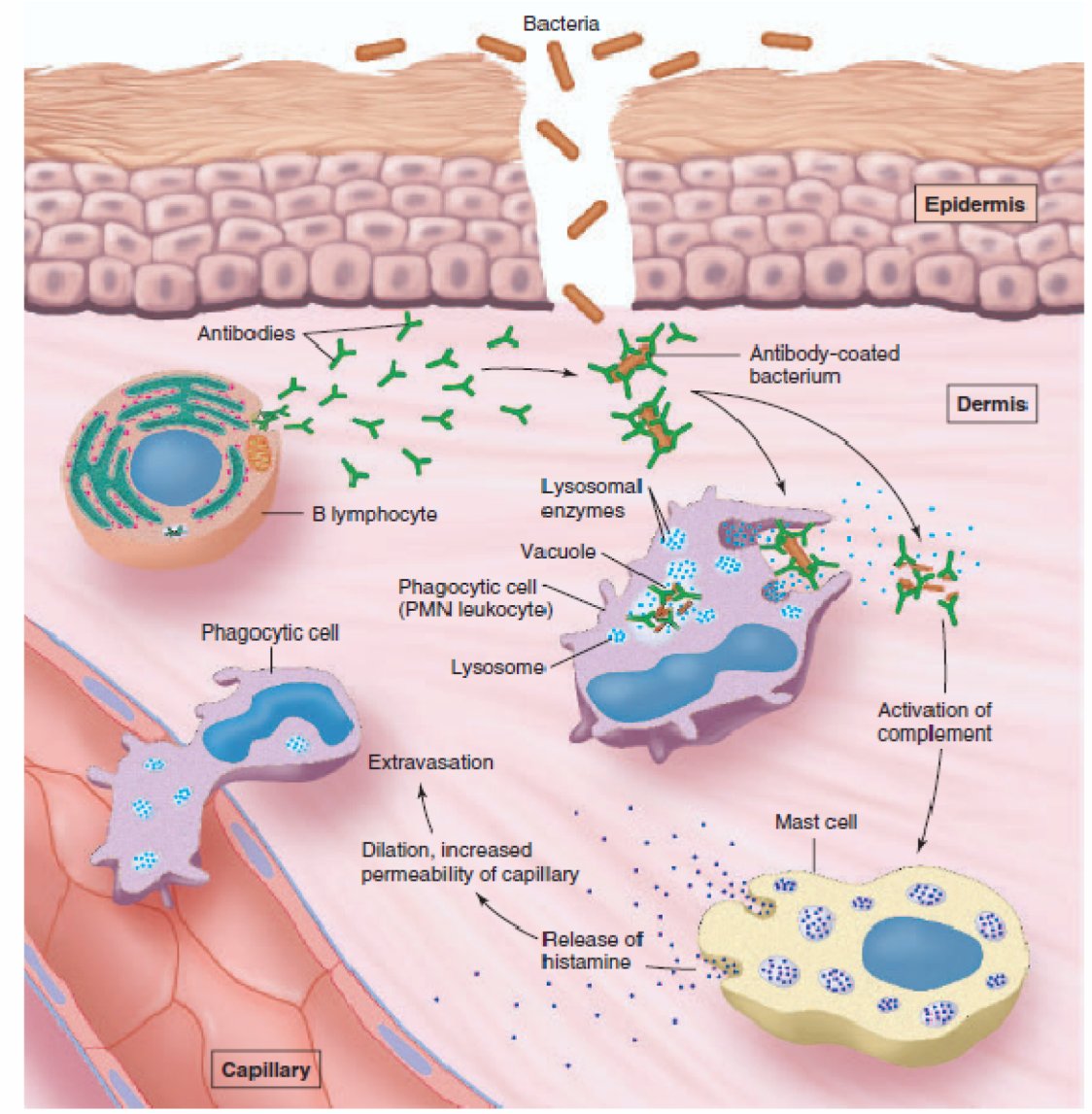


Figure 2 - Cellular and molecular events triggered during skin inflammation. Explained in detail in the text above (Fox S.I., 2003).

These inflammatory events involve chemical mediators that can be derived from the microorganism, released from damaged cells in response to tissue injury, generated by several plasma enzyme systems or products of various white blood cells. These chemical mediators can be acute-phase proteins, like C-reactive proteins, which cause complement-mediated lyses or complement-mediated increase in phagocytosis, histamine, which causes vasodilation and increased permeability, and kinins (for example: bradykinin).

When MΦs are activated they release reactive oxygen intermediates (ROIs), nitric oxide (NO) and proteolytic enzymes, in order to kill the pathogen. In order to augment NO levels, MΦs begin to express high levels of inducible nitric oxide synthase (iNOS) that oxidizes L-arginine and originates L-citrulline and NO. NO has a potent antimicrobial activity and when combined with the superoxide anion, it gives rise to more potent antimicrobial mediators. Activated MΦs also release growth factors (GFs) and cytokines, such as Granulocyte-macrophage colony-stimulating factor (GM-CSF), Granulocyte colony-stimulating factor (G-CSF), Macrophage colony-stimulating factor (M-CSF), Tumor necrosis factor (TNF)-α, Interferon (IFN)-α, Interleukin (IL)-1 and IL-6, as well as, complement proteins and chemokines, like CCL5. These inflammatory mediators activate other immune cells and induce the recruitment of neutrophils, monocytes and effectors T lymphocytes to the site of injury. MΦs also produce platelet-derived growth factor (PDGF) to stimulate the proliferation of ECs and the formation of blood vessels (angiogenesis), as well as, FBs proliferation.

The inflammatory response is followed by the repair and regeneration process. This phase is characterized by decreased capillary permeability and vasodilation, to ensure the activation of enzymes for blood-clotting and activate a cascade, which results

in the deposition of insoluble strands of fibrin and, as a result, the formation of a blood clot (Abbas A. & A.H. Lichtman, 2004; Goldsby *et al*., 2003).

2.2. Adaptive immune system

The first and quicker response to microorganisms occurs by the innate immune response, which then activates the adaptive immune system. The innate immune system not only initiates but also determines the degree and the specific ending of the adaptive immune response, which takes days to develop and provoke durable immunologic memory (Hammad H. & B.N. Lambrecht, 2008). Adaptive immunity is characterized by specificity, diversity, memory, self/nonself recognition and life-long immunologic memory to the infectious agents. Adaptive immunity is essentially mediated by B cells, T cytotoxic and T helper (Th) lymphocytes. These cells produce and display antigen binding cell-surface receptors that become activated (effectors cells) upon interaction with the antigen, mediating a final specific immune response, which leads to cytokines secretion. The adaptive immune response also regulates the innate response, minimizing tissues damage provoked by the innate response. Accordingly, regulatory T cells are able to repress the innate responses *in vitro* and *in vivo* (ex: Th2 cytokine response) (Lu *et al*., 2006; Ralainirina *et al*., 2007).

Endogenous and exogenous factors may activate KCs and LCs, leading to cytokine release that consequently activates the immunity of LCs, dermal DCs and MCs (resident innate immune system). These cells get mature and migrate to the draining lymph node. When reaching lymphoid organs, these cells present the antigen to naïve Th cells which, in turn, acquire a Th17, Treg, Tr1, Th3, Th1 or Th2 phenotype.

2.2.1. Lymphocyte acquisition of Th1/Th2 phenotype

The activation of the Th1 or Th2 response determines the outcome of the infection (Figure 3). Some invading pathogens stimulate the Th1 response, instead of the Th2 response; otherwise, others pathogens may stimulate higher Th2 cytokine differentiation than Th1. The predominant anti-inflammatory Th2 response inhibits MΦs, inhibiting parasites death which provokes pathogenicity (Abbas A. & A.H. Lichtman, 2004; Goldsby *et al*., 2003).

Th1 and Th2 cells release different cytokines that are important to regulate the cell's immune response, in order to elicit a pro or anti-inflammatory response,

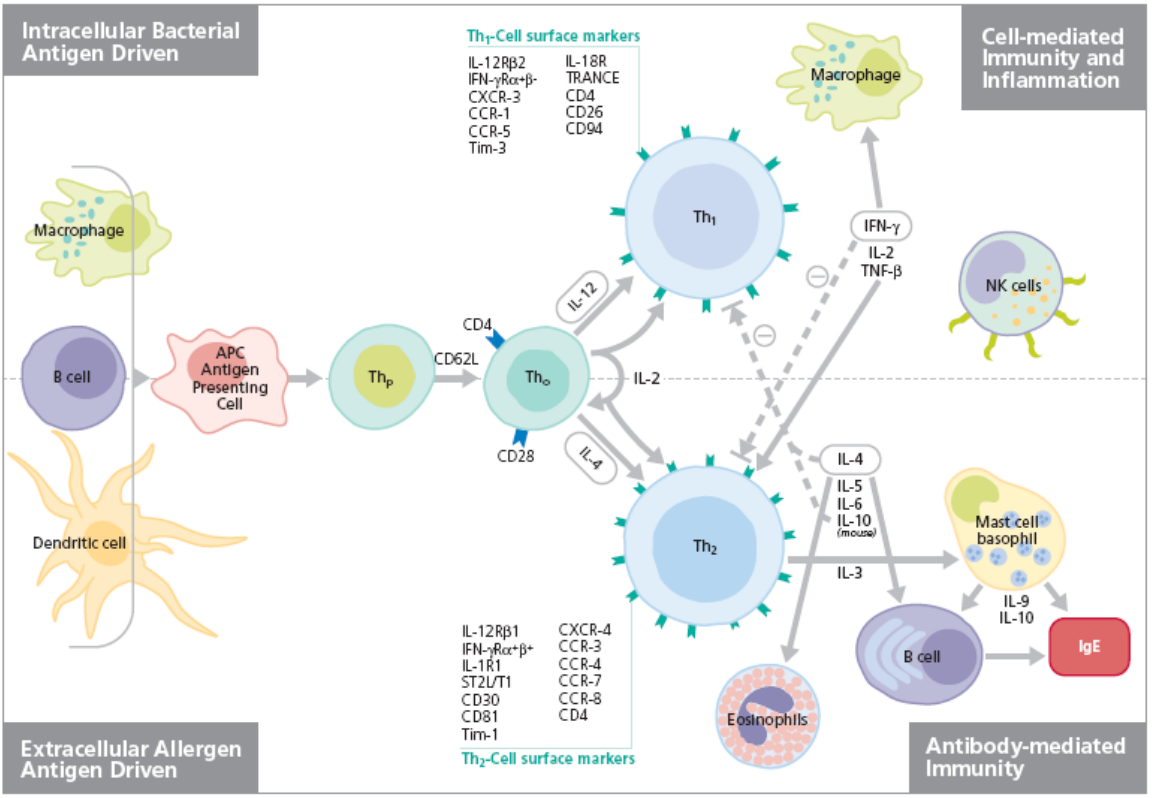


Figure 3 – Antigen-presenting cells induce T cells maturation and differentiation into Th1 or Th2 cells.
Tho cells express the cytokine IL-12 to induce Th1 maturation and express the cytokine IL-4 to induce Th2 maturation. Th1 cells mediate pro-inflammatory response by releasing IFN-δ, TNF-β, GM-CSF and IL-1; Th2 cells mediate anti-inflammatory response by releasing IL-4, IL-5, IL-6, IL-10 and IL-13 (BD Biosciences).

respectively. These cytokines trigger the specific Th response, as well as, inhibit the development and activity of the opposite response, inducing a cross-regulation of Th response. The Th1 response is mediated by the expression of pro-inflammatory cytokines – IFN-δ, TNF-β and GM-CSF – that are responsible for the elimination of intracellular parasites and the development of autoimmune responses. Indeed, in Th1 response, MΦs are intensely activated. This response is largely connected with the promotion of excessive inflammation and tissue injury. The Th2 response induces the production of specific cytokines: IL-4, IL-5, IL-13 and also IL-10. IL-4 stimulates the production of IgE antibody, IL-5 activates eosinophils, IL-4, IL-10 and IL-13 inhibit MΦ activation and consequently, terminate the Th1 response. The Th2 response also supports some types of allergic reactions.

2.2.2. Leukocyte migration

After leukocyte activation, these cells must migrate to the tissue containing the stimulus. Indeed, to make possible leukocytes departure from lymphoid organs, cells must adhere to and pass between ECs lining the walls of blood vessels, through a process denominated extravasation. Extravasation occurs in four phases: rolling, activation by chemoattractant stimulus (essentially IL-8 and MIP-1β), arrest and adhesion plus transendothelial migration. Activated leukocytes start expressing G-protein coupled receptors (GPCR) to chemoattractant stimuli released by ECs of the blood vessels. Indeed, when chemoattractant molecules are released, lymphocytes start migrating into the blood vessels (transendothelial migration). Chemoattractant stimuli include platelet-activating factor (PAF), complement split products (C5a, C3a and C5b67) and chemokines. These chemokines belong to different classes: CXC chemokines (or CXC ligands – CXCLs), CC chemokines, C chemokines or CX3C chemokines (Butcher E. & L.J. Picker, 1996; Cinamon *et al*., 2001).

Chemokines can be divided into inducible or ‘‘inflammatory’’ chemokines and constitutively expressed or ‘‘homeostatic’’ chemokines. Inflammatory chemokines are critical for attracting a diverse set of effectors leukocytes, as well as, neutrophils, monocytes/MΦs, DCs, and NKs cells, to the inflammatory sites, playing a key role in the innate immune response. Inflammatory chemokines typically bind to more than one chemokine receptor, suggesting redundancy in the inflammatory chemokine network. Homeostatic chemokine receptor/ligand pairs, mediated by CXC chemokine receptor–CXC chemokine ligand, are important for migration of antigen-presenting cells (APC) and lymphocytes into the lymph node, whereas other homeostatic chemokine receptor/ligand pairs are important for a specific traffic of effectors T cells. Both actions are critically important for an effective adaptive immune response (Rot A. & U.H. vonAndrian, 2004).

The binding of chemoattractant molecules to GPCRs of activated leukocytes induces the intracellular signaling pathway nuclear factor-κB (NF-κB) pathway that triggers neutrophil migration through the vessel wall into the tissues and upregulates leukocyte-specific adhesion molecules (CAMs) expression by ECs, such as E-selectin, P-selectin and intercellular adhesion molecule (ICAM)-1. These adhesion molecules are responsible for the binding to CAM receptors of the activated and migrating leukocytes (Kupper T. & R.C. Fuhlbrigge, 2004).

After extravasation, antigen presenting cells (APCs) are able to present the antigen to naive and memory T cells, inducing the recruitment of other immune cells from the blood vessels – monocytes, basophils, neutrophils, eosinophils, MCs, T and B lymphocytes and DCs.

3. Wound healing (WH)

WH is a dynamic and complex process that involves several cells, including a variety of immune cells and inflammatory mediators. WH can be divided in four overlapping phases: homeostasis/coagulation, inflammation, migration-proliferation and remodeling. Homeostasis lasts 2–3 h, the fibrin plug is formed and aggregated platelets release pro-inflammatory mediators such as cytokines and GFs. Cytokines recruit neutrophils and monocytes to the wound site activating the inflammatory phase of WH, lasting from hours to days. The proliferation phase occurs in different cell types such as epithelial cells, ECs and fibroblasts. After cytokine stimulation, these cells migrate to the wound site – migration phase. Extracellular matrix (ECM) is deposited and begins the angiogenesis and re-epithelialisation, contracting and closuring of the wound. The remodeling phase (can last several weeks) and is characterized by ECM adjustment and formation of scar tissue. Wound healing involves the activation of different cells, namely KCs, FBs, ECs, LCs, MΦs, MCs and platelets, inflammatory mediators, such as GFs, chemokines and cytokines, and it also requires complex biological and molecular events that induce cell migration, cell proliferation and ECM deposition (Singer *et al*., 1999).

4. Growth factors (GFs) in wound healing

In the skin, GFs are important mediators in the complex process of WH. These mediators are released by the majority of the skin cells and bind to their receptors which are essentially transmembrane tyrosine kinase receptors, involved in the activation of different signaling pathways, such as the MAPK and the Akt, which *per se* activate transcription. EGF, VEGF, fibroblast growth factor (FGF), PDGF, nerve growth factor (NGF) and transforming growth factor-β (TGF-β) are important growth factors in the skin. PDGF is chemotactic for migration of cells into the healing skin wound, as well

as, it enhances the proliferation of FBs and induced fibrosis. FGFs stimulate the proliferation of mesodermal, ectodermal and endodermal cells origin, as well as, regulate the differentiation of their target cells. EGF promotes epidermal thickness and cellularity, stimulated by epidermal KCs proliferation and differentiation, as well as, enhances KCs migration. VEGF is a major regulator of vasculogenesis and angiogenesis during WH. NGF is essential for development and survival of some sympathetic and sensory neurons with a relevant role in the skin as they release neuropeptides that mediate inflammation. TGF-β is important for reepithelization and tissue formation, for the expression of integrins necessary to KCs migration, as well as, to stimulate angiogenesis, fibroblast proliferation, myofibroblasts differentiation and matrix deposition. Considering all these GFs functions, it is implicit that GFs are essential for the later phases of wound healing, the proliferation and remodeling phases (Werner *et al*., 2003).

5. Diabetic wound healing (DWH)

Currently, diabetes affects over 285 million adults. As a consequence, diabetic people may develop coronary heart disease, chronic kidney disease, diabetic ketoacidosis, diabetic retinopathy and obesity. Additionally, diabetic people can develop wound healing difficulties, persisting in three main body areas: the eye, skin and bones.

Diabetic keratopathy is a consequence of defective healing in corneal epithelium injuries, which is important as a barrier against ocular infection in the eyes. Patients with keratopathy have abnormalities in corneal re-epithelialization associated with persistent epithelial defects, infectious corneal ulcers, decreased corneal sensitivity, increased epithelial fragility, secondary scaring, punctuate keratopathy, edema and loss of vision (Cisarik-Fredenburg P., 2001).

Diabetic foot ulcer (DFU) is a painless and harmful disease that also affects diabetic people and is characterized by delayed wound healing, consequence of differential causes. Sensory denervation - neuropathy - is an emerging risk of the development of DFUs. In addition to neuropathy, these patients have impaired angiogenic response and decreased blood flow supply to the site of injury (Galiano *et al*., 2004; Singer *et al*., 1999). Delayed wound healing can also be explained by cytokines deregulation, inhibiting the last phase of wound healing: migration-proliferation and remodeling (Goren *et al*., 2006). Accordingly, TNF-α, IL-1, IL-6 and CCL5 are produced in large amounts by immune cells and adipocytes, in type 2 diabetic patients (Bogdanski *et al*., 2007). Nevertheless, other inflammatory mediators like GFs, specifically bFGF, PDGF and VEGF are diminished in expression or their activities are impaired. Diabetic wounded skin also presents impaired expression of ECM proteins and misbalance between the accumulation of ECM components and their remodeling by matrix metalloproteinases (MMPs) (Lobmann *et al*., 2002).

Focusing on skin cells from DFUs, like KCs and FBs, these cells have deregulated migration and proliferation (Loots *et al*., 2002). In hyperglycemia and inflammatory conditions, leukocytes (mainly neutrophils) also have defective migration to the wound (Goren *et al*., 2006; Singer *et al*., 1999). Neutrophils have deficient chemotactic, phagocytic and microbicidal activities, augmenting the susceptibility and severity of infection in diabetes (Stegenga *et al*., 2008). MΦs undergo morphological transformations and decrease inflammatory mediators release, like TNF, IL-1 and VEGF (Zykova *et al*., 2000). Lack of upregulation of chemokines, cytokines and GFs in KCs and dermal ECs, in the margin of chronic DFUs, associated with the reduced influx of immune cells, may lead to poor formation of granulation tissue and chronicity of ulcer epithelialization (Galkowska *et al*., 2006).

Considering all these deficiencies in the skin of diabetic people, an extra disturbance, such as a physical stress, can be a potential cause of foot ulcers or even bone healing problems, like the Charcot neuroarthropathy foot (characterized by weakened bones that can fracture) (Singer *et al*., 1999), that may lead to a final foot amputation.

6. Skin as a neuroimmunoendocrine organ

Neuropeptides circulate between the brain and peripheral organs, through the CNS, ANS and Sensorial Nervous System (SNS). SNS includes sensory receptors that sense internal or external, chemical or physical alterations, namely chemoreceptors, mechanoreceptors, noniceptors, photoreceptors and thermoreceptors, as well as, afferent sensory neurons that transmit the perceived information. Sensory neurons have the cell body located in the dorsal root ganglia of the spinal nerve, in the spinal cord, or in the brain and can have different conducting fibers, namely A fibers (α, β and δ) and C fibers: A fibers are myelinated and high conducting although C fibers are unmyelianted and slow conducting. The autonomous nervous system includes parasympathetic ganglia (close to the target organ), efferent parasympathetic motor neurons, sympathetic ganglia (located in the spinal cord) and efferent sympathetic motor neurons (Roosterman *et al*., 2006). To understand the relevance and function of these nerves, the pungent agent from chilli peppers – capsaicin – that results in the depletion of sensory neuropeptides and permanent degeneration of small-diameter C-fibers by binding to the transient receptor potential vanilloid 1 (TRPV1), as well as, it's antagonist – capsazepine - have been used in research (Bevan *et al*., 1992).

Endogenous factors, such as pH, cytokines, kinins, histamine, proteases and exogenous agents, like allergens, bacteria and UV radiation (Wollenberg *et al*., 2001) activate sensory nerve endings. After injury, MCs release histamine (Figure 4A) which activates sensory neurons via H1 receptors, inducing orthodromic stimulation to spinal cord (Figure 4B) and antidromic stimulation to surrounding skin cells (Figure 4C), triggering the release of neuropeptides (Figure 4D) (Maggi C., 1991). Orthodromic stimulation is characterized by the transmission of stimuli through unmyelinated C or myelinated A primary afferent nerve fibers, via dorsal root ganglia to the spinal cord and then to specific areas of the CNS, resulting in the perception of pain, burning or other symptom (Figure 4E). Consequently, it results in the release of neuropeptides in the site of the skin stimuli by efferent autonomous motor neurons.

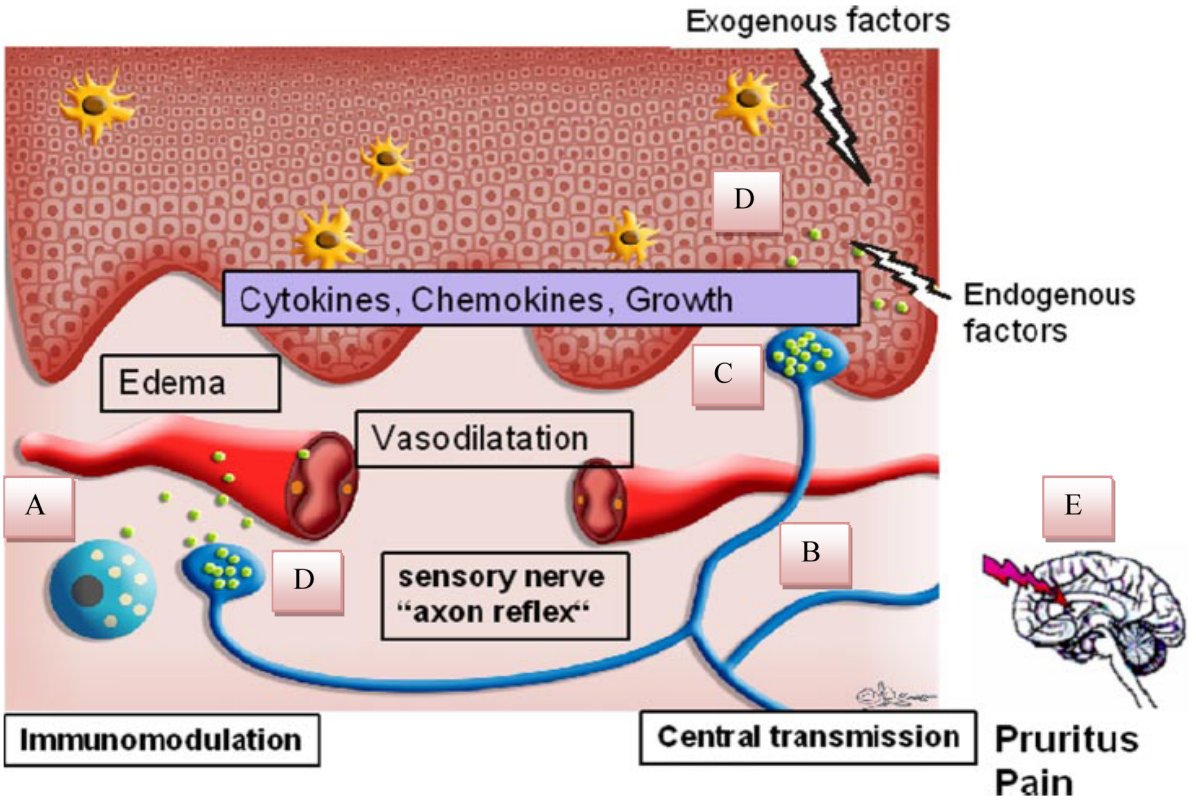


Figure 4 – Neuroimmunoendocrine organ. Read text above for detailed information (Adapted from Roosterman *et al*., 2006). Legend: A- Histamine release by MCs; B – Orthodromic stimulation of spinal cord; C – Antidromic stimulation of skin cells; D – Neuropeptides release; E – Perception of pain, burning or other symptom.

Antidromic stimulation is characterized by axon reflex in the neighboring afferent nerve fibers with a final release of neuropeptides. The analgesic morphine can activate opioid receptors located on primary afferent sensory nerve terminals, reduce the excitability of these neurons and suppress the antidromic release of pro-inflammatory neuropeptides, like Substance (SP) and Neurokinin (NKA). Consequently, providing this analgesic to ulcer patients delays wound healing (Rook *et al*., 2009). Finally, neuropeptides released from cutaneous nerves act on target cells via a paracrine, juxtacrine and endoncrine pathways, similar to cytokines.

In the last three decades, neuropeptides received further attention by the scientific community. These molecules play an important role in wound healing, not only in the adult stage, but also in fetal skin wound healing. In a study with fetal rats during skin development, nerves and neuropeptides were upregulated and contributed to the survival and regeneration of peripheral neurons and modulated an ideal wound healing process - scarless wound healing (Antony *et al*., 2010). However, higher levels of nerves and neuropeptides with reduced neutral endopeptidase (NEP) levels were shown in human and porcine hypertrophic scar with exuberant inflammation (Scott *et al*., 2007). These results reveal the importance of neuropeptides in wound heal, particularly, their effect on skin cells and in the modulation of skin inflammation.

6.1. Neuropeptides in diabetic wound healing

6.1.1. Denervation of diabetic wounded skin

Neuropathy is a possible cause of delayed diabetic wound healing and this may be caused by altered metabolism of glucose via the polyol pathway resulting in sorbitol accumulation, ischemia, superoxide-induced free-radical formation and impaired axonal transport (Apfel S.C., 1999). Diabetic skin has motor, sensory and autonomic fibers denervation: motor neuropathy causes weakness and waste of small intrinsic muscles;

sensory neuropathy limits the sensations of pain, pressure, temperature, and others; and autonomic denervation causes arteriovenous shunting, leading to vasodilation in small arteries (Dinh *et al*., 2004). Chronic nerve compression in addition to neuropathy, may lead to amputation. Recent approaches in clinical surgery - neurosurgical prevention - showed relief of pain in 88% and restoration of sensation in 79% of patients, preventing ulceration and amputation (Dellon A.L., 2007).

Diabetic neuropathy contributes to the impairment of wound healing. Accordingly, the work of Gibran *et al*. (2002) demonstrated that there were fewer nerves in the epidermis and papillary dermis of skin from diabetic human patients and diabetes (db/db) murine skin (Gibran *et al*., 2002). Ever since, db/db murine skin became a model to study the role of nerves in cutaneous injury, as these mice developed neuropathy and delayed healing. Additionally, diminished release of neuropeptides from nerves may be the possible cause of impaired wound healing, as Engin *et al*. (1996) demonstrated delayed wound contraction and loss of neuropeptide secretion from nerve endings in denervated tissues.

Indeed, further studies were done to identify which neuropeptides are decreased due to the denervation and what function they had in the skin that turned impaired. However, some other neuropeptides may have higher release and improved function. In the next sections, several studies will be presented, describing the state of the art about the importance of neuropeptides in wound healing.

6.1.2. Decreased SP, CGRP, NPY and POMC peptides in DWH

SP promotes a pro-inflammatory environment, inducing vasodilation, angiogenesis, leukocyte chemotaxis and adhesion to ECs, which ensures the extravasation, migration and subsequent accumulation of leukocytes into the site of injury. Some studies performed in mice show that in normal wound healing, SP release

induced by stress augments the growing nerves and connection of SP to MCs, leading to a rise in apoptotic cells in the skin (Peters *et al*., 2005). Additionally, SP upregulates NO production, enhancing wound closure kinetics and epithelialization (Muangman *et al*., 2009), it also increases the density of neutrophils, DCs, ECs and MΦs, and triggers the production of TNF-α, IL-1-β, IL-2 and IL-6 by T-lymphocytes (Delgado *et al*., 2003). In contrast, in skin biopsies of diabetic wounds, SP levels are reduced due to elevated levels of the degrading enzyme NEP (Antezana *et al*., 2002) and reduced SP-positive nerve fibers (Lindberger *et al*., 1989). Even though, exogenous SP treatment improves wound healing kinetics in animal models (Gibran *et al*., 2002).

CGRP is known to induce neurogenic inflammation, inducing a pro-inflammatory response in different cells, including the formation of new vessels, important during physiological and pathological wound healing (Toda *et al*., 2008). In WH, inflammation may induce CGRP release, and CGRP is able to activate inflammatory pathways (Ambalavanar *et al*., 2006). Diabetes decreases the expression, release and action of CGRP, resulting in reduced CGRP-mediated vasodilation (Sheykhzade *et al*., 2000).

In type 1 and type 2 diabetic patients, NPY levels are increased in the hypothalamus, however NPY levels in the skin are reduced (Wallengren *et al*., 1995). NPY binding to Y2 receptors is important in angiogenesis and this binding induces the formation of microvessels with distinct vascular tree-like structures. As a result, the deletion of Y2 receptor in diabetic mice results in the blockage of NPY-induced angiogenesis and delayed WH (Ekstrand *et al*., 2003). These consequences might explain a possible cause of delayed WH in diabetic patients, reinforcing the importance of NPY in both the inflammatory and angiogenic phases of WH.

In diabetic rats, POMC peptides, like ACTH and α-MSH, were less expressed. However, Gohshi *et al*. (1998) demonstrated contradictory results in short-time (1 week) diabetic rats, detecting an increase of ACTH (POMC peptide) release, via cAMP pathway. In long-term diabetic rats (8 weeks), the results were already concordant, since ACTH release decreases, which may be explained by an alteration in the properties of the L-type Ca^{2+} channel coupled with the CRF receptor, or with the CRF receptor itself. Knowing the anti-inflammatory role of POMC peptides, less is known about how its decreased levels affect wound healing.

6.1.3. Increased CRF and NT levels in diabetic wound healing

Type 2 diabetic patients have subclinical hypercortisolism. This is a consequence of Hypothalamic-pituitary-adrenal axis hyperactivation, corticotropin-releasing factor stimulation and pituitary adrenal peptides release. Impaired stress response is responsible for the decrease of pituitary and adrenal sensitivity, as well as, the glucocorticoid negative feedback sensitivity (Chan *et al*., 2002). Accordingly, chronic stress delays wound healing in humans and rodents (Detillion *et al*., 2004). In a study performed with a rhesus monkey model of wound healing, stress decreased IL-8 and CCL3 expression (Kalin *et al*., 2006), as well as, IL-1β and PDGF, in a stress model of mouse wound healing. Thus, the authors infer that glucocorticoids release mediated by stress is responsible for delayed wound healing.

Studies with pancreas and intestines from diabetic mice have shown elevated levels of NT. In fact, NT may be implicated in the pathogenesis of DWH (Fernstrom *et al*., 1981). However, studies concerning the involvement of NT in DWH have not been performed yet.

6.2. Neuropeptides in skin inflammation

Neuropeptides have appeared as key regulators of inflammation and inflammatory processes *per se*, giving new insights in the understanding of the skin's immune system. Neuropeptides may be released in the skin by the Autonomous Nervous System and also by skin cells, such as KCs, ECs, FBs, epidermal DCs and LCs (Leung M. & C.C. Wong, 2000; Wang *et al*., 2002). Neuropeptides may induce the activation of immune cells (neutrophils, MΦs, antigen presenting DCs and lymphocytes). As a result, T-lymphocytes may release specific anti or pro-inflammatory mediators (cytokines, chemokines and GFs), B cells can produce antibodies and LCs may modulate antigen presentation (Roosterman *et al*., 2006). Accordingly, inflammatory mediators are able to start and end skin inflammation and determine important immune functions, such as proliferation/differentiation/apoptosis of skin cells, vasodilation/vasoconstriction, vessels permeability, angiogenesis, T cells chemotaxis to the site of injury, MC degranulation and histamine release, pro/anti-inflammatory cytokines release and improvement of WH. Neuropeptides may regulate cytokines release and the inflammatory response, as well as, its own function, by inducing its degradation with cell-associated neuropeptide-degrading peptidases, like NEP, and by controlling nerve growth (Olerud *et al*., 1999).

Neuropeptides predominantly interact with GPCR. These receptors exist in different cell types in the skin, including in KCs, microvascular ECs, merkel cells, FBs and all immune cells of the skin; and activate different signaling pathways and mediators, namely cyclic adenosine monophosphate (cAMP), the phosphoinositol/calcium, the diacylglicerol (DAG)/protein Kinase C (PKC), the NF-kB, the Phosphoinositide 3-kinase (PI3-K)/Akt and also the Mitogen-activated protein kinases (MAPK) pathways.

6.3. Signaling pathways activated by neuropeptides

Inside the skin cells, different signaling mechanisms can be activated when a ligand binds to its receptor. Different ligands may activate different signaling pathways, such as pathogens, metabolites from pathogens, GFs, cytokines, neuropeptides and other physiologic stimuli. The activation of skin cells by neuropeptides involves several intracellular signaling cascades that are mainly initiated by a seven membrane GPCRs.

GPCRs are heptahelical hydrophobic segments which form seven-transmembrane-spanning domains (TM7), with an extracellular N terminus and an intracellular C terminus (Figure 5). These receptors received this name because they associate with intracellular heterotrimeric G proteins (Gαβδ), which become activated when an extracellular ligand binds to a GPCR. This binding induces a conformational change in the receptor, promoting heterotrimeric G protein activation. G proteins are divided in four families, based on the similarities functions to the α-subunit, namely Gs, Gi, Gq and $G_{12/13}$ (Oldham W. & H.E. Hamm, 2008; Wilkie *et al*., 1992). G-protein downstream signaling may activate different signaling pathways and mediators, namely cAMP, phosphoinositol/calcium, DAG/PKC, NF-κB pathway, PI3-K/Akt pathway, and MAPK pathways.

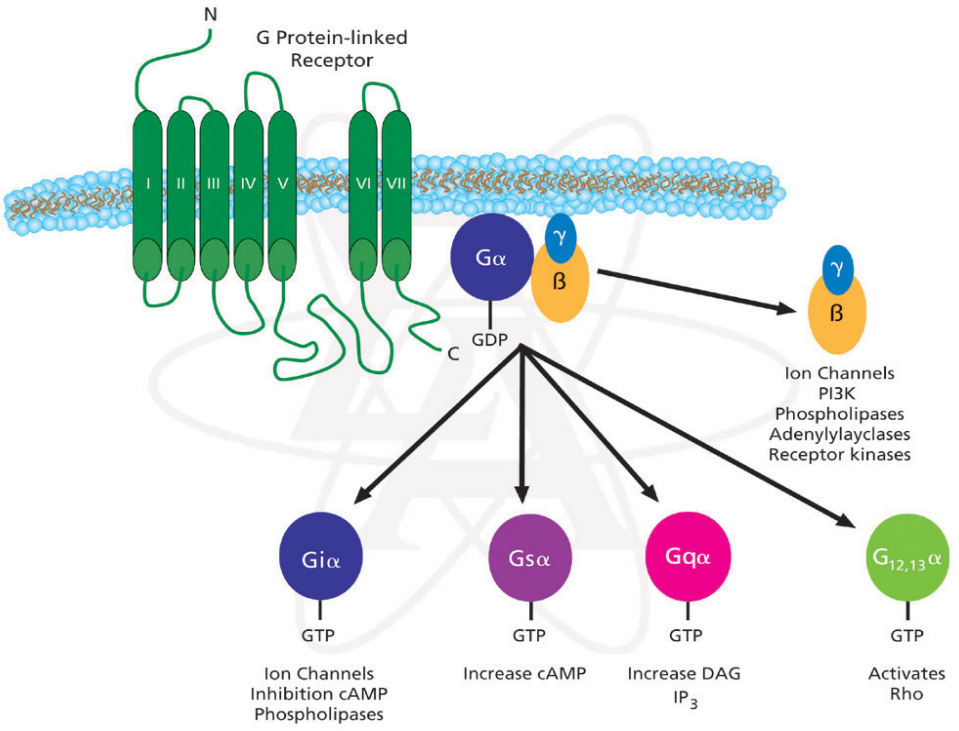


Figure 5 - Diversity of G protein-coupled receptor signal transduction pathways (SIGMA-ALDRICH).

The DAG/PKC/NF-κB pathway also involves the Gq protein, responsible for the generation of IP_3 and DAG, by PLC. DAG activates PKC, a multifunctional kinase that phosphorylates many different targets. Indeed, PKC may activate the NF-κB pathway. The NF-κB signaling pathway can be activated through two different mechanisms, a pathway dependent on the NF-kB inhibitor, IκBα – the classical pathway of NF-κB activation – and independent on IκBα – the alternative pathway of NF-κB (Senftleben *et al*., 2001). Here will be discussed the classical pathway of NF-κB and innate immunity activation (Figure 6). NF-κB is activated upon IκBα phosporylation, which is an NF-κB inhibitory protein localized in the cytosol and complexed with the NF-κB. This phosphorylation leads to its ubiquitination and degradation, by proteasome. Consequently, NF-κB is then translocated into the nucleus where it binds to specific

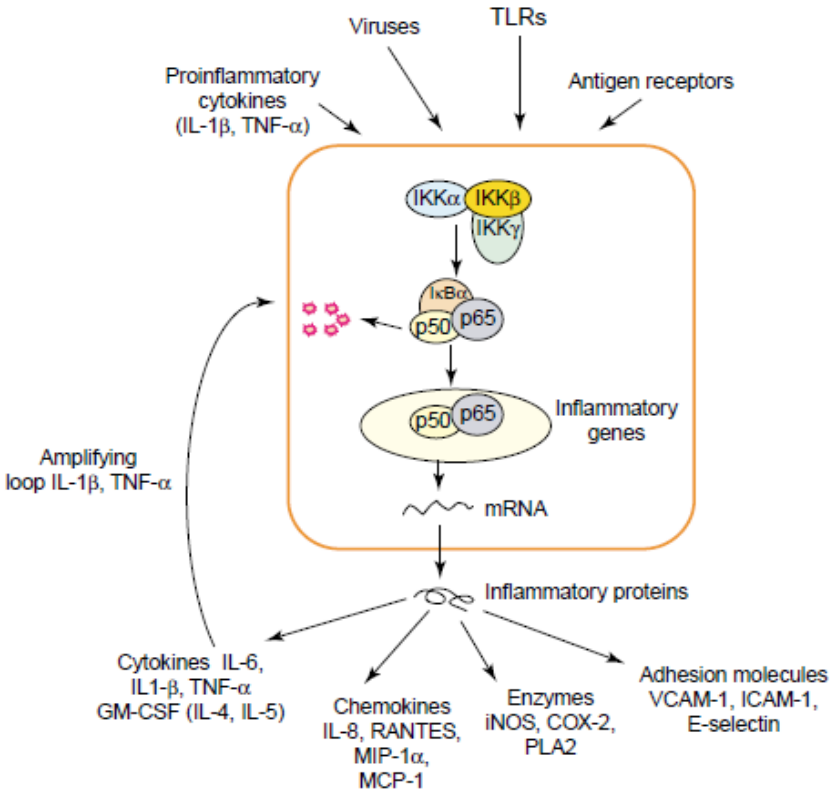


Figure 6 – NF-κB signaling pathway (Bonizzi G. & M. Karin, 2004).
Read text for detailed explanation.

sequences of DNA called response elements, activating transcription. NF-κB is involved in diverse cells immune responses, increasing the transcription of genes like chemokines (IL-8, CCL5, MIP-1 and MCP-1), cytokines (IL-6, IL-1β, TNF-α, GM-CSF, IL-4 and IL-5), adhesion molecules (VCAM-1, ICAM-1 and E-selectin), enzymes that produce secondary inflammatory mediators (iNOS, COX-2 and PLA2) and inhibitors of apoptosis (Ghosh *et al*., 1998) .

GPCR activates PI3-K either through βγ subunits associated to G-proteins or via Receptor Tyrosine kinase (RTK) and integrin transactivation. PI3-K activates Akt (Figure 7) that phosphorylates phosphatidylinositols of the cell membrane, generating phosphatidylinositol (3,4,5)-trisphosphate (PIP_3) from phosphatidylinositol-4,5-bisphosphate (PIP_2). At the cell membrane, PIP_3 recruits the serine/threonine protein kinase Akt, also known as protein kinase B (PKB) or RAC-PK, and phosphoinositide-

dependent protein kinase (PDK)-1. Akt binding to PIP_3 induces a conformational change, making Akt accessible to phosphorylation by PDK-1, which culminates with Akt activation. PKB/Akt further activates or inhibits different molecules (for example, mTOR and NF-κB) mediating different cell mechanisms, like cell growth, cell cycle, transcription, translation, cell proliferation, apoptosis, cytoskeletal reorganization and glycogen synthesis (Brazil *et al*., 2002).

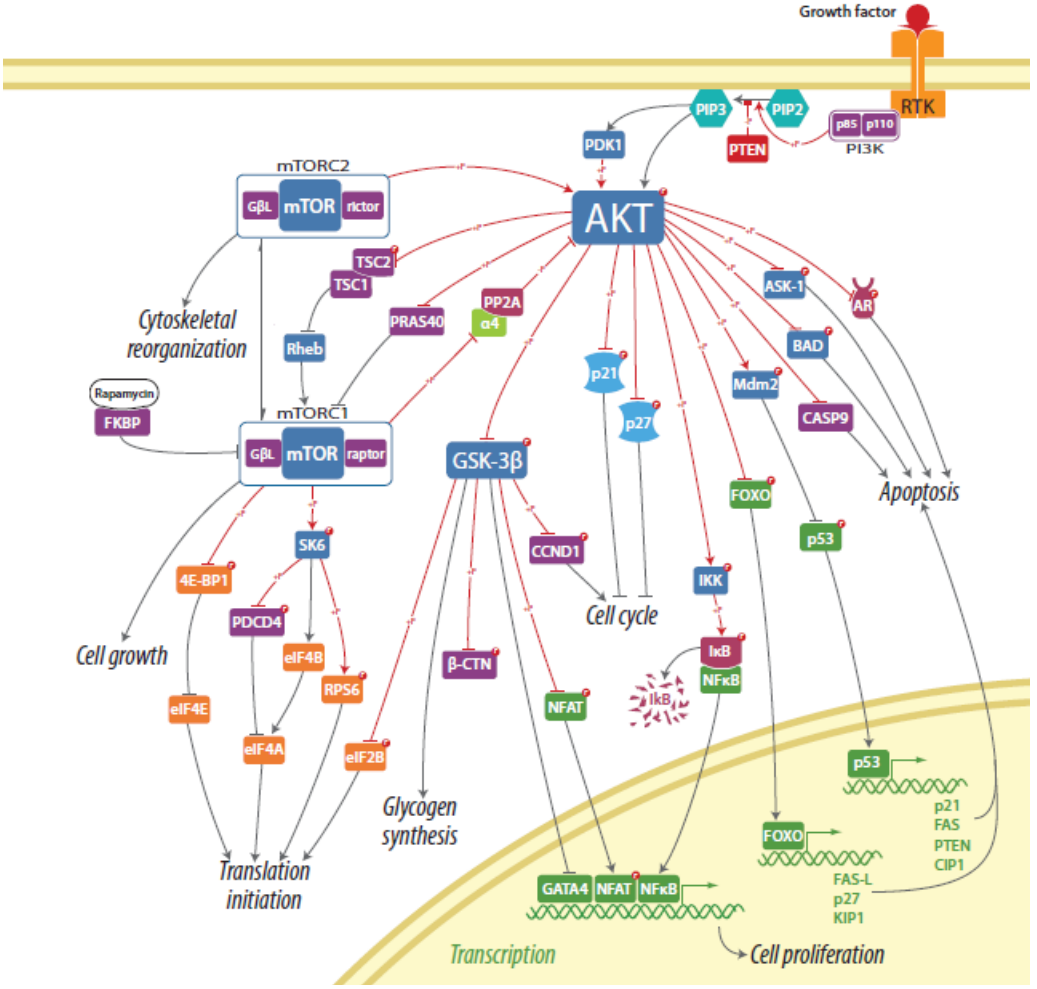


Figure 7 – Akt signaling pathway, downstream activation/inhibition molecules and final cell effects (Invitrogen). Read text for detailed explanation.

Mitogen-activated protein kinases are a family of serine/threonine protein kinases involved in different cell functions, such as cell growth, development, proliferation, differentiation and apoptosis. MAPK signaling cascades are organized hierarchically through the action of three kinases. MAPKs are phosphorylated and activated by MAPK-kinases (MAPKKs), which in turn are phosphorylated and activated by MAPKK-kinases (MAPKKKs). The MAPKKKs are in turn activated by upstream interaction with the family of small GTPases and/or other protein kinases.

The MAPK essentially involves Ras, which is a small G protein whose activation by GTP initiates the MAPK cascade. Figure 8 shows Ras downstream cascade of phosphorylations, which ends in the ERK1/2, c-Jun N-terminal kinases (JNK) 1/2/3 and p38 MAPK phosphorylation and activation. These last molecules regulate the expression of diverse genes involved in inflammation (Zhang *et al*., 2007).

The activation of MAPKs, the magnitude and duration of their activation, and their inactivation are critical in mediating appropriate physiological responses. Indeed, MAPK phosphorylation, that trigger its activation, is highly regulated by MAPK phosphatases (MKPs) or dual specificity phosphatases (DUSPs), that function as negative regulators, responsible for MAPK dephosphorylation (Farooq A. & M.M. Zhou, 2004; Theodosiou *et al*., 2002). These phosphatases have identical structural features: a C-terminal catalytic domain containing a highly conserved signature motif HCXXXXXR and two Cdc25-like domains. The dephosphorylation occurs in threonine and/or tyrosine residues within the signature sequences -Thr-X-Tyr- located in the activation loop of MAPKs. To date, thirteen members of this family had been identified, with different substrate affinities and distinct subcellular localizations and types of regulation.

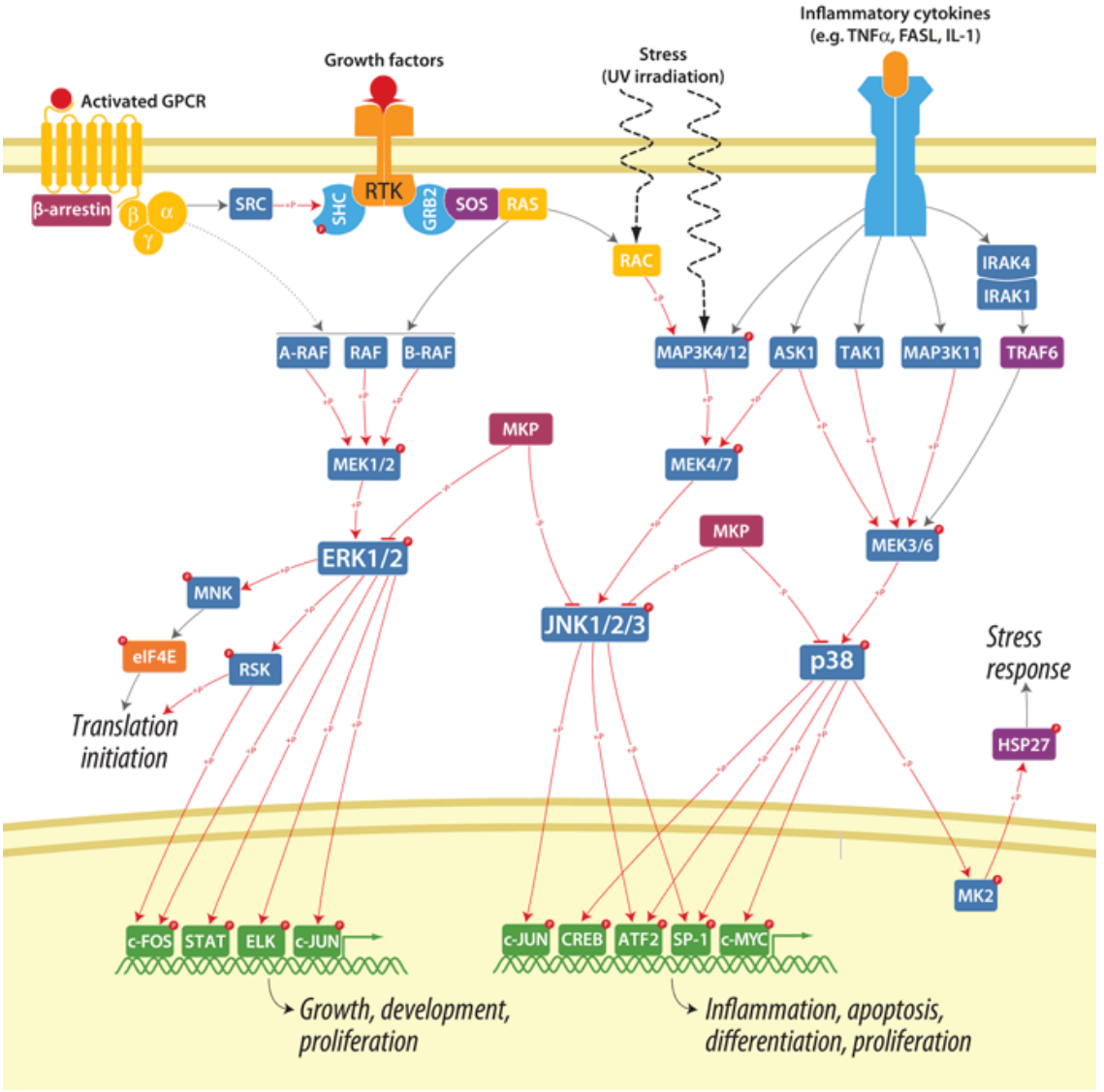


Figure 8 – MAPK signaling cascades (Invitrogen). Read text for detailed explanation.

Neuropeptides activate different signaling pathways that regulate the immune responses, degranulation of mast cells, the synthesis/release of pro or anti-inflammatory cytokines and chemokines, the control of vasodilation/vasoconstriction and the proliferation/differentiation of cells. For that, neuropeptides bind to specific receptors in different skin cells, like KCs, LCs, FBs, MΦs and MCs, and they also recruit lymphocytes from the lymph nodes. Diverse neuropeptides mediate all these cell responses, namely SP, Vasointestinal Polypeptide (VIP), Calcitonin gene-related

peptide (CGRP), Pituitary adenylate cyclase activating polypeptide (PACAP), Neuropeptide Y (NPY), Corticotropin Releasing Hormone (CRH), Proopiomelanocortin peptides (POMC peptides), Melanocyte-stimulating hormone (MSH), Secretoneurin (SN), Urocortin and Neurotensin (NT). This work will address the role of NT in DCs and FBs.

6.4. Neuropeptides signaling in dendritic cells (DCs)

DCs received this name because of the long membrane extensions they possess, that are similar to dendrites of nerve cells. There are many types of DCs: LCs, interstitial DCs, myeloid cells and lymphoid DCs; even though, the main function of mature DCs remains unchanged - the presentation of the antigen to the Th cells. In inflammation, mature and immature forms of LCs and interstitial DCs capture the antigen, by phagocytosis or endocytosis, and then migrate into draining lymph nodes, functioning as APCs which, in turn, initiate Th cells response. DCs give important information to T and B cells about inflammation, such as the nature of the pathologic insult and the type of tissue in which the inflammation occurred (Goldsby *et al*., 2003). Taking in consideration these essential functions of DCs, it is easy to comprehend that impairment of these cells leads to immune diseases.

The role of neuropeptides in the regulation of DCs is well documented at different levels, essentially in the density of LCs, DCs migration and maturation, in the antigen-presenting function and in the inhibition/upregulation of the immune system. Subsequently, in the next section, it will be addressed the immune regulation of DCs by different neuropeptides.

6.4.1. SP, CGRP, VIP, Secretin, SN and stressor neuropeptides induce migration and maturation of LCs

Some neuropeptides play a chemotactic role in the migration and maturation of blood-derived DCs. CGRP, VIP, Secretin and SN induce immature DC migration, while SP stimulates its migration only slightly (Dunzendorfer *et al*., 2001).

SP receptors (NK-1) are present in normal murine and human DCs and can elicit transcription factor activation in DCs (Marriott I. & K.L. Bost, 2001). Mathers *et al.* (2007) used the gene gun to deliver transgenic (tg) Ag to the skin of C57BL/6 mice and the selective NK-1R agonist. The administration of NK-1R agonist induced NF-κB mediated migration of activated LCs to skin-draining lymph nodes, increasing the expression of tg Ag in the epidermis and stimulating LCs to induce both Th1 and Tc1 cellular responses and antibody mediated responses (Mathers *et al*., 2007).

Well-established murine sound stress model (frequency of 300 Hz and an intensity of 75 dB, during 24h, at intervals of 15 sec) induces DCs maturation and migration. Accordingly, some activation related molecules are upregulated, such as MHC class II and CD11c, inducing cellular activation and triggering neutrophil respiratory burst and langerin expression. Langerin is an intradermal transmembrane cell surface antigen receptor that binds to mannose cell wall of pathogens, inducing its internalization into LCs Birbeck granules and providing access to a nonclassical antigen-processing pathway. Not only maturation is stimulated, but also migration, as it can be observed by the upregulation of the adhesion molecule ICAM-1, in murine skin (Joachim *et al*., 2008). Since sound stress may enhance stressor neuropeptides, like CGRP, SP, NT, CRH and α-MSH, these can be responsible for DC maturation and migration to blood vessels.

6.4.2. CGRP, VIP, PACAP and α-MSH mediate anti-inflammatory and Th2 responses in LCs

Tori *et al.* (1998) determined the anti-inflammatory properties of CGRP, in LCs (Tori *et al*., 1998). Indeed, CGRP inhibits antigen-presenting function and enhances anti-inflammatory cytokines like IL-10. Since CGRP acts in DCs, mature and immature DCs express type 1 CGRP receptors that regulate the interaction of LCs with T cells. Indeed, CGRP downregulates CD86 (a cell surface ligand that provides co-stimulatory signals essential for T cell activation and survival), HLA-DR (involved in antigen presenting cell function to T cells) and T cell proliferation, in human DCs (Carucci *et al*., 2000). In a recent study performed in murine DCs, the authors verified that CGRP also inhibits TLR-stimulated production of inflammatory mediators, mediated by the cAMP/PKA pathway, leading to a rapid up-regulation of the transcriptional repressor, the inducible cAMP early repressor (ICER). Additionally, CGRP also attenuated serum TNF-α levels (Harzenetter *et al*., 2007).

α-MSH also induced an anti-inflammatory function on DCs, inducing IL-10 release. In addition, PACAP and VIP suppress LC antigen presentation and modulate cytokine production. In a LPS-induced inflammatory study, NF-κB is activated in both a LC-like cell line (XS52) and an epidermal LC. Treatment of cells with these neuropeptides suppresses the phosphorylation of p-IKKβ and consequently inhibits the normal activation of the NF-κB pathway (Ding *et al*., 2007). Indeed, the Th1 response is inhibited, while the Th2 immune response is enhanced (Ding *et al*., 2008).

6.5. Neuropeptides signaling in fibroblasts (FBs)

Bayreuther *et al.* (1988) concluded that human FBs differentiate along a terminal lineage, in parallel with hemapoietic cells. This lineage comprises different phenotypic FBs: resident FBs, circulating precursors and transforming epithelial cells. In addition to

migrating surrounding FBs to the site of injury, resident FBs are important in rapid proliferation after injury. Circulating precursors, also called fibrocytes, are blood borne cells expressing the hematopoietic cell surface markers $CD34^+$, $CD45^+$ and collagen, and are able to leave the blood, enter into the tissue and become a FB. These cells are able to migrate to wounds to promote wound healing and fibrotic tissue repair (Yang *et al*., 2002). Transforming epithelial cells are epithelial cells that can give rise to FBs, in a process called epithelial-mesenchymal transition (EMT).

FBs are present in different tissues and their main function resides in ECM production and turnover. In the skin, FBs are present in the dermis, in the papillary, reticular dermis and dermal papilla region of the follicle (Sorrell J. & A.I. Caplan, 2004), and produce and organize the ECM, supported by FB communication with each other and other cell types, like KCs. Moreover, FBs regulate skin immune function, as these sentinel cells produce and release chemokines (like IL-8 and CCL5), COX and prostanoids after activation by infectious agents (like LPS of bacteria) and factors emanating from infiltrating hematopoietic cells (Smith *et al*., 1997).

FBs are essential cells in wound healing and differentiate, migrate and increase ECM synthesis in the proliferation and remodeling phase of WH (Takehara *et al*., 2000). Skin FBs proliferation is regulated by various GFs, like PDGF, b-FGF, TGF-β and connective tissue growth factor (CTGF). Additionally, PDGF and CTGF *per se* stimulate chemotaxis of skin FBs; b-FGF stimulates angiogenesis; and TGF-β stimulates ECM production. FBs differentiation involves a change in FBs phenotype, from a relatively quiescent state, in which FBs are only responsible for the slow turnover of ECM, to a proliferative and contractile phenotype termed myofibroblasts. Myofibroblasts have prominent cortical microfilament bundles with dense bodies and have a distinct alpha-smooth muscle actin (α-SMA) important for *in vivo* contraction.

These cells are important during WH, as they can contract to promote wound closure, as well as, induce fibrogenesis (ECM production), fundamentally fibronectin and collagen. ECM molecules constitute the newly granulation tissue and give structure integrity to the granulation tissue. Also, proteinases (MMPs and plasminogen activator/plasmin) activity and consequent degradation of ECM is also important in tissue remodeling after new ECM deposition - immature matrix is remodeled as part of scarring and scar contraction. In the end, in physiological conditions, myofibroblastic cells die via apoptosis forming the scar, while in pathological conditions, these cells are responsible for fibroses, as these cells persist in the tissue and augment ECM (Jeon *et al*., 2007).

Neuropeptides regulate FBs proliferation and migration, as well as, modulate FB immune function. In fact, this modulation is largely documented for SP, but poorly explored for others neuropeptides. In the next section, it will be reviewed the current knowledge of the neuropeptides effects on FBs.

6.5.1. SP and enkephalins enhance FBs proliferation

FBs proliferation is mediated by diverse neuropeptides, namely enkephalins and SP. A unique study from Weisinger *et al.* (1995) showed that enkephalins mediate cultured embryonic FBs proliferation, shifting FBs from their normal G1 restriction point to one in the S-phase (Weisinger *et al*., 1995). Considering SP, several authors reported FBs proliferation mediated by SP. Indeed, SP by itself and in synergy with IL-1α and PDGF induce bone marrow (BM) FBs proliferation, in which SP, IL-1α and PDGF-BB receptors induction is important to their synergistic effects. Moreover, as IL-1α induces NK-1 mRNA, synergism of SP with IL-1α is favored (Rameshwar *et al*., 1997). In addition, the release of IL-3 from BM-derived MCs induces FBs proliferating activity, partially abrogated by the SP antagonists, indicating that SP or histamine release (provoked by SP) augment the fibrogenic cytokine IL-3 and consequently, FBs

proliferation (Katayama I. & K. Nishioka, 1997). The proliferative effect of SP was also determined in cultured granulation tissue FBs *in vitro*, in a remarkable dose-dependent fashion, and partly mediated by bFGF (Jiang *et al*., 2004). Accordingly, SP may induce *in vitro* expression of bFGF mRNA and bFGF protein in FBs of granulation tissue, significant in wound healing (Jiang *et al*., 2003). Furthermore, immunoreactive stain for SP in granulation tissue is correlated with gene expression of EGF, FGF-2 and their receptors (Lai *et al*., 2003). Moreover, in wounds of scalded rats, SP levels are increased and mediate proliferative and anti-apoptotic effects by elevating the expression of proliferating cell nuclear antigen and BCL-2 in FBs, with greater influence on keloid FBs than on hypertrophic scar FBs (Jing *et al*., 2010), mediating WH and scar formation.

6.5.2. Neuropeptides induce FBs migration

SP, SN, bombesin and CGRP are involved in FBs migration, in different tissues. In accordance, SP chemoattracts human FBs *in vitro*, in a concentration-dependent migratory response mediated by the C-terminus of the neuropeptide through NK1 receptor but not NK2 (Kähler *et al*., 1993). SN, by the C-terminal fragment of the peptide, also triggers the selective migration of human skin FBs *in vitro*, but does not stimulate their proliferation (Kahler *et al*., 1996). Bombesin and CGRP induce IMR-90 FB migration, essential in repair and healing of airway injury (Yule *et al*., 1999).

6.5.3. Neuropeptides regulate FBs immune function

Neuropeptides are also implicated in FBs immune function. In accordance, SP mRNA augments in response to the transforming growth factor (TGF)-β and bFGF in rheumatoid arthritis FB-like synoviocyte and osteoarthritis FB-like synoviocyte (Inoue *et al*., 2001). Higher SP levels induce proliferation and enzyme secretion in FB-like synoviocytes present in joints. In addition, SP mediates the expression of MHC class II,

ICAM-1, vascular cell adhesion molecule (VCAM)-1, LFA-3, CD40, B7.1 or B7.2 molecules, on rheumatoid arthritis FB-like synoviocyte incubated with IFN-γ or IL-1β or TNF-α. As a result, SP potentiates the effect of pro-inflammatory cytokines on the expression of VCAM-1 on rheumatoid arthritis FB-like synoviocyte (Lambert *et al*. 1998). Moreover, SP, but not VIP or SN, induces human neutrophil adhesion and consequent migration in a 5-mμ pore polycarbonate filter system covered by human lung FB monolayers, representative of subendothelial barrier of FBs and extracellular matrix towards lung inflammatory sites, in acute lung injury (Kähler *et al*., 2001). Neuropeptides are also important in inflammatory processes of angiogenesis. In fact, SP, CGRP, VIP and NPY alter angiogenic GFs expression, namely angiogenin, angiopoietin-2, PDGF, VEGF, EGF, bFGF, HE-EGF, hepatocyte growth factor (HGF), leptin and placental growth factor, in *in vitro* pulp FBs, suggesting neuropeptides importance in pulpal inflammation and repair (El Karim *et al*., 2009).

6.6. Neurotensin (NT) and neurotensin receptors (NTRs)

NT is a tridecapeptide essentially localized in the CNS and specialized endocrine cells (N-cells) of the small bowell (Reinecke M., 1985). NT response is mediated by its binding to one of the three existing NTRs: neurotensin receptor 1, 2 and 3 (NTR1, NTR2 and NTR3). NTR1 and NTR2 are GPCRs and NTR3 is an intracellular receptor with a single transmembrane domain, which is 100% homologous to gp95/sortilin. NTR1, rather than NTR2, exhibits high affinity for NT (Chalon *et al*., 1996) and NT effects are essentially mediated by this receptor, as NTR3 is predominantly localized in the trans Golgi network although the mature protein can also be present in the plasma membrane (Nielsen *et al*., 1999).

NTRs undergo molecular and cellular regulation after acute and chronic agonist exposure. Indeed, like other GPCRs, upon agonist stimulation, the receptor is

uncoupled, internalized, endocytosed, and the cells are completely desensitized. At the same time, NT message is transduced and NT cellular effects occur. Accordingly, this desensitization process is dependent on the amount of free and functional NTR1 at the surface and this state remains until NTR1 is recycled to the membrane or new receptors reach the cell surface, or both. Also, NTR1 responses are dependent on the time and the dose of agonist exposure. Chronic stimulation with high agonist doses induces permanent cell sensitivity to NT. Summarizing, the cellular response of NT is determined by NT release, NT degradation and the dynamic regulation of NTR1 (Sorrell J. & A.I. Caplan, 2006).

NTRs mediate the activation of different signaling pathways. NTR1 induces intracellular signaling through PLC and the inositol phosphate signaling pathway and is also known to function through the production of cGMP, cAMP and arachidonic acid, as well as, the MAP kinases pathways and inducing inhibition of Akt activity. NTR2 receptor internalizes receptor–ligand complexes and activates ERK1/2 but does not induce calcium mobilization. NTR3 may be involved in the migration of human microglial cells, via the stimulation of both MAP and PI3-K pathways (St-Gelais *et al*., 2006).

6.6.1. NT in the CNS, gastrointestinal tract and skin

NT is involved in inflammatory processes occurring in different organs, by its release through peripheral nerves or by its production in tissues. In the nervous system, NT has a pro-inflammatory role, inducing vasodilatation, vascular permeability increase, MCs degranulation and enhancement of directional migration and phagocytosis of neutrophils (Goldman *et al*., 1983). NT also induces the expression of several cytokines/chemokines, including IL-1 and MIP-2, in microglia cells (Dicou *et*

al., 2004). This expression is mediated by a mechanism dependent on both PI3-K and MAP kinases pathways, via NTR3 (Martin *et al*., 2003).

In the gastrointestinal tract, NT is involved in the physiopathology of colonic inflammation, a tissue rich in NTR1 (Castagliuolo *et al*., 1999), stimulating NF-κB dependent IL-8 secretion and IL-8 gene expression in human colonocyte cells (Zhao *et al*., 2003). However, in a recent research study performed in the gastrointestinal, NT treatment induces an anti-inflammatory cytokine profile in colitis, characterized by the diminishment of IL-6 and TNF-α (Assimakopoulos *et al*., 2008).

Less is known about the role of NT in skin inflammation. NT-positive fibers and NT mRNA are present in the skin and dorsal root ganglia (Donelan *et al*., 2006; Hartschuh *et al*., 1983). The prohormone convertase 5, an enzyme that cleaves pro-NT into its active form, is expressed in dorsal root ganglia. Additionally, NT is also involved in MCs histamine release and granule extrusion (Carraway *et al*., 1982). Knowing the role of NT in the CNS and gastrointestinal tract inflammation, much information is still missing considering skin inflammation. In fact, NT presence in the skin has been implicated in the pathogenesis of skin disorders, exacerbated by stress (Singh L., 1999).

6.6.2. The effects of NT on LCs and FBs

In accordance with the present literature, the effect of NT on LCs and FBs was not explored until the current date. Knowing the potential effects of NT in inflammation and the important role of neuropeptides in the regulation of these cells function, it can be stated that this poorly explored subject is undoubtedly a prospective area of research.

7. Objectives

This work attempts to address the role of NT in skin inflammation and its possible functions in important processes of WH. Indeed, this thesis will be focused on signal transduction, inflammatory mediators and pro/anti-inflammatory function of cells, and cell migration occurring in FSDCs and BJ cells, key cells of the epidermis and dermis, respectively. Also, these studies aim to determine if different glucose levels alter the metabolism and transcription machinery of FSDCs or BJ cells. Finally, after knowing the role of NT in skin inflammation, a possible connection between NT effects in skin inflammation and WH will be determined. Additionally, it will be described the role of NT in pathological conditions, determining its repercussions in DWH. Understanding the role of neuropeptides in the skin, its application on skin wounds could be a potential therapy for skin pathologies, like DFUs, a problematic and prevalent skin disease that can lead to a final amputation.

Chapter II. Materials & Methods

1. Reagents

Lipopolysaccharide (LPS) from Escherichia coli (serotype 026:B6) was obtained from Sigma Chemical Co. (St. Louis, MO, USA), NT was obtained from Bachem (Weil am Rhein, Germany) and NTR1 inhibitor SR48692 was obtained from Axon Medchem (Groningen, The Netherland). The protease inhibitor cocktail (Complete Mini) and the phosphatase inhibitor cocktail (PhosSTOP) were obtained from Roche (Carnaxide, Portugal). Bicinchoninic acid (BCA) kit assay was obtained from Novagen. 30% Acrylamide/BisSolution 29:1 (3.3% c), TEMED and SYBR green were obtained from BioRAD, High Capacity cDNA Reverve Transcription kit was obtained from Applied Byosistems.

The polyvinylidene difluoride (PVDF) membranes and the antibody against β-actin were purchased from Millipore Corporation (Bedford, MA). The antibodies against pAkt (Thr 308) and NTR were purchased from Santa Cruz (Frilabo), the antibodies against total JNK was obtained from UpState (Tape Group), the antibodies against phopho-(p-)JNK, p-ERK, p-p38 MAPK and total Akt were purchased from Cell Signaling and the antibody against total p38 MAPK was purchased from Biolegend (Tape Group). The alkaline phosphatase-linked secondary antibodies (anti-mouse and anti-rabbit) and the enhanced chemifluorescence (ECF) reagent were obtained from GE Healthcare (Carnaxide, Portugal). The Vectashield mounting medium was purchased from Vector, Inc. (Burlingame, CA, USA) and the Alexa Fluor 555 phalloidin antibody was purchased from Invitrogen (Barcelona, Spain).

TRIzol® was obtained from Invitrogen, diethyl pyrocarbonate (DEPC) was acquired from AppliChem. Methanol, ethanol, isopropanol were obtained from Merck. All primers were obtained from MWG Biotech (Ebersberg, Germany). All other reagents were purchased from Sigma Chemical Co.

2. Cell culture

The fetal mouse skin-derived dendritic cell (FSDC) line was kindly supplied by Dr. G. Girolomoni (Department of Biomedical and Surgical Science, Section of Dermatology and Venereology, University of Verona, Italy). This cell line is a skin DC precursor with antigen presenting capacity. This cell line was previously characterized and had a surface phenotype consistent with a LC progenitor (H-$2^{d.b+}$, I-$A^{d.b+}$, $CD54^+$, $CD11b^+$, $CD11c^+$, $MHCII^+$, $MHCI^+$, $B7.2^+$, $B7.1^-$, $CD44^+$, $B220^-$, $CD3^-$) (Girolomoni *et al*., 1995), a phenotype that was confirmed in our lab for the most important of these surface markers (data not shown). FSDCs are adherent and present a morphology consistent with a DC type, having an irregular shape and many surface projections arising from the cell.

FSDCs were cultured in serum-containing endotoxin-free Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 10% (v/v) of inactivated fetal calf serum, 3.02 g/l sodium bicarbonate, 100 U/ml penicillin, 100 g/ml streptomycin and 30 mM of glucose, in a humidified incubator with 5% CO_2/95% air, at 37◦C. Indeed, FSDCs did not require exogenous GFs for their continued proliferation.

FSDCs have a doubling time of about 48h and were used after reaching 80-90% confluence, occurring every 7 days. Cells were subcultured (1:5) in plastic flasks of 75 or 150cm^2, using trypsin to detach the cells from the flasks after a previous wash with phosphate buffered saline (PBS). Afterwards, cells were centrifuged at 230g for 4min and resuspended in fresh IMDM medium.

The BJ cell line was kindly supplied by Paula Marques and João Malva (Life Sciences Department and Center for Neurosciences and Cell Biology, Coimbra University, Portugal). This cell line was established from skin taken from normal newborn human foreskin fibroblasts and is telomerase negative. Telomerase is a

reverse-transcriptase enzyme that elongates the telomeres. Cells transfected with the catalytic subunit of human telomerase (hTERT), although they are not transformed, do not display replicative senescence when considered as cell populations (Bodnar *et al*., 1998). Accordingly, the BJ cell line has the capacity to proliferate to a maximum of 72 population doublings before the onset of senescence.

BJ cells were cultured in serum-containing endotoxin-free Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) of inactivated fetal calf serum, 3.02 g/l sodium bicarbonate, 100 U/ml penicillin, 100 g/ml streptomycin and 30 mM of glucose, in a humidified incubator with 5% CO_2/95% air, at 37◦C. No exogenous GFs were required for their continued proliferation.

BJ fibroblasts have a doubling time of about 72h and were used after reaching 80-90% confluence, which occurs approximately every 7 days. Cells were subcultured (1:3) in plastic flasks of 150cm^2 and identically detached, centrifuged and resuspended as FSDCs. Along the experiments, cells were monitored by microscopic observation in order to detect any morphological change.

3. Chemicals preparation

NT was dissolved in ultra-pure MiliQ H_2O in a final concentration of 6 mM (10 mg/mL); LPS was dissolved and aliquoted in sterile PBS to achieve a final concentration of 1 mg/mL and 0.1 mg/mL; the NTR inhibitor was dissolved and aliquoted in sterile DMSO into a final concentration of 3.41 mM (2 mg/mL). Daily dilutions were diluted in ultra-pure MiliQ H_2O. These stocks solutions were aliquoted and diluted into different concentrations and stored at -20ºC.

4. MTT and griess reaction assays

4.1. MTT assay

The MTT assay is a standard colorimetric assay to measure the reduction capacity of mitochondria and the activity of the mitochondrial dehydrogenases. MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is a tetrazole yellow compost that when cleaved by mitochondrial dehydrogenases converts into formazan, a purple compost which is insoluble in water (Figure 9). After formazan dissolution in a solubilisation solution, like isopropanol, the intensity of the colour of the purple solution can be determined in a spectrophotometer, at a wavelength of 500-600nm.

Only living, viable and metabolic cells, with active dehydrogenases, are able to reduce MTT. Accordingly, the amount of purple formazan produced by the cells will be correlated with more viable cells, which means that there are more cells (in comparison to a control situation) or that there are the same amount of cells, but with increased dehydrogenase activity. Thus, this assay measures cell death or cells changing metabolism. This method is usually used to determine composts cytotoxicity, as some composts can have a great chemical potential but provoke cell toxicity (Mosmann T., 1983).

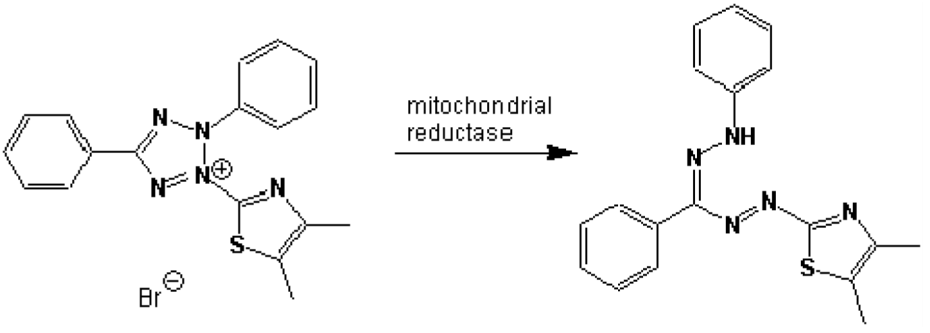


Figure 9 – MTT reduction reaction (Mosmann T., 1983).

4.2. Griess reaction

NO is a pleiotropic biological mediator, regulating diverse activities ranging from neuronal function to immune system regulation. NO is produced via the reaction of L-Arginine, NADPH, and O_2 to NO and citrulline (Bredt D.S. & S.H. Snyder, 1990; Moncada *et al*., 1989; Palmer *et al*., 1988a; Palmer *et al*., 1988b) and is catalyzed by enzymes of the nitric oxide synthase (NOS) family, the neuronal (nNOS/NOS1), endothelial (eNOS/NOS3), and inducible (iNOS/NOS2) nitric oxide synthase (Alderton *et al*., 2001). The names of the enzymes are related to the local where these enzymes are largely expressed. Usually, eNOS and nNOS are constitutively expressed and regulated by Ca^{2+}/Calmodulin, while iNOS is induced by endotoxin and inflammatory cytokines, exhibiting some insensitivity to calcium (Alderton *et al*., 2001; Kone *et al*., 2003).

NO is not stored but is synthesized *de novo* and freely diffuses across lipid membranes. Indeed, its action occurs essentially on target cells, via different mechanisms. For example, it can activate the enzyme guanylyl cyclase and generate the second messenger guanosine 3',5'-cyclic monophosphate (cGMP), implicated in diverse biological functions, like the regulation of smooth muscle contractility, cell survival, proliferation, axon guidance, synaptic plasticity, inflammation, angiogenesis, and the activity of cyclic nucleotide-gated channels (Arancio *et al*., 2001; Arancio *et al*., 1996; Guzik *et al*., 2003; Hamad *et al*., 2003; Hood J. & H.J. Granger, 1998; Kronemann *et al*., 1999; Lev-Ram *et al*., 1997; Suhasini *et al*., 1998; Zhuo *et al*., 1994). In the present work, as FSDCs were treated with LPS, NO measurement gives essentially the state of inflammation in cells. NO is measured through an indirect way, by measuring the levels of NO metabolites, nitrite (NO^{2-}) and nitrate (NO^{3-}) (Marletta *et al*., 1988; Tsikas *et al*., 2005; Wennmalm *et al*., 1993 and 1992), as they are more stable metabolites. The assay is based on a chemical reaction of diazotization described by

Griess (Griess P., 1879), which uses sulfanilamide and N-1-napthylethylenediamine dihydrochloride (NED) under acidic (phosphoric acid) conditions (Figure 10).

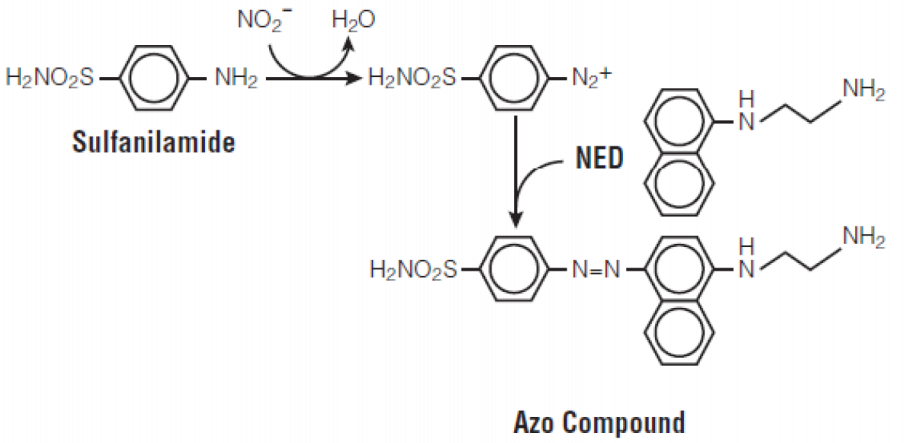


Figure 10 – Griess Reaction (Corporation, 2009).

4.3. Cells treatment

FSDCs were stimulated with 1µg/ml of LPS, an *in vitro* model of inflammation, and treated with different NT concentrations (10, 50 and 100 nM), for 5min to 1h and during 1 to 7 days. Cell viability was determined by the reduction of MTT (Mosmann T., 1983). NO production was evaluated by the colorimetric griess reaction that detects nitrite in the supernatants of stimulated cells.

Briefly, FSDCs were plated at 2×10^5 cells/well in 48-well plates, in a final IMDM volume of 600µL. LPS or/and NT stock solutions were added to obtain the different final in-well concentrations studied. Subsequent to the incubations, 170µl of the medium was collected to perform the griess reaction and then, 43µl of MTT solution (5 mg/ml) was added to each well. Microplates were further incubated at 37ºC for 1h, in a humidified atmosphere of 95% air and 5% CO^2. Following, 300µl of acidic isopropanol (0.04N HCl in isopropanol) was added to each well and mixed in order to dissolve the dark blue crystals of formazan. Acidic isopropanol was collected to an

ELISA microplate and formazan quantification was performed using an ELISA automatic microplate reader (SLT, Austria) at 570nm, with a reference wavelength of 620nm.

The collected medium was placed in 96-well plates and diluted with equal volume (170µl) of Griess reagent [0.1% (w/v) N-(1-naphthyl)ethylenediamine dihydrochloride and 1% (w/v) sulphanilamide containing 5% (w/v) H_3PO_4], during 30min, in the dark. Finally, the absorbance was measured at 550nm. Culture medium was used as blank and nitrite concentration was determined from a regression analysis using serial dilutions of sodium nitrite as standard.

5. Microscopy

Microscopy can give us visible information about the state of cells. The morphology of cells is crucial to determine if cells are viable/death, (in)activated or with morphologic alterations. These parameters may direct us to a possible understanding of the biological state of the cells. Indeed, cell morphology was regularly observed using an optical microscope. FSDCs morphology was also analyzed when stimulated with 1µg/ml of LPS, or with LPS in the presence of 10, 50 or 100 nM of NT, or maintained in IMDM medium containing 2% of inactivated fetal calf serum, during 1, 3, 5 and 7 days. Following incubations, photographs were taken using a coupled AxioCamMR3 camera with PALM reflector and 20X objective, using an inverted Axiovert 200 microscope with transmitted and reflected light transmission microscopy. Furthermore, the FSDCs and BJ cytoskeleton, as well as, nucleus morphology after 24h of LPS/NT treatment and BJ cell migratory properties after 24h of LPS/NT treatment were also determined and will be subsequently described.

5.1. Scratch Assay

The *in vitro* scratch assay is a cost-effective method to study cell migration *in vitro*. This method involves the formation of a new artificial gap - ‘‘scratch’’ - on a confluent cell monolayer, where cells from the confluent non-gapped area move to the newly created gap through cell migration, until cell–cell contacts are established again. The principal steps of this method are: formation of a ‘‘scratch’’ on the cell monolayer, image capturing in the beginning and the end of cell migration (closure of the scratch), and finally, comparison of the images to determine the rate of cell migration (Liang *et al*., 2007).

BJ cells ($4x10^5$) were resuspended in 3mL of DMEM medium in μ-Dish$^{35mm, high}$ (Ibidi) and left in the incubator overnight to stabilize. After stabilization, the medium was changed and, with a p200 pipette tip, a ‘‘scratch’’ was made in the cell monolayer in a straight line to create a ‘‘scratch’’ area without cells. The medium was immediately removed and cells were washed two to three times with PBS 1X. Following, DMEM medium containing 2% of inactivated fetal calf serum was added to the cells to diminish cell proliferation. Photographs were captured with a coupled AxioCamMR3 camera with PALM reflector and 20X objective, using an inverted Axiovert 200 microscope with transmitted and reflected light, choosing a specific and numbered/lettered area to permit later recognition of the photographed area. Afterwards, BJ cells were incubated with 1μg/ml of LPS, or with LPS in the presence of 10/100nM of NT, or maintained in DMEM medium containing 2% of inactivated fetal calf serum (control) and allowed to migrate during 24h. After the incubation period, photographs were taken in the same area where the first photograph was taken. Photographs were analyzed and the number of cells in the scratch area was counted.

5.2. Immunocytochemistry assay

Immunocytochemistry assay was performed to analyze actin network of the cells. Accordingly, cells were fixed to preserve normal cell structure and permeabilized to permit the antibody entry into the cells. Cells were incubated with antibodies that target specific peptides or protein antigens in the cell via specific epitopes, like the antibody used in this work for actin - phalloidin. Phalloidin is an antibody which uses Phallotoxins properties, which are a group of bicyclic heptapeptides from *poisonous mushrooms* that bind actin filaments (Cooper *et al*., 1987). Usually, after incubation with the primary antibody, cells are incubated with a secondary antibody, conjugated with a fluorofore that binds to the primary antibody. However, the antibody used in the present work was already conjugated with the fluorofore Alexa Fluor 555. Moreover, cells were stained with 4',6-diamidino-2-phenylindole (DAPI), which is a blue fluorescent probe that fluoresces brightly upon selectively binding to the minor groove of double stranded DNA (Kapuscinski J., 1995).

FSDCs (5×10^5) and BJ cells (2×10^5) were cultured in 24-well plates with lamella and stimulated with 1µg/ml LPS, or treated with 10/100nM of NT, or stimulated with 1µg/ml LPS and treated with 10/100 nM of NT, or left untreated (control), during 24h. After incubation, cells were washed with ice-cold PBS, fixed with 4% paraformaldehyde in PBS for 10min, and then permeabilized with 0.1% Triton X-100 in PBS containing 200 mM glycine, during 5min. After a blocking step with 1% BSA, for 30min, cells were incubated with Alexa Fluor 555 phalloidin antibody (1:500) for 30min at room temperature and washed three times with PBS. Then, cells were incubated for 1min with DAPI (0.1 µg/ml in PBS) and mounted with Vectashield medium to reduce photobleaching. For image acquisition, fluorescence labelling was visualized using a fluorescence microscope - Zeiss Axiovert 200 - and images captured

with a coupled AxioCamHR camera. In each experiment, the optimal acquisition parameters were defined for the control cells and then maintained for all the other conditions within the same experiment. The filter set used included an excitation filter of 560nm and an emission filter of 575nm for Alexa Fluor 555 and an emission filter of 420nm for DAPI. The settings for contrast, brightness, pinhole, acquisition mode and scanning time were maintained throughout the work (Neves *et al*., 2009).

6. Western blot (WB)

6.1. Cell lysates preparation for WB analysis

FSDCs cells ($7{,}5x10^5$) were plated in 12-well plates and incubated overnight to adhere to the bottom of the wells. Then, cells were treated with 10nM of NT or/and 1µg of LPS during 5, 15, 30 and 60min. After incubation, cells were washed with ice-cold PBS and harvested in a sonication buffer containing 50 mM Tris HCl pH 8, 150 mM NaCl, 1% NP-40 (Nonidet P-40), 0.5% Sodium Deoxycholate, 0.1% SDS, 2 mM EDTA, proteases inhibitor cocktail, fosfatases inhibitor cocktail and DTT 1 mM. Cell lysates were sonicated three times, during 4sec, at 40µm in a Vibra Cell sonicator (Sonics & Material INC), to disrupt cells. Following, cell lysates were submitted to centrifugation (14,000g for 10min) and the supernatant was collected. Protein concentration was determined using the bicinchoninic acid (BCA) method and then, cell lysates were denatured at 95ºC, for 5min, in sample buffer (0.5 M Tris HCl pH 6.8; 10% (w/v) SDS; 0.6 M DTT; 30% (v/v) glycerol and 0.01% bromophenol blue).

6.2. Protein quantification by the bicinchonininc acid (BCA) method

BCA method is a copper-based protein assay used for protein quantification, sustained on the well-known "biuret reaction", whereby peptides containing three or more aminoacid residues form a colored chelate complex with Cu^{2+}, in an alkaline

environment containing sodium potassium tartrate. The BCA method involves two different step reactions: the biuret reaction, whose blue color results from the reduction of Cu^{+2} to Cu^{+1} by proteins; and the BCA chelation with Cu^{+1}, resulting in an intense purple color (Figure 11). The purple colored reaction product is formed by the chelation of two molecules of BCA with one cuprous ion. The purple product absorbs strongly at 540-570nm, which absorption can be read in a spectrophotometer. Since the production of copper in this assay is a function of protein concentration and incubation time, the protein content of unknown samples may be determined spectrophotometrically by comparison with known protein standards. Indeed, at the same time, the absorption of Bovine serum albumin (BSA) of increasing concentrations between 12.5 µg/mL and 800µg/mL was determined and used as a standard linear curve to determine protein concentration. Indeed, equal amounts of diluted protein samples (1:9) and water or BSA dilutions and the sample buffer RIPA in a final volume of 50µL were added to an Elisa plate. Then, 200µL of BCA reagent was added to the wells and the plate was incubated in the dark for 30min, at 37ºC. After incubation, the absorption was measured in ELISA

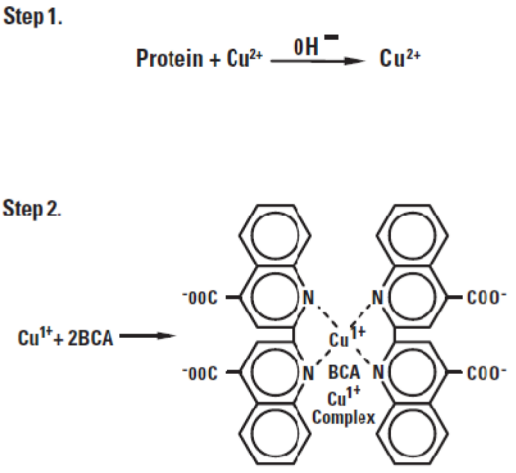


Figure 11 – Schematic reaction for the bicinchoninic acid (BCA)-containing protein assay (Pierce , 2009).

automatic microplate reader (SLT, Austria) at 570nm. This method is not affected by a range of detergents and denaturing agents such as urea and guanidinium chloride, although it is more sensitive to the presence of reducing sugars (Pierce, 2009).

6.3. SDS-PAGE, PVDF transfer and WB analysis

WB was used to determine proteins levels inside of the cell: the total amount of a determined protein or the phosphorylated amount of a determined protein inside of the cell. This technique gives us information about the levels of proteins and also phosphorylated proteins, which can indicate the activation of signaling pathways.

WB was performed to evaluate the levels of IκB-α, p-ERK1/ERK2, p-p38 MAPK, p-SAPK/JNK and p-Akt protein levels. Each gel was loaded always with the same amount of protein, which was 20 to 80µg (depending on the quantity needed to observe the protein band). Proteins were separated by electrophoresis (140 Volt during 60 to 70min) on a 10% (v/v) sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to polyvinylidene fluoride (PVDF) membrane. After electrophoresis and transfer, membranes were blocked with 5% (w/v) fat-free dry milk in Tris-buffered saline containing 0.1% (v/v) Tween 20 (TBS-T), for 1h at room temperature. Subsequently, blots were incubated overnight at 4ºC (p-ERK, p-JNK and IKB-α) or at room temperature (p-p38 and p-Akt) with the primary antibodies against the different proteins to be studied: IkBα (1:1000); p-ERK1/ERK2 (1:1000); p-p38 MAPK (1:1000); p-JNK (1:1000) and p-Akt (1:500). After incubation, membranes were washed three times for 15min with 0.1% TBS-T and incubated for 1h at room temperature, with alkaline phosphatase-conjugated anti-rabbit antibody (1:5000), or alkaline phosphatase-conjugated anti-mouse antibody (1:5000). The immune complexes were detected by membrane exposure to the ECF reagent, during 4 to 6min, followed by

scanning for blue excited fluorescence on the VersaDoc (Bio-Rad Laboratories, Amadora, Portugal). The generated signals were analyzed using the Image-Quant TL software.

To test whether similar amounts of protein for each sample were loaded, the membranes were stripped and reprobed with antibodies for total ERK1/2, SAPK/JNK, p38 MAPK and Akt, or with an antibody for β-actin, and blots were developed with alkaline phosphatase-conjugated secondary antibodies and visualized with ECF. The stripping was performed after PVDF membrane activation: 10sec in methanol, twice washing for 15min, incubation with water, 0.2 M of NaOH and water for 5min; finally, incubation with the blocking solution and reprobing the total protein antibodies were performed.

7. Real-time reverse transcription by polymerase chain reaction (real-time RT-PCR or qRT-PCR)

7.1. RNA extraction

FSDCs (2×10^6) or BJ cells (8×10^5) were plated in 6-well plates or 60mm dishes in a final volume of 4 or 6ml, respectively. Cells were treated with 1µg/ml of LPS during 6h and/or 10/100 nM of NT during 6h, or pre-treated with 10/100nM of NT during 24h and stimulated with 1 µg/ml of LPS during 6h, or left untreated (control). Total RNA was isolated from these cells with the TRIzol® reagent according to the manufacturer's instructions, to later determine the transcription levels of different molecules in the cell. Briefly, cells were washed with ice-cold PBS, harvested and homogenized in 1ml of Trizol by pipetting vigorously. After addition of 200µl of chloroform, samples were vortexed, incubated for 2min at room temperature and centrifuged at 12,000g, for 1min, at 4◦C. The aqueous phase containing RNA was transferred to a new tube, and then RNA precipitated with 500µl of isopropanol after

10min centrifugation at 12,000g. The pellet was washed with 1ml of 75% ethanol and resuspended in 100µl of 60◦C heated RNase free water. The RNA concentration was determined by OD260 measurement using a Nanodrop spectrophotometer (Wilmington, DE, USA). RNA was stored in RNA Storage Solution (Ambion, Foster City, CA, USA) at −80◦C.

7.2. Real-time RT-PCR

In the real-time RT-PCR technique total RNA is initially converted into cDNA, by reverse transcription. A specific sequence of the cDNA (the gene in study) is amplified using specific primers, and the inclusion of a fluorescent dye allows the detection of the reaction progress in real time. Finally, real-time RT-PCR allows the measurement of gene amplification, important to compare the amount of gene expression in the cell, in a control or treatment situation.

One microgram of total RNA was reverse transcribed using High Capacity cDNA Reverse Transcription (RT), from Applied Byosistems. Briefly, 2µl of 10X RT Buffer, 0.8µl of 25X dNTP Mix, 2µl of 10X RT random primers, 1µl of Multiscribe™ Reverse Transcriptase and 4.2µl of nuclease free H_20 were added to 10µl of RNA (1µg) sample. A protocol for cDNA synthesis was run on all samples (10min at 25◦C, 120min at 37◦C, 5min at 85◦C and then put on hold at 4◦C). After the cDNA synthesis, the samples were diluted with RNase-free water up to a volume of 100µl and concentration of 0.01 µg/µl.

Real-time RT-PCR was performed in a 20µl volume containing 2.5µl cDNA (25ng), 10µl 2X Syber Green Supermix, 2µl of each primer (250 nM) and 1µl of H_2O PCR grade. Samples were denatured at 95◦C during 3min. Subsequently, 40 cycles were

run for 10sec at 95◦C for denaturation, 30sec at the appropriate annealing temperature and 30sec at 72 ◦C for elongation.

Real-time RT-PCR reactions were run in duplicate for each sample on a Bio-Rad My Cycler iQ5. Primers were designed using Beacon Designer® Software v7.2, from Primier Biosoft International and thoroughly tested. Primer sequences are given in Table I. On each real-time RT-PCR plate there was a non-template control present for each pair of primers analyzed. For determination of primer-pair specific efficiencies, a four point dilution series of the control sample for each pair of primers was run on each experiment. Amplification reactions were monitored using a SYBR-Green assay. After amplification, a threshold was set for each gene and Ct-values were calculated for all samples. Gene expression changes were analyzed using the built-in iQ5Optical system software v2. The software enables analyzing the results with the Pfaffl method (Pfaffl, M., 2001), a variation of CT method corrected for gene-specific efficiencies, and to report gene expression changes as relative fold changes compared to the control samples. The results were normalized using a reference gene, hypoxanthine phosphoribosyltransferase 1 (HPRT-1), an enzyme that primarily function to salvage purines from degraded DNA to renewed purine synthesis, acting as a catalyst in the reaction between guanine and phosphoribosyl pyrophosphate to form GMP. This reference gene was previously validated in our lab (Neves *et al*., 2009) and its expression did not suffer variations upon FSDCs and BJ cell lines stimulation with LPS and NT.

Table I - Primer sequences for targeted cDNAs.

Primer	5'-3'Sequence (F:Forward; R-Reverse)	RefSeqID	Source
HPRT1	F: GTTGAAGATATAATTGACACTG R: GGCATATCCAACAACAAAC	NM_013556	Mouse
NT	F: AATGTTTGCAGCCTCATAAATAAC R:TGCCAACAAGGTCGTCATC	NM_024435	Mouse
NTR1	F: GGCAATTCCTCAGAATCCATCC R: ATACAGCGGTCACCAGCAC	NM_018766	Mouse
NTR2	F: GCCATTACTAACAGTCTAAGC R: GCAATTCGTCCTATTCTACAC	NM_008747	Mouse
NTR3	F: ATGGCACAACTTCCTTCTG R: AGAGACTTGGAGTAGACAATG	NM_019972	Mouse
IL-1β	F: ACCTGTCCTGTGTAATGAAAG R: GCTTGTGCTCTGCTTGTG	NM_008361	Mouse
IL-6	F: TTCCATCCAGTTGCCTTC R: TTCTCATTTCCACGATTTCC	NM_031168	Mouse
IL-10	F: CCCTTTGCTATGGTGTCCTTTC R: ATCTCCCTGGTTTCTCTTCCC	NM_010548	Mouse
TNF-α	F: CAAGGGACTAGCCAGGAG R: TGCCTCTTCTGCCAGTTC	NM_013693	Mouse
IFN-δ	F: CTTCTTGGATATCTGGAGGAACTG R: GGTGTGATTCAATGACGCTTATG	NM_008337	Mouse
G-CSF	F: TCATTCTCTCCACTTCCG R: CTTGGTATTTACCCATCTCC	NM_009971	Mouse
CCL5	F: CACTCCCTGCTGCTTTGC R: CACTTGGCGGTTCCTTCG	NM_013653	Mouse
VEGF-A	F: CTT GTT CAG AGC GGA GAA AGC R: ACA TCT GCA AGT ACG TTC GTT	NM_001025250	Mouse
EGF	F: GCA CAG TTT GTC TTC AAT GGC R: TGT TGG CTA TCC AAA TCG CCT TGC	NM_010113	Mouse
PDGF-BB	F: ATT AGA GGT GCA GTG TGC GTG TGA R: AGG GCA CAT GAG GAA GAA GAC ACA	NM_011057	Mouse
IRS-1	F: ACC GAC GCA TGG TCT ATG TTG CTA R: AAA CCT GGC CTG GTA CCC ATT ACA	NM_010570	Mouse
GLUT4	F: AAC GGA TAG GGA GCA GAA ACC CAA R: GTG CAA AGG GTG AGT GAG GCA TTT	NM_009204	Mouse
HPRT1	F: TGACACTGGCAAAACAATG R: GGCTTATATCCAACACTTCG	NM_000194	Human
NT	F: GCATACATCAAAGATTAGT R: TAAAGCAGTAGGAAGTTT	NM_006183	Human
NTR1	F: GTCGTCATACAGGTCAAC R:GATGATGGTGTTCAGGAC	NM_002531	Human
NTR2	F: GCAAGAATGAACAGAACA R: GAATGATTAGTGATGAGGTT	NM_012344	Human
NTR3	F: TGGGTTGGAGATAGCACTGG R: ACGACTTCCTCCAGACACCT	NM_002959	Human
IL-1β	F: GCTTGGTGATGTCTGGTC R: GCTGTAGAGTGGGCTTATC	NM_000576	Human
IL-6	F: TCTGGATTCAATGAGGAGACTTG R: TCACTACTCTCAAATCTGTTCTGG	NM_000600	Human
IL-8	F: CTTTCAGAGACAGCAGAG R: CTAAGTTCTTTAGCACTCC	NM_000584	Human
CCL11	F: ACCAGAGCCTGAGTGTTG R:TGCCCTTTGGACTGATAATGA	NM_002986	Human
CCL4	F:CGCCTGCTGCTTTTCTTACAC R:CAGACTTGCTTGCTTCTTTTGG	NM_002984	Human

CCL5	F: CAGTGAGCTGAGATTGTG R:TTTGTTGTTGTTGTTGTGA	NM_002985	Human
VEGF-A	F:CACTAGCTTATCTTGAAC R:ATAGTATGTAGATGTATATTGAA	NM_001025366	Human
EGF	F: TCAGAAGATAACATTACAGAAT R: AATACACCGAGCATACAT	NM_001178130	Human
IRS-1	F: AGTCCCAGCACCAACAGAAC R: TCATCCGAGGAGATGAAACC	NM_005544	Human
GLUT4	F: AGGTGAAACCCAGCACAGAACTTG R: AGAGGGTTAAAGTGCTGCAGAGGA	NM_001042	Human

Legend: Hypoxanthine phosphoribosyltransferase 1 (HPRT1); Neurotensin (NT); Neurotensin receptor (NTR) Interleukin (IL); Tumor necrosis factor (TNF); Interferon (IFN); Granulocyte colony-stimulating factor (G-CSF); Vascular endothelial growth factor (VEGF); Epidermal growth factor (EGF); Platelet-derived growth factor (PDGF); Insulin receptor substrate-1 (IRS-1); Glucose transporter 4 (GLUT4).

7.3. Calculation of real-time RT-PCR results

Since the real-time RT-PCR results are presented as ratios of treated samples over untreated (control) or LPS treated cells, the distribution of the data does not follow a normal distribution. Therefore, a two-base logarithmic transformation was therefore used to make observations symmetric and closer to a normal distribution. If x represent the fold change of a gene in one sample, then the two-base logarithmic transformation is: $\log2(x) = \ln(x1)/\ln(2)$. This way, fold changes of 2 and 0.5 correspond to mean log2 values of 1 and −1, respectively.

8. Statistical analysis

The results are presented as mean ± S.D. of the indicated number of experiments, and the statistic difference between two groups was determined by the two-sided unpaired Student's t test. For multiple group comparisons, the One-Way ANOVA test, with a Dunnett's or Bonferroni´s Multiple Comparison post-test was used. The tests were performed using GraphPad Prism, version 5.02 (GraphPad Software, San Diego, CA, USA). Statistically significant values are as follows: $*P<0.05$, $**P<0.01$, $***P<0.001$.

Chapter III. Results on FSDCs

1. Neurotensin and neurotensin receptors

The presence of NTRs, namely NTR1, NTR2 and NTR3, was determined in untreated cells (control) and in cells exposed to LPS during 30min. It was observed that NTR1 and NTR3 were constitutively expressed in FSDCs (Figure 12), as it can be observed by WB analysis. In contrast, the NTR2 seemed not to be expressed by FSDCs, as it was not detectable by WB analysis. Additionally, LPS did not modify the protein levels of NTR1 and NTR3 (Figure 12). As WB analysis gave essentially qualitative information about the presence of the receptors, we next confirmed the absence of NTR2 by real-time RT-PCR analysis in untreated cells (control) and in cells exposed to LPS during 6h. Furthermore, as these receptors are present at the cell surface, in cell membranes, or internalized as a receptor–ligand complex, the methods used to obtain cell extracts could not had been effective enough to achieve these receptors and quantify them. In addition, NTRs mRNA levels in normal and stimulated conditions can be evaluated with more accuracy by real-time RT-PCR. Indeed, accordingly to WB results, NTR1 and NTR3 were expressed in FSDCs, while NTR2 was almost absent. Comparing the relative amounts of the transcripts, NTR3 appeared to be the most expressed among the analyzed NTRs receptors (Figure 13). The treatment of cells with LPS during 6h caused a decrease on the transcription of all studied NTRs, by -1.14 ± 0.55 (n=3), -1.88 ± 0.77 (*$p<0.05$, n=3) and -0.85 ± 0.78 (n=3) fold, for NTR1, NTR2 and NTR3 expression relatively to the control (Figure 14). The effect of NT in NTR3 expression was also analyzed by RT-PCR in untreated cells (control) and cells exposed to 10 nM of NT during 6h. It was observed that NT increased NTR3 mRNA expression by 0.94 ± 0.68 (n=3) fold (data not shown). Furthermore, NT was not constitutively expressed in FSDCs (Figure 13), however, LPS caused a 3.75 ± 0.50 (***$p<0.0001$, n=3) fold-increase relatively to the control (Figure 14).

Figure 12 – NTRs (NTR1 and NTR3) expression in FSDCs by WB analysis. NTR2 expression was not detectable by WB analysis.
FSDCs were maintained in IMDM medium (control), or incubated with 1µg/ml of LPS during 30min. Total cell extracts were subjected to SDS-PAGE, transferred to PVDF membranes and subjected to WB analysis, using the NTR1, NTR2 and NTR3 antibodies. The blot shown is representative of 3 independent experiments yielding similar results.

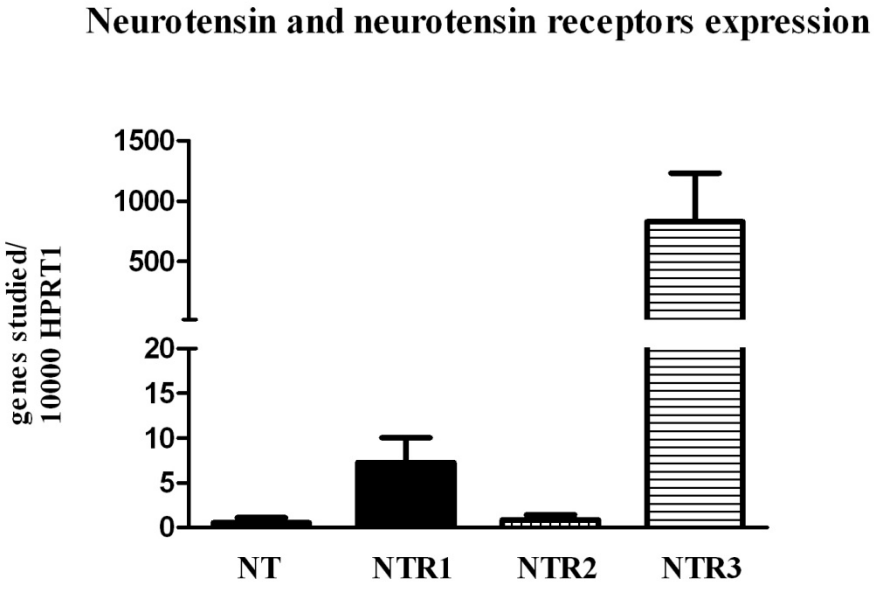


Figure 13 – NT and NTRs (NTR1, NTR2 and NTR3) were differentially expressed in FSDCs.
Cells were plated at 2×10^6 cells/well in 6-well microplates in a final volume of 6 ml of medium, at 37◦C, with 5% CO^2. Total RNA was isolated and retrotranscribed as indicated in experimental procedures. The mRNA levels were assessed by quantitative real-time RT-PCR. Gene expression is indicated as genes studied/10 000 molecules of the reference gene HPRT1. Each value represents the mean±S.D. from three independent experiments.

The effect of LPS on neurotensin and neurotensin receptors expression

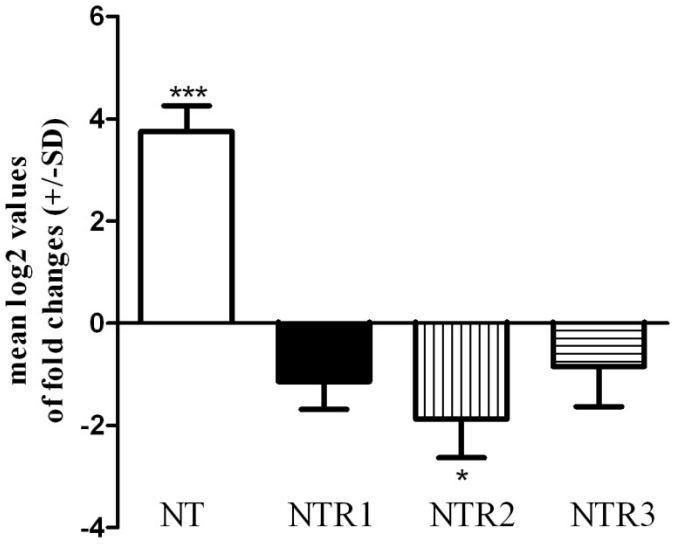


Figure 14 – NT and NTRs (NTR1, NTR2 and NTR3) were differentially expressed in inflammatory conditions, in FSDCs.
FSDCs were maintained in IMDM medium (control) or were incubated with 1µg/ml of LPS during 6h. Total RNA was isolated and retrotranscribed as indicated in experimental procedures. The mRNA levels were assessed by quantitative real-time RT-PCR. Gene expression is indicated as mean log2 values of fold changes relatively to the control. Each value represents the mean±S.D. from three independent experiments (*$p < 0.05$; ***$p < 0.001$ unpaired two-tailed t-student test).

2. The effect of neurotensin on FSDCs viability

FSDCs were incubated with 10, 50 and 100 nM of NT, from 5 to 60min, in order to evaluate if NT provoked toxicity to the cells. None of the three concentrations tested caused mortality to the cells. Even so, 10 nM of NT was able to slightly increase FSDCs viability (28% for all time exposures) (Figure 15A). Cells were also incubated with LPS alone or in the presence of different concentrations of NT. Cell viability was not affected by these compounds; furthermore, 10 and 50 nM of NT slightly increased cell viability, after 5min of incubation (Figure 15B).

FSDCs were also incubated with 10, 50 and 100 nM of NT, from 1 to 7 days, to determine if NT was toxic to the cells during long-time periods of incubation (Figure

16A). All NT concentrations barely diminished FSDCs viability. However, 10 nM of NT was the harmless concentration at the first day (cell viability increased 5% and 13% relatively to the 50 and the 100 nM concentrations), although 50 nM of NT was the most efficient concentration at the third day (cell viability increased 12% and 6% relatively to the 10 and 100 nM concentrations) and, 100 nM at the fifth day (cell viability increased 17% of the mean, versus the 10 and 50nM concentrations) and at the seventh day (cell viability increased 15% and 8% relatively to the 10 and the 50 nM concentrations). At the seventh day, the viability was maintained similar to the control (100 ± 17%) (Figure 16A). FSDCs were also incubated with LPS and different concentrations of NT from 1 to 7 days (Figure 16B) and the results demonstrated that along the 7 days, cells treated with LPS and 10 nM of NT showed a decrease on cell viability (from 4% to 14%) than cells only treated with LPS. However, cells treated with LPS and 100 nM of NT demonstrated a tendency to more viability when compared to cells treated with only LPS at short (31% at day 1) and long-time incubation periods (6% and 7% at day 5 and 7). Taking into account the long period of incubation, none of these compounds significantly affected cell viability.

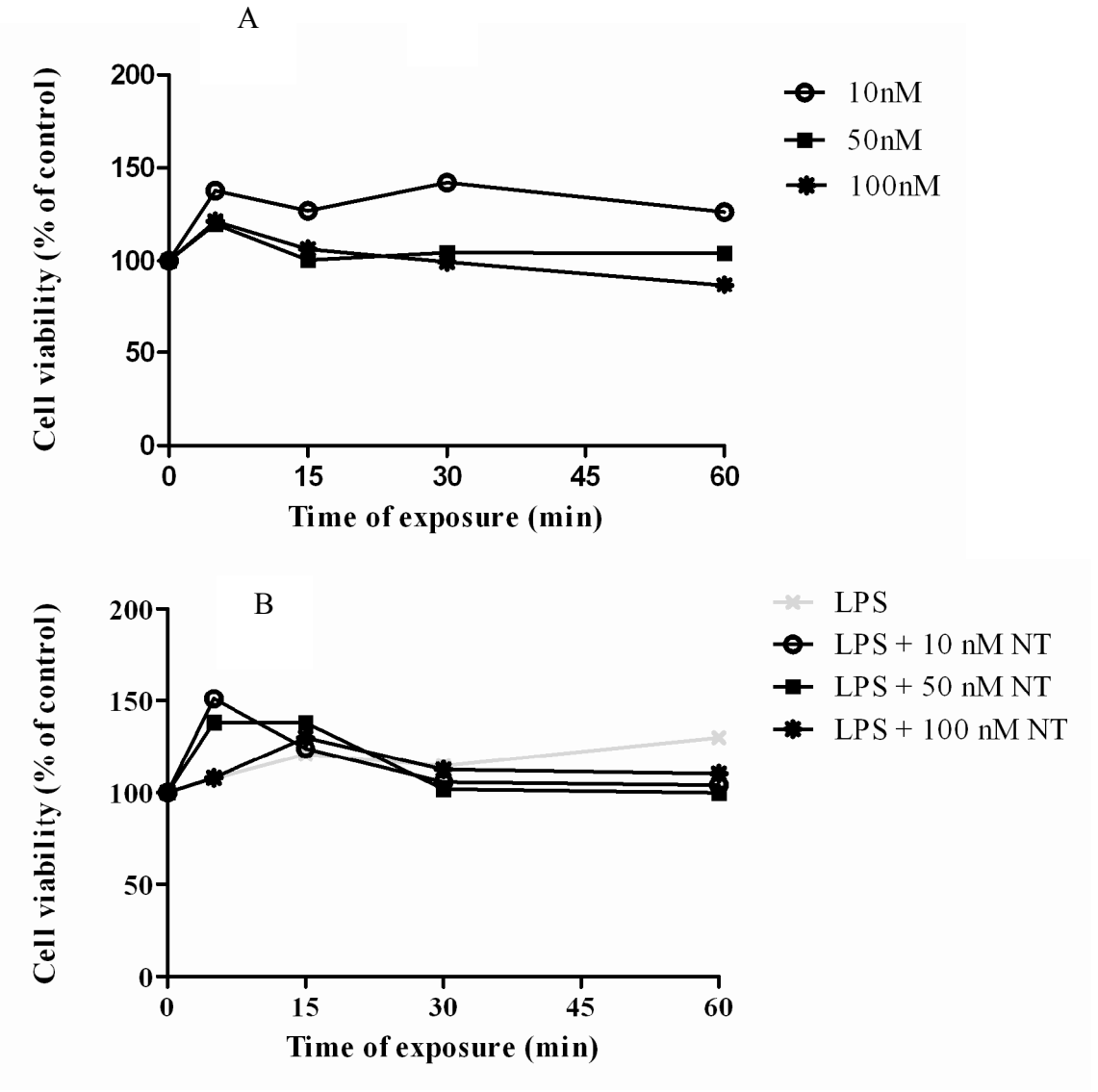


Figure 15 – NT did not affect FSDCs viability during short-time periods of incubation.

FSDCs were maintained in IMDM medium (control), or were incubated with 10, 50 or 100 nM of NT, or with 1µg/ml of LPS in the presence of 10, 50 or 100 nM of NT, during 5, 15, 30 or 60min. Cell viability was determined by the MTT reaction. The results were expressed as % of MTT reduction relatively to the control. Each value represents the mean from 2 experiments, performed in duplicate.

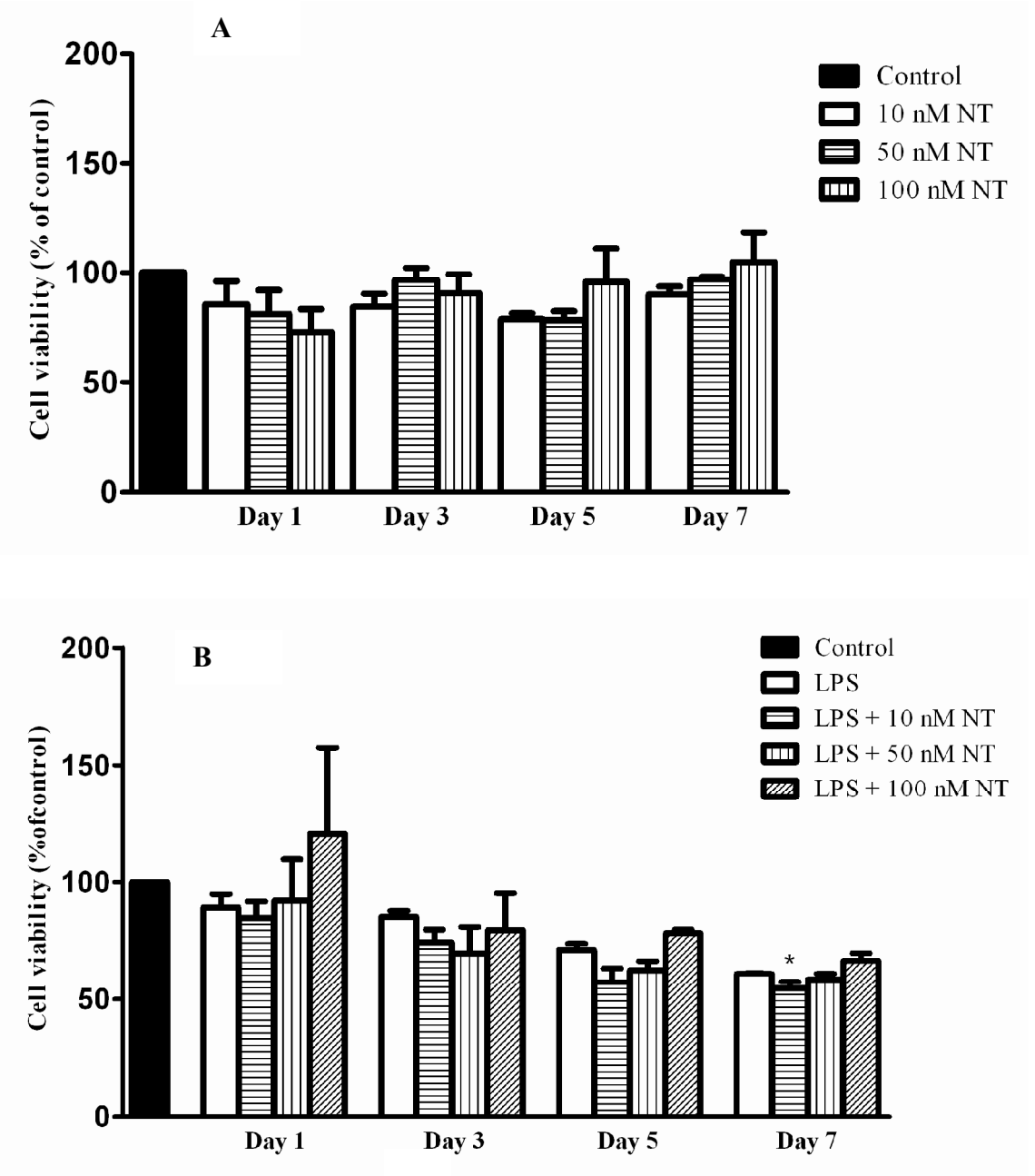


Figure 16 – FSDCs viability was slightly and differentially modulated by distinct NT concentrations, during long-time periods of incubation. FSDCs were maintained in IMDM medium (control), or incubated with 10, 50 or 100 nM of NT, or with 1µg/ml of LPS in the presence of 10, 50 or 100 nM of NT, during 7 days. In the last day, the viability of the cells was determined by the MTT reaction. The results were expressed as % of MTT reduction relatively to the control. Values were compared using one-way ANOVA followed by the Dunnett's comparison test for each day relatively to LPS. Each value represents the mean ± SD from 3 experiments, performed in duplicate (*$p<0.05$).

3. iNOS expression and nitrite production

FSDCs were maintained in IMDM medium or incubated with 1 µg/mL of LPS during 6h. After incubation, total RNA was isolated from cells, quantified and reverse transcribed to cDNA, to finally perform real-time RT-PCR. FSDCs highly expressed iNOS which was upregulated by the LPS stimulus (data not shown). Thus, iNOS expression might have represented an elevated iNOS production and, *per se*, high iNOS-regulated NO production. Furthermore, NO production was analyzed in NT treaded cells, with or without LPS, during 1 to 7 days.

FSDCs were incubated with 10, 50 and 100 nM of NT to determine nitrite levels. All NT treatments diminished nitrite production (data not shown). Cells were also incubated with LPS and LPS-treated cells were incubated with different concentrations of NT, from 1 to 7 days. In comparison with control cells, LPS increased nitrite levels to 4484 ± 1454% (n=3) relatively to the control in the first day, followed by a decrease till the seventh day. Additionally, it was observed that at day 1, in LPS-treated cells simultaneously exposed to NT, all NT concentrations tended to increase nitrite levels, being this increase lower for 10 nM and higher for 100 nM. In the 3rd, 5th and 7th days, 10 nM of NT tended to diminish nitrite production, while 50 and 100 nM of NT tended to increase nitrite production. These effects were not statistically significant (n=3) (Figure 17).

Taken together, FSDCs treatment with NT decreased nitrite production and in the presence of LPS, 10nM of NT was able to decrease nitrite levels, in contrast to 50 and 100 nM of NT that increased nitrite levels.

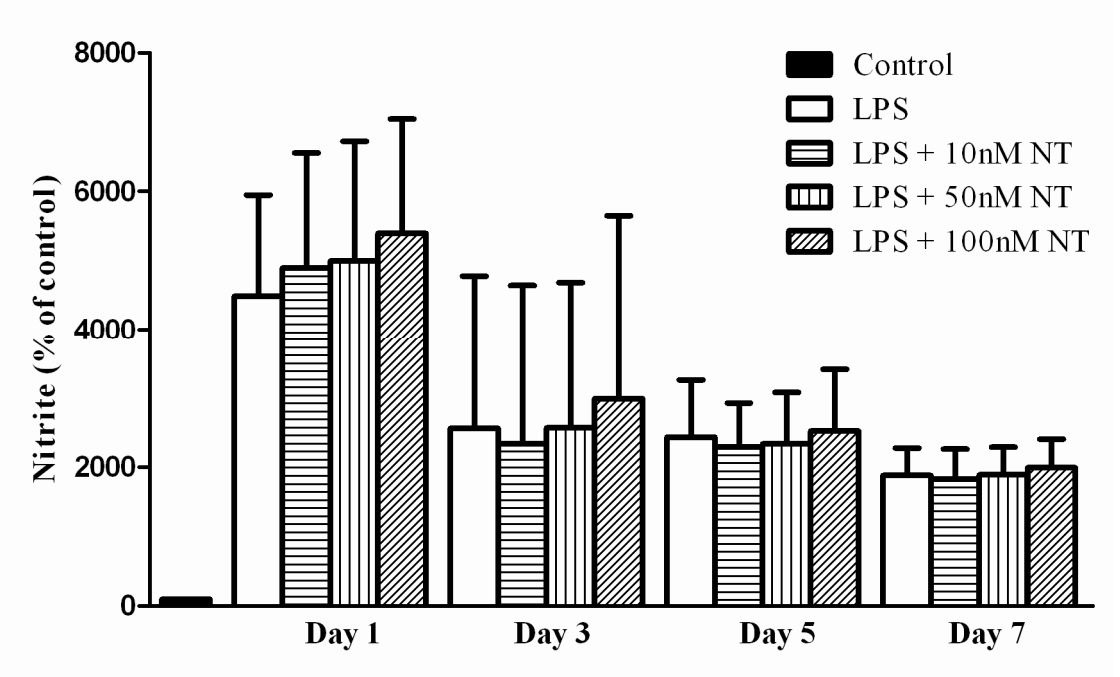


Figure 17 – NT modulated nitrite production in FSDCs.
FSDCs were maintained in IMDM medium (control), or with 1 µg/mL of LPS, or with LPS in the presence of 10, 50 or 100 nM of NT, during 7 days. Culture medium was collected every 2 days and used for nitrite determination, by the griess reaction. The results were expressed as % of nitrite production by control cells. Values were compared using one-way ANOVA followed by the Dunnett's comparison test. Each value represents the mean ± SD from 3 independent experiments, performed in duplicate.

4. Activation of intracellular signaling cascades

FSDCs were incubated with 10 nM of NT during 5, 15, 30 and 60min, to evaluate the effect of the neuropeptide on intracellular signaling pathways activation. WB analysis was performed to detect the phosphorylated form of the MAP kinases (ERK1/ERK2, SAPK/JNK and p38 MAPK) and Akt. The involvement of the transcription factor NF-κB was also evaluated by the determination of protein levels of its inhibitory protein IκB-α.

NT upregulated IκB-α levels, activated Akt and slightly activated p38 MAPK. By contrast, ERK and SAPK/JNK MAP kinases signaling pathways were not significantly activated (Figure 18). The NF-κB inhibitory protein – IκBα – was not

degraded and it even seemed to be increased in a time-dependent manner till 30min, indicating the inhibition of NF-κB (Figure 19). FSDCs were also incubated with 1 μg/ml of LPS and 10 nM of NT during 5, 15, 30 and 60min, to determine the effect of NT on LPS-induced intracellular signaling pathways activation. The results obtained with 100 nM of NT were similar to those obtained with 10 nM, therefore the experiments were performed with 10 nM of NT. Upon LPS stimulation, maximal ERK1/ERK2, SAPK/JNK, p38 MAPK and Akt phosphorylation was observed between 15 and 30min, being this activation more notorious in the ERK1/ERK2 signaling pathway as it appeared to be more activated than the others. The inhibitory protein of NF-κB – IκBα – diminished in a time-dependent manner until 30min, indicating the activation of NF-κB. When NT was added simultaneously as a second stimulus, p38 MAPK and ERK1/ERK2 signaling pathways were upregulated essentially at 30min, while the SAPK/JNK signaling pathway was downregulated at 30min, when compared with the results obtained with LPS alone. Between 5 and 15min, NT also provoked an increase of the inhibitory protein of NF-κB – IκBα – diminishing NF-κB activation. No alteration was observed in the Akt signaling pathway when cells were incubated with NT and LPS, in comparison with LPS alone. Results are represented in Figures 20 to 24. The same results were obtained when NT was added 30min before LPS or with cells incubated with 100 nM of NT (data not shown). When NTR1 was inhibited with SR48692, inactivation/activation of the signaling pathways returned to normal. WB bands were quantified for the experiments in which NT modulated the signaling pathways studied.

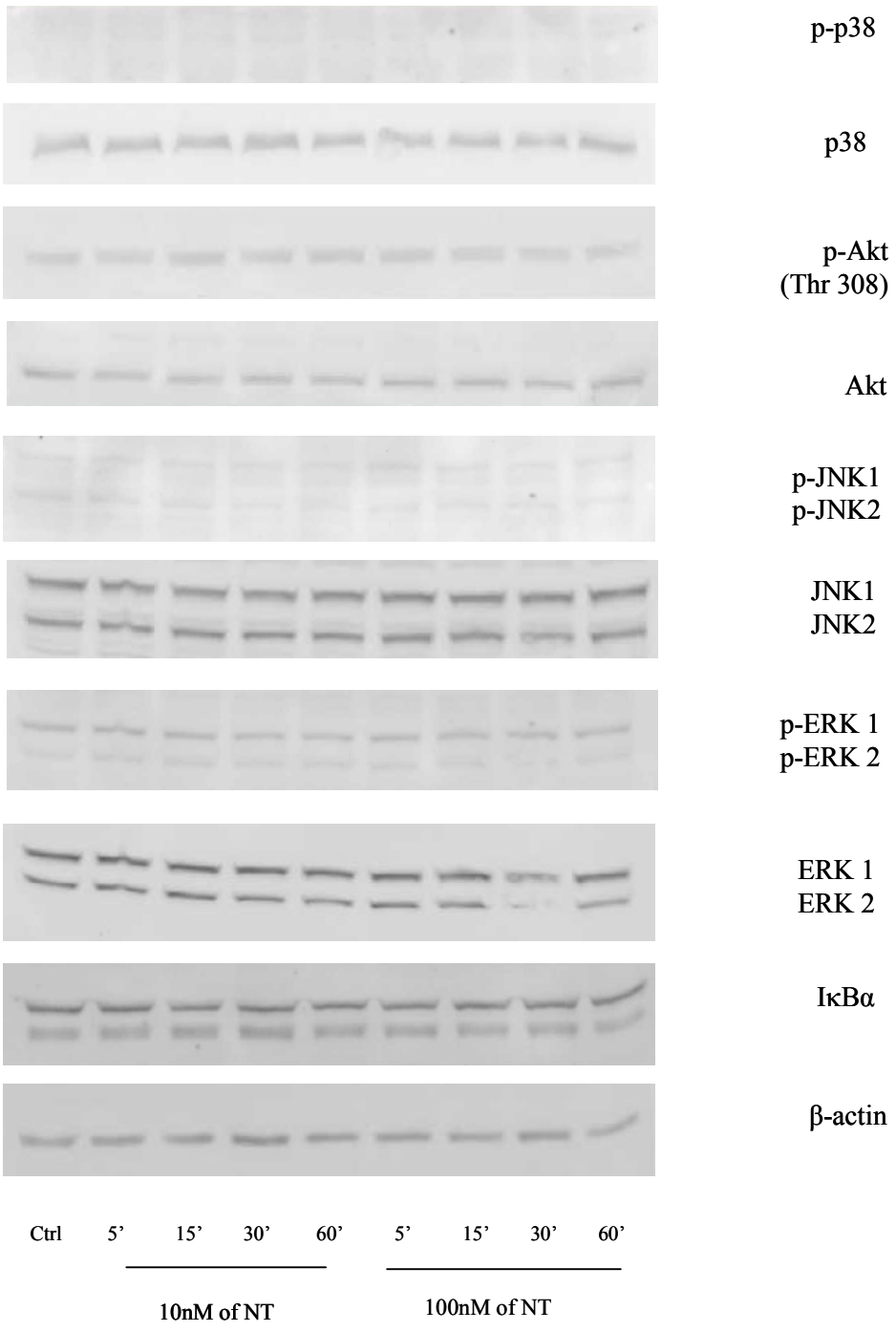


Figure 18 – NT modulated the activation of significant inflammatory signaling cascades in FSDCs.
FSDCs were maintained in IMDM medium (control), or were incubated with 10nM of NT, during 5, 15, 30 or 60min. Total cell extracts were subjected to SDS-PAGE, transferred to PVDF membranes and subjected to WB analysis, using antibodies to p-ERK1/2, p-JNK, p-p38 and p-Akt. The activation of NF-κB was evaluated by the determination of the levels of its inhibitory protein, IkB-α (WB band on the bottom). Equal loading was evaluated with antibodies to ERK1/2, SAPK/JNK, p38 MAPK and Akt or with an anti-actin antibody. The results were expressed as % of intensity relatively to the control. The blots shown are representative of at least three independent experiments yielding similar results.

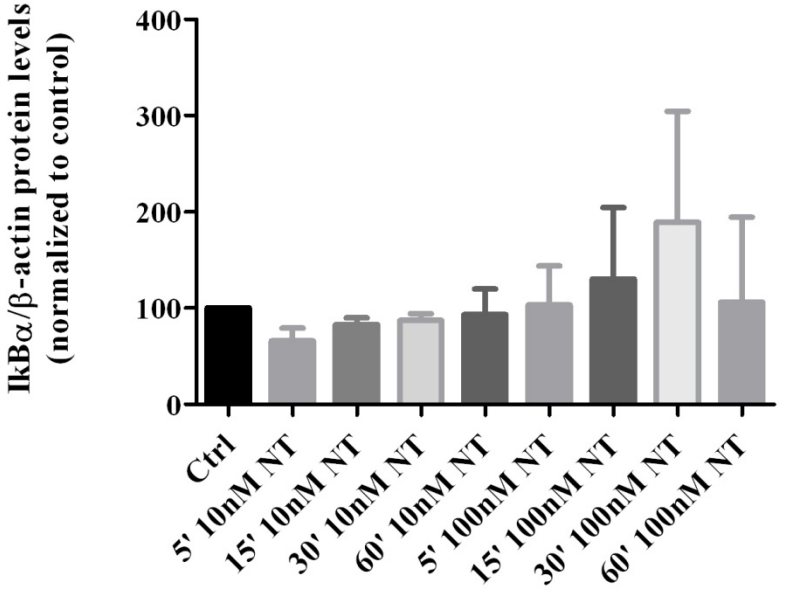


Figure 19 – Quantification of the expression levels of IκB-α by WB, normalized to β-actin antibody.
FSDCs were maintained in IMDM medium (control), or were incubated with 10 nM of NT, during 5, 15, 30 or 60min. Total cell extracts were subjected to SDS-PAGE, transferred to PVDF membranes and subjected to WB analysis. The activation of NF-κB was evaluated by the determination of the levels of its inhibitory protein, IκB-α, and normalized to β-actin antibody. The results were expressed as % of IκB-α production relatively to the control. The graphic shown is representative of at least 3 independent experiments yielding similar results. Each value represents mean±SD.

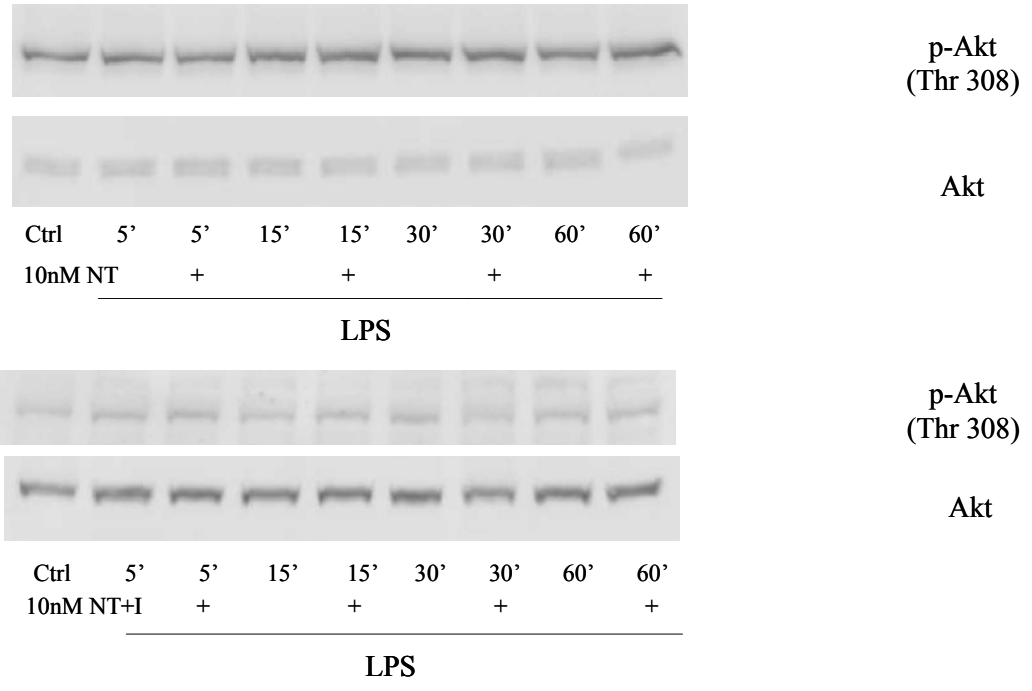
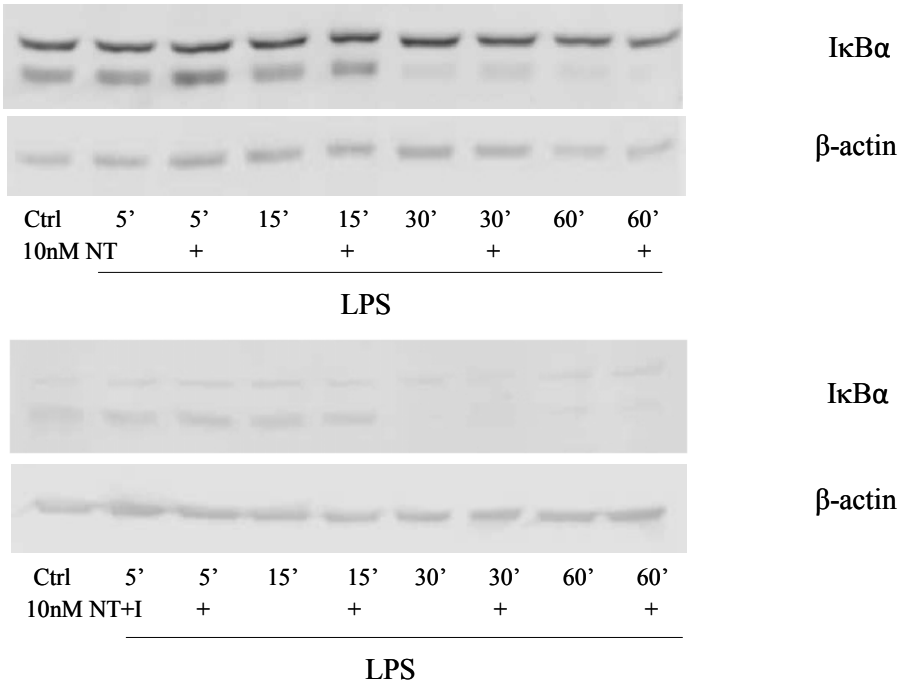


Figure 20 - Determination of p-Akt/Akt expression by WB analysis.

Figure 20 (continued) - FSDCs were maintained in IMDM medium (control), or were incubated with 1µg/ml of LPS, or with LPS in the presence of 10 nM of NT, or in the presence of 10 nM of NT and NTR1 inhibitor (I), during 5, 15, 30 or 60min. Total cell extracts were subjected to SDS-PAGE, transferred to PVDF membranes and subjected to WB analysis, using p-Akt antibody, normalized to Akt antibody, respectively. The blot shown is representative of at least 3 independent experiments yielding similar results.



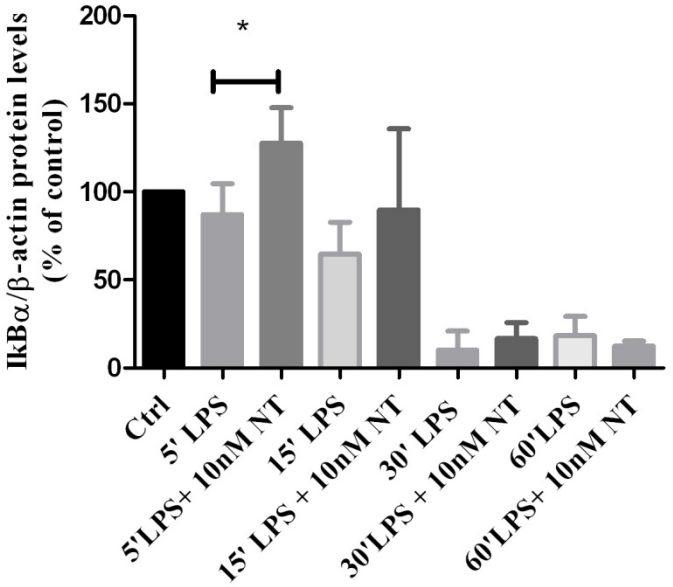


Figure 21 – Determination of IκB-α/β-actin expression by WB analysis. Quantification of WB bands was evaluated with normalization to β-actin antibody.

Figure 21 (continued) - FSDCs were maintained in IMDM medium (control), or were incubated with 1µg/ml of LPS, or with LPS in the presence of 10 nM of NT, or in the presence of 10 nM of NT and NTR1 inhibitor (I), during 5, 15, 30 or 60min. Total cell extracts were subjected to SDS-PAGE, transferred to PVDF membranes and subjected to WB analysis. The activation of NF-κB was evaluated by determination of the levels of its inhibitory protein, IκB-α (WB band on the bottom), and normalized to β-actin antibody. The blot shown is representative of at least 3 independent experiments yielding similar results. The results were expressed as % of IκB-α expression relatively to the control. Each value represents mean±SD (*p < 0.05 t-student test, comparing treatments and stimulus of the same time set).

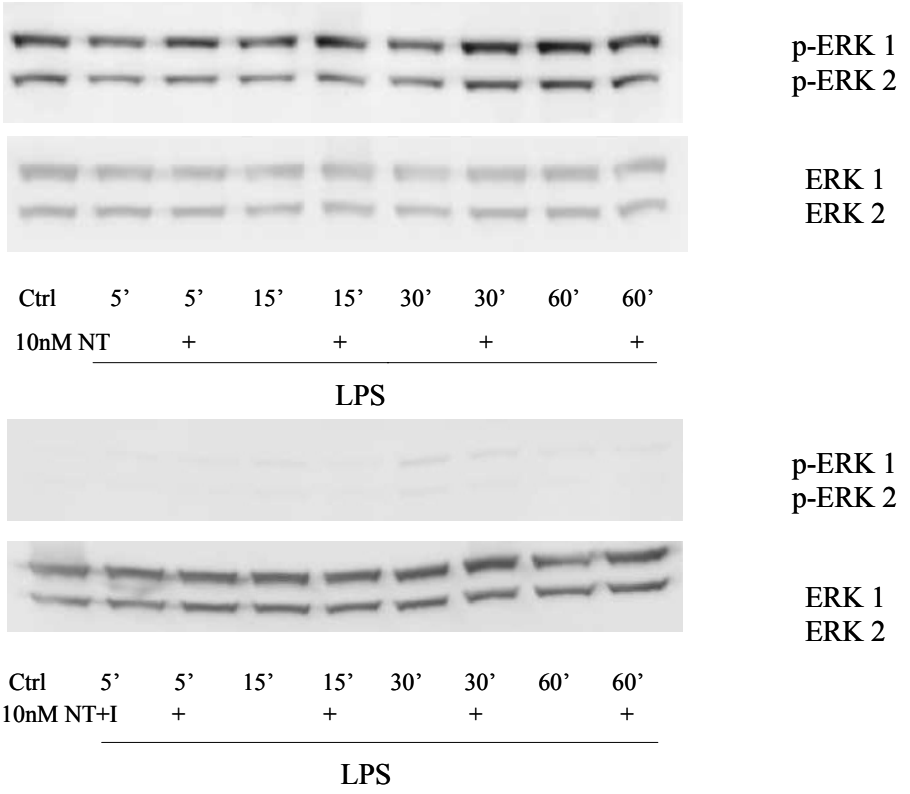


Figure 22 - Determination of p-ERK/ERK bands by WB analysis. Quantification of WB bands was evaluated with normalization to ERK antibody.

FSDCs were maintained in IMDM medium (control), or were incubated with 1µg/ml of LPS, or with LPS in the presence of 10 nM of NT, or in the presence of 10 nM of NT and NTR1 inhibitor (I), during 5, 15, 30 or 60min. Total cell extracts were subjected to SDS-PAGE, transferred to PVDF membranes and subjected to WB analysis, using p-ERK antibody, normalized to ERK antibody. The blot shown is representative of at least 3 independent experiments yielding similar results. The results were expressed as % of p-ERK expression relatively to the control. Each value represents mean±SD (t-student test, comparing treatments and stimulus of the same time set).

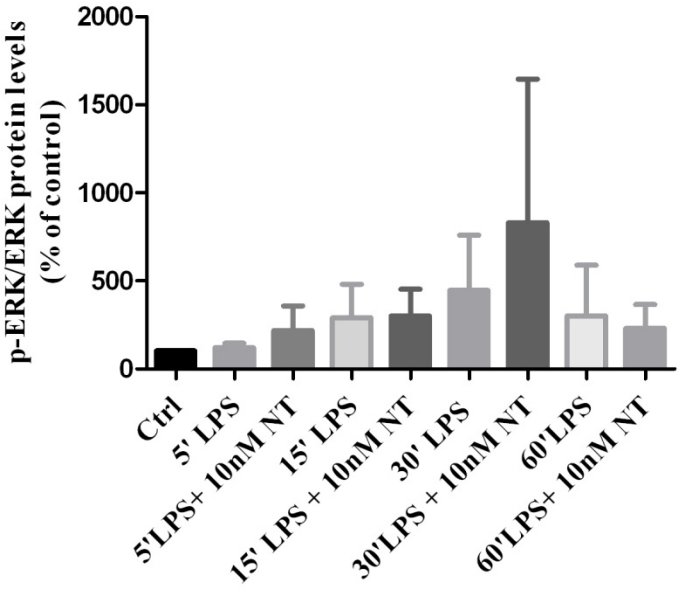


Figure 22 (continued).

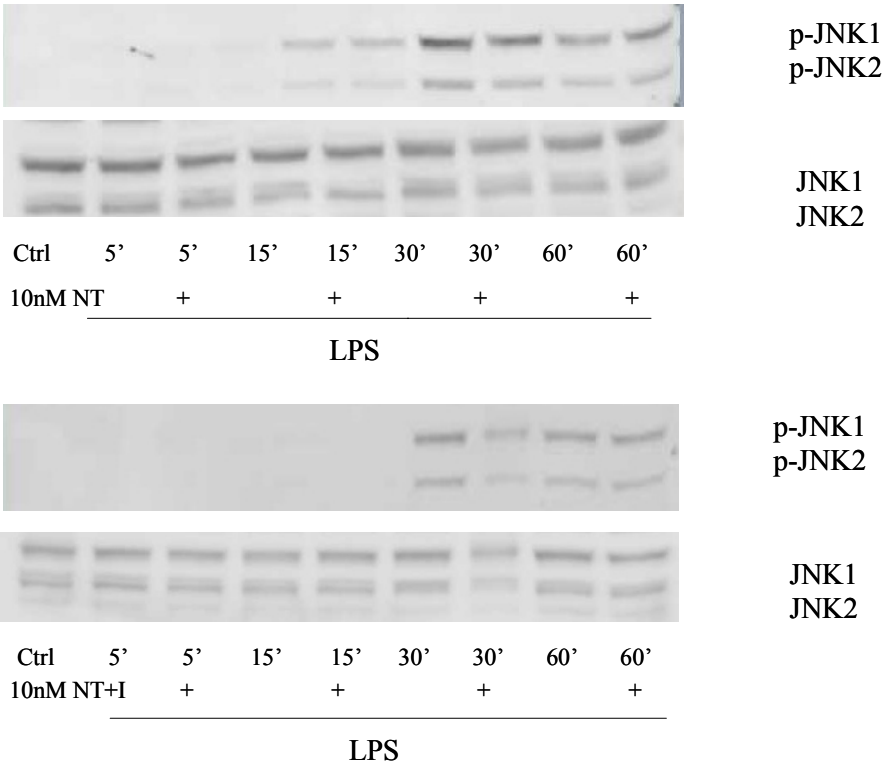


Figure 23 - Determination of p-JNK/JNK bands by WB analysis. Quantification of WB bands was evaluated with normalization to JNK antibody.

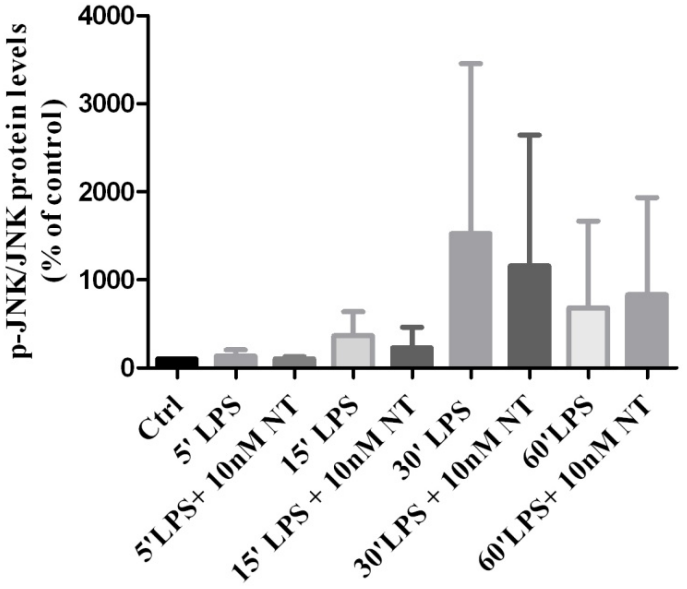


Figure 23 (continued) - FSDCs were maintained in IMDM medium (control), or were incubated with 1µg/ml of LPS, or with LPS in the presence of 10 nM of NT, or in the presence of 10 nM of NT and NTR1 inhibitor (I), during 5, 15, 30 or 60min. Total cell extracts were subjected to SDS-PAGE, transferred to PVDF membranes and subjected to WB analysis, using p-JNK antibody, normalized to JNK antibody. The blot shown is representative of at least 3 independent experiments yielding similar results. The results were expressed as % of p-JNK expression relatively to the control. Each value represents mean±SD (t-student test, comparing treatments and stimulus of the same time set).

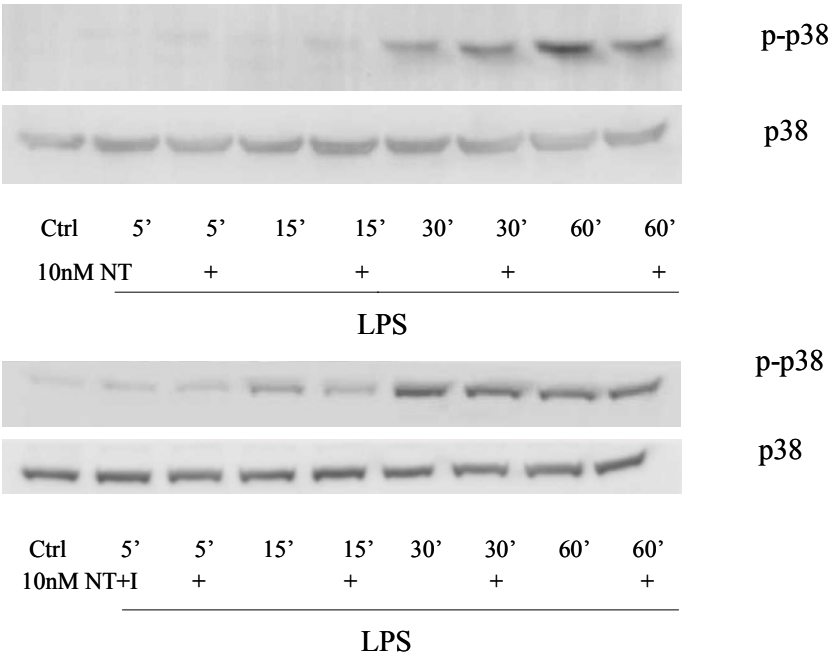


Figure 24 - Determination of p-p38/p38 expression by WB analysis. Quantification of WB bands was evaluated with normalization to p38 antibody.

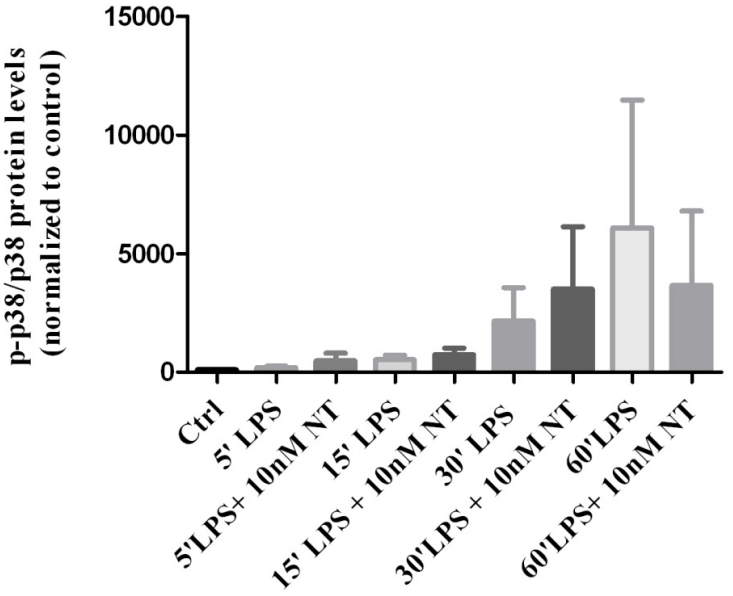


Figure 24 (continued) - FSDCs were maintained in IMDM medium (control), or were incubated with 1µg/ml of LPS, or with LPS in the presence of 10 nM of NT, or in the presence of 10 nM of NT and NTR1 inhibitor (I), during 5, 15, 30 or 60min. Total cell extracts were subjected to SDS-PAGE, transferred to PVDF membranes and subjected to WB analysis, using p-p38 antibody, normalized to p38 antibody. The blot shown is representative of at least 3 independent experiments yielding similar results. The results were expressed as % of p-p38 expression relatively to the control. Each value represents mean±SD (t-student test, comparing treatments and stimulus of the same time set).

5. The effect of glucose and LPS on FSDCs

FSDCs were cultured in IMDM medium with 30 mM of glucose to reproduce hyperglycemic conditions observed in diabetic patients. To determine if an increase in glucose levels alter Insulin receptor substrate 1 (IRS-1) and Glucose transporter type 4 (GLUT4) expression in FSDCs, cells were also cultured at 25 mM of glucose. Additionally, FSDCs response to different glucose levels was also analyzed in an inflammatory environment, by stimulating FSDCs with LPS, during 6h.

The expression of IRS-1 and GLUT4 was studied in cells cultured in the presence of either 25 or 30 mM of glucose. Firstly, FSDCs cultured in 25mM of glucose exhibited both IRS-1 and GLUT4 expression, showing 17.22 ± 24.78 and 33.21 ± 22.93 (n=3) expression per reference gene, indicating that insulin signaling and GLUT4

trafficking may occur in these cells. Secondly, cells cultured in higher glucose levels (30 mM) had higher IRS-1 and GLUT4 expression, by 82.50 ±74.04 and 110.83 ± 85.62 (n=3) expression per reference gene, when compared to cells cultured with 25 mM of glucose (Figure 25A). However, the mechanism by which this process occurred was not elucidated in this work. The expression of IRS-1 and GLUT4 was also studied in cells cultured with 25 and 30 mM of glucose, in inflammatory conditions (6h of LPS stimulus). The main conclusions to retain from this study were a tendency in IRS-1 to decrease by -2.60 ± 1.46 (n=3) fold in cells cultures with 30mM of glucose and in inflammatory conditions relatively to the control (Figure 25B). Indeed, during inflammatory conditions, insulin signaling might be affected, especially in higher glucose conditions (tendency for IRS-1 expression to decrease).

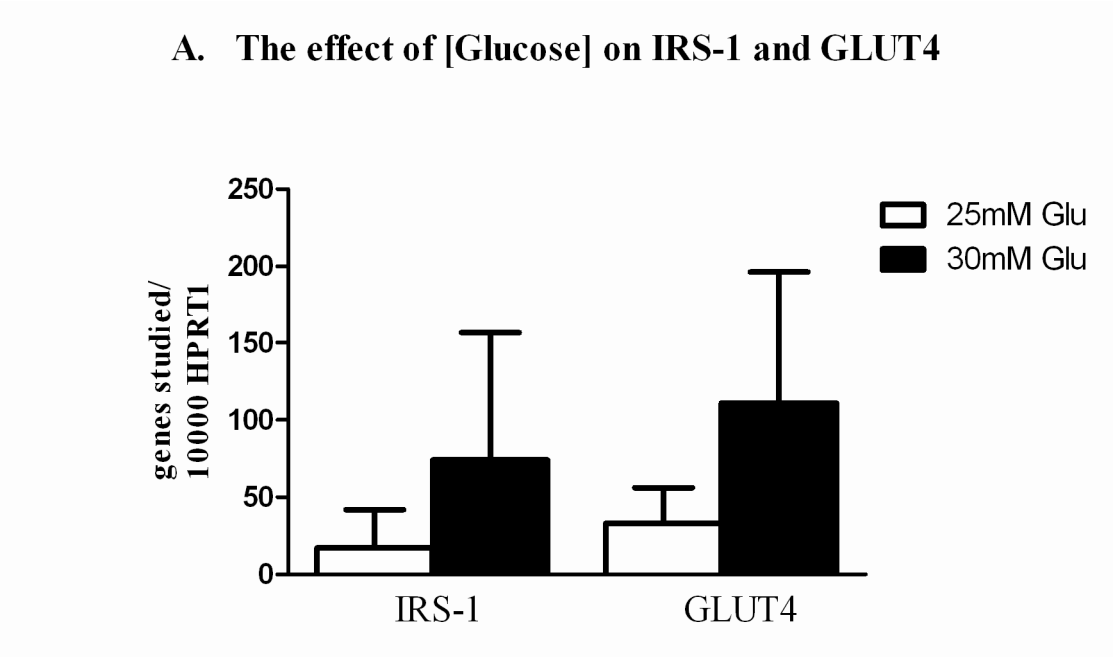


Figure 25 – The expression of IRS-1 and GLUT4 was differentially modulated by glucose in FSDCs.

B. The effect of LPS on IRS-1 and GLUT4, in different [Glucose]

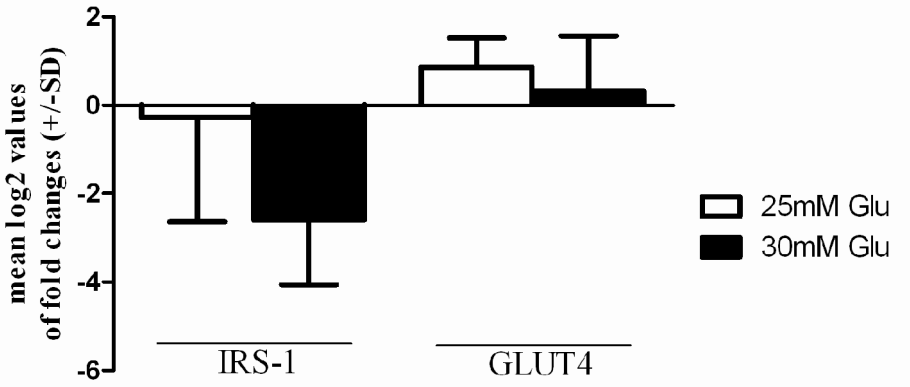


Figure 25 (continued) Cells were plated at 2×10^6 cells/well in 6-well microplates in a final volume of 6 ml of medium, at 37◦C, with 5% CO^2. Cells were cultured in IMDM medium in a final concentration of 25 mM of glucose (white bars) and supplemented with 5 mM of glucose (black bars), during at least 1 weak; treated with (B) or without (A) a stimulus of LPS, during 6h. Total RNA was isolated and retrotranscribed as indicated in experimental procedures. The mRNA levels were assessed by quantitative real-time RT-PCR. Gene expression is indicated as genes studied/10 000 molecules of the reference gene HPRT1 (A) and mean log2 values of fold changes (B) relatively to the control. Each value represents the mean±S.D. from three independent experiments (one way ANOVA multiple comparison Dunnett's test between 25 and 30mM of glucose with corresponding control, in Figure 25B).

6. Cytokine, chemokine and growth factor's profile.

FSDCs were maintained in IMDM medium or incubated with 1 µg/mL of LPS during 6h. After incubation, total RNA was isolated from cells, quantified and reverse transcribed to cDNA, to finally perform real-time RT-PCR. The FSDCs' cytokine/chemokine profile was determined and is represented in Table II.

FSDCs expressed a diverse range of cytokines and chemokines, at different levels. FDSC constitutively expressed the cytokines IL-1β, IL-6, TNF-α, G-CSF and IL-10, the chemokine CCL5, and the growth factors EGF, VEGF and PDGF, while the cytokine IFN-γ was not expressed in these cells. The pro-inflammatory cytokines IL-1β, IL-6 and TNF-α and the chemokine CCL5 were constitutively and largely expressed,

while the pro-inflammatory cytokine G-CSF and the anti-inflammatory cytokine IL-10 showed a lower expression profile. With respect to GFs, FSDCs highly expressed VEGF and PDGF, while EGF was less expressed (Table II – Ctrl).

FSDCs stimulated with LPS during 6h completely modified the expression levels of various cytokines. Indeed, this inflammatory stimulus highly induced the expression of the pro-inflammatory cytokines TNF-α, IL-1β, IL-6 and G-CSF, as well as, the chemokine CCL5. The anti-inflammatory cytokine IL-10 was slightly induced. GFs were almost unaffected by LPS (Table II – LPS).

Table II - Expression of cytokines, chemokines and growth factors in FSDCs.

Gene	Ctrl (Genes studied/10 000 HPRT1)	LPS (Mean log2 values of fold changes relatively to the control)
IL-1β	1099.55	4.94 ± 3.18 (***p< 0.001, n=14)
IL-6	663.33	8.00 ± 3.22 (***p< 0.001, n=17)
TNF-α	6131.69	3.58 ± 1.17 (***p< 0.001, n=16)
IFN-γ	0.07	0.72 ± 1.34 (n=4)
G-CSF	60.81	6.65 ± 3.13 (**p < 0.01, n=5)
CCL5	4069.67	7.52 ± 2.44 (***p< 0.001, n=16)
IL-10	115.50	2.28 ± 2.29 (***p< 0.001, n=17)
EGF	14.16	-0.35 ± 1.26 (n=16)
VEGF	2229.72	-0.15 ± 1.38 (n=16)
PDGF	2100.44	0.73 ± 0.74 (**p < 0.01, n=10)

Cells were plated at 2×10^6 cells/well in 6-well microplates in a final volume of 6 ml of medium and treated with 1µg of LPS during 6h (LPS), or left untreated (Ctrl), at 37◦C, with 5% CO^2. Total RNA was isolated and retrotranscribed as indicated in experimental procedures. The mRNA levels were assessed by quantitative real-time RT-PCR. Gene expression is indicated as genes studied/10 000 molecules of the reference gene HPRT1 (left column) or mean log2 values of fold changes (right column) relatively to the control, as indicated in Table II. Values from the right column represent the mean±S.D. from at least three independent experiments (**p < 0.01; ***p < 0.001 unpaired two-tailed t-student test relatively to the corresponding control). Legend: IL – Interleukin; TNF – Tumor necrosis factor; G-CSF – Granulocyte-colony stimulating factor; EGF – Epidermal growth factor; VEGF – Vascular endothelial growth factor; PDGF– Platelet-derived growth factor.

7. Modulation of FSDCs' cytokine and chemokine profile by neurotensin

FSDCs were subjected to different NT and LPS incubation protocols, namely: incubation with 10 or 100 nM of NT for 6h or 30h; incubation with LPS or simultaneously with 10 or 100 nM of NT for 6h; and incubation with 10 or 100 nM of NT 24h before an additional stimulus of LPS for 6h; with LPS alone during 6h. After these incubation periods, total RNA was isolated from cells, quantified and reverse transcribed to cDNA, to finally perform real-time RT-PCR.

NT altered the expression of different cytokines in an opposite dose-related manner, both at short and long-time periods. FSDCs incubated with 10 nM of NT for short-time periods (6h), tended to diminish the expression of the majority of the cytokines, namely IL-6, TNF-α and IL-10 by -0.34 ± 0.26 (n=3), -1.10 ± 0.62 (*$p<0.05$, n=3) and -1.77 ± 0.91 (*$p<0.05$, n=3) fold, while IL-1β expression was slightly increased by 0.42 ± 0.57 (n=4) fold, relatively to the control (Figure 26A). Incubation of FSDCs with NT during long-time periods (30h) exerted similar effects but at a less degree. In addition, the slight IL-1β increase in expression observed with NT after 6h of incubation, diminished after 30h of incubation by -0.19 ± 0.46 (n=4) fold relatively to the control. However, the chemokine CCL5 presented a significant increase of 0.74 ± 0.50 (*$p<0.05$, n=4) fold relatively to the control, after 30h of NT incubation, demonstrating a slower activation in the expression of this chemokine, as it only increased after 30h of NT incubation (Figure 26B). Controversially, the effects produced by 10 nM of NT, were opposite to the effects observed with 100 nM of NT in these cells. Indeed, 100 nM of NT tended to increase the expression of IL-6, TNF-α and IL-10 by 0.53 ± 0.83 (n=3), 0.35 ± 0.26 (n=3) and 1.00 ± 0.28 (n=3) fold and decrease IL-1β expression by -0.37 ± 0.39 (n=3) fold, relatively to the control and during the short-time incubation period of 6h (Figure 26A); during the long-time incubation period

of 30h, the tendency to increase was clearly diminished and occurred an increase of IL-1β expression by 0.56 ± 0.69 (n=5) fold, relatively to the control (in contrast to 6h of incubation) (Figure 26B). In conclusion, 10 nM of NT significantly decreased the expression of crucial inflammatory mediators, such as TNF-α and IL-10 at 6h and augmented the expression of the chemokine CCL5 at 30h; 100 nM of NT induced the opposite response, although without significance.

NT altered the expression of different cytokines, in LPS-stimulated FSDCs, both at short and long-time incubation periods. When cells were simultaneously incubated with LPS and 10 nM of NT during 6h, the cytokine profile was identical to the profile induced by the NT stimulus alone. There was a tendency to decrease IL-6 and TNF-α expression by -0.57 ± 1.21 (n=4) and -0.97 ± 0.89 (n=3) fold and a tendency to an earlier (after 6h of LPS and NT incubation relatively to the increased expression of CCL5 only observed after 30h of NT incubation) CCL5 increase by 0.54 ± 1.37 (n=3) fold, relatively to the control; IL-1β and IL-10 expression was unaltered (Figure 27A). Furthermore, when cells were pre-treated with 10 nM of NT during 24h and then incubated with LPS for 6h, the cytokine profile appeared to completely change. There was an upregulation of the expression of all cytokines, with a significant increase of IL-1β expression by 1.18 ± 0.42 (***$p < 0.001$, n=4) and a clear tendency in the upregulation of IL-6 expression by 0.70 ± 1.66 (n=6), relatively to the control (Figure 27B). Pre-treatment (24h) of FSDCs with NT concentrations simulated an *in vivo* and artificial treatment with NT immediately after skin injury (before the start of inflammation). Simultaneous cell treatment with LPS and NT (6h) simulated a treatment with NT after injury where the inflammation had already started. In inflammatory conditions, this situation may be represented by the release of NT by skin

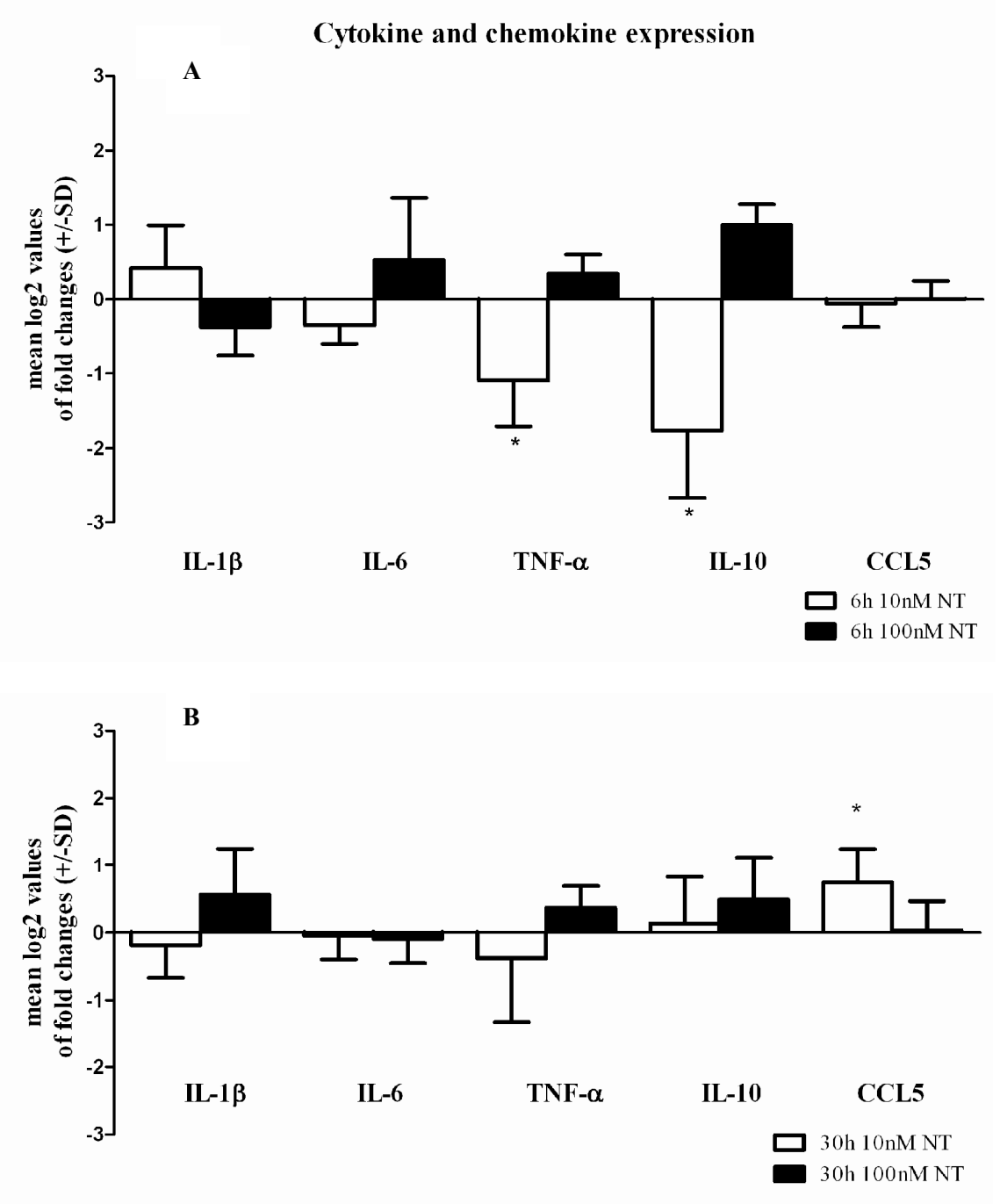


Figure 26 – The expression of cytokines and chemokines was differentially modulated by NT (10 and 100 nM), after 6 and 30h of incubation, in FSDCs.

FSDCs were plated at 2×10^6 cells/well in 6-well microplates in a final volume of 6 ml of medium and treated with 10 nM (white bars) and 100 nM (black bars) of NT, during 6h (A) and 30h (B), at 37◦C, with 5% CO^2. Total RNA was isolated and retrotranscribed as indicated in experimental procedures. The mRNA levels were assessed by quantitative real-time RT-PCR. Gene expression is indicated as mean log2 values of fold changes relatively to the control. Each value represents the mean±S.D. from at least three independent experiments (*$p < 0.05$ one way ANOVA multiple comparison Dunnett's test between 10 and 100 nM of NT stimuli with corresponding control).

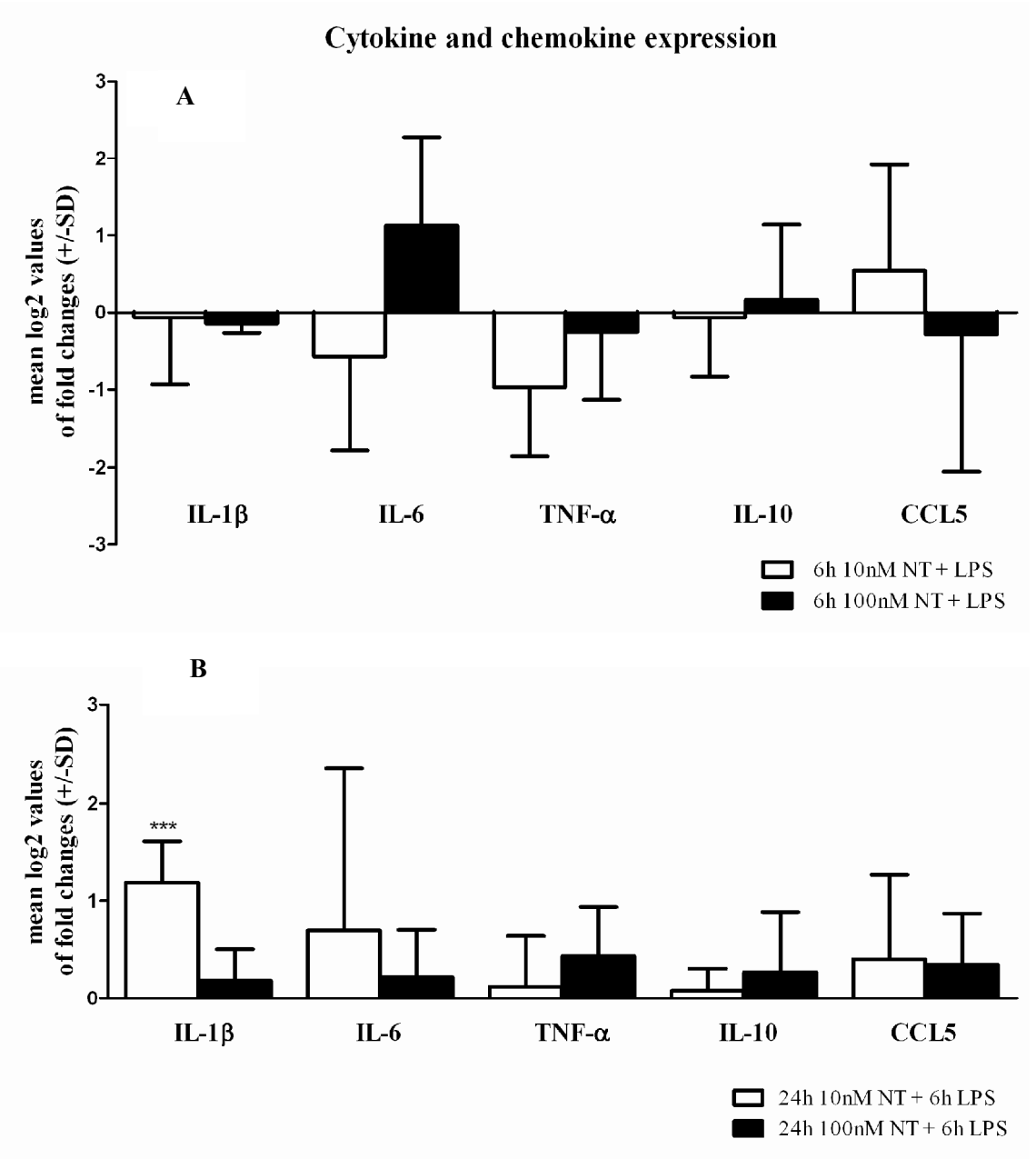


Figure 27 – The expression of cytokines and chemokine was differentially modulated by LPS (1µg) and NT (10 and 100 nM), after 6 and 30h of incubation, in FSDCs.

FSDCs were plated at 2×10^6 cells/well in 6-well microplates in a final volume of 6 ml of medium and treated with 1µg of LPS and 10 nM (white bars) and 100 nM (black bars) of NT, during 6h (A) and 30h (B), at 37◦C, with 5% CO^2. Total RNA was isolated and retrotranscribed as indicated in experimental procedures. The mRNA levels were assessed by Quantitative real-time RT-PCR. Gene expression is indicated as mean log2 values of fold changes relatively to LPS. Each value represents the mean±S.D. from at least three independent experiments (***$p < 0.001$one way ANOVA multiple comparison Dunnett's test between 10 and 100 nM of NT stimuli with corresponding control).

cells or nerves. Indeed, this pre-treatment adjusted cytokine profile to a more pro-inflammatory output. Cells treated with 100 nM of NT did not evoke the same response as did 10 nM of NT after 6h. Although the expression of TNF-α remained similar to that of 10 nM of NT, the expression of IL-6 showed a tendency to increase, while CCL5 to diminish (Figure 27A). At long-time incubation periods of these cells with both 10 and 100 nM of NT, the expression of cytokines and CCL5 was upregulated (Figure 27B).

8. Modulation of the growth factor's profile by neurotensin

FSDCs were incubated with 10 or 100 nM of NT, 1 μg/mL of LPS or LPS and 10 or 100 nM of NT during different time incubation periods: incubation with 10 or 100 nM of NT for 6 or 30h; incubation with LPS or simultaneously with 10 or 100 nM of NT for 6h; incubation with 10 or 100 nM of NT 24h before an additional stimulus of LPS for 6h; incubation with LPS alone during 6h. After these incubation periods, total RNA was isolated from cells, quantified and reverse transcribed to cDNA, to finally perform real-time RT-PCR.

As seen previously, FSDCs constitutively expressed lower levels of EGF and higher levels of VEGF. FSDCs stimulated with 10 nM of NT decreased EGF expression when incubated at short-time periods while its expression was increased after 30h of NT incubation; while the expression of VEGF tended to diminish after 6 and 30h of incubation (Figure 28A). When FSDCs were simultaneously treated with NT and LPS, EGF expression tended to be increased at both incubation periods, although VEGF expression was almost unaltered (Figure 28B).

In FSDCs stimulated with 100 nM of NT the results had opposite tendencies at 6h of NT incubation – increased EGF and VEGF expression – while no differences were detected in long-time incubation periods (Figure 28A). In FSDCs stimulated with LPS and 100 nM of NT, EGF and VEGF expression did not vary to a large extent, only

VEGF expression had a slight tendency to decrease (Figure 28B). In summary, the results demonstrated that NT increased EGF levels, while VEGF levels decreased, in these conditions.

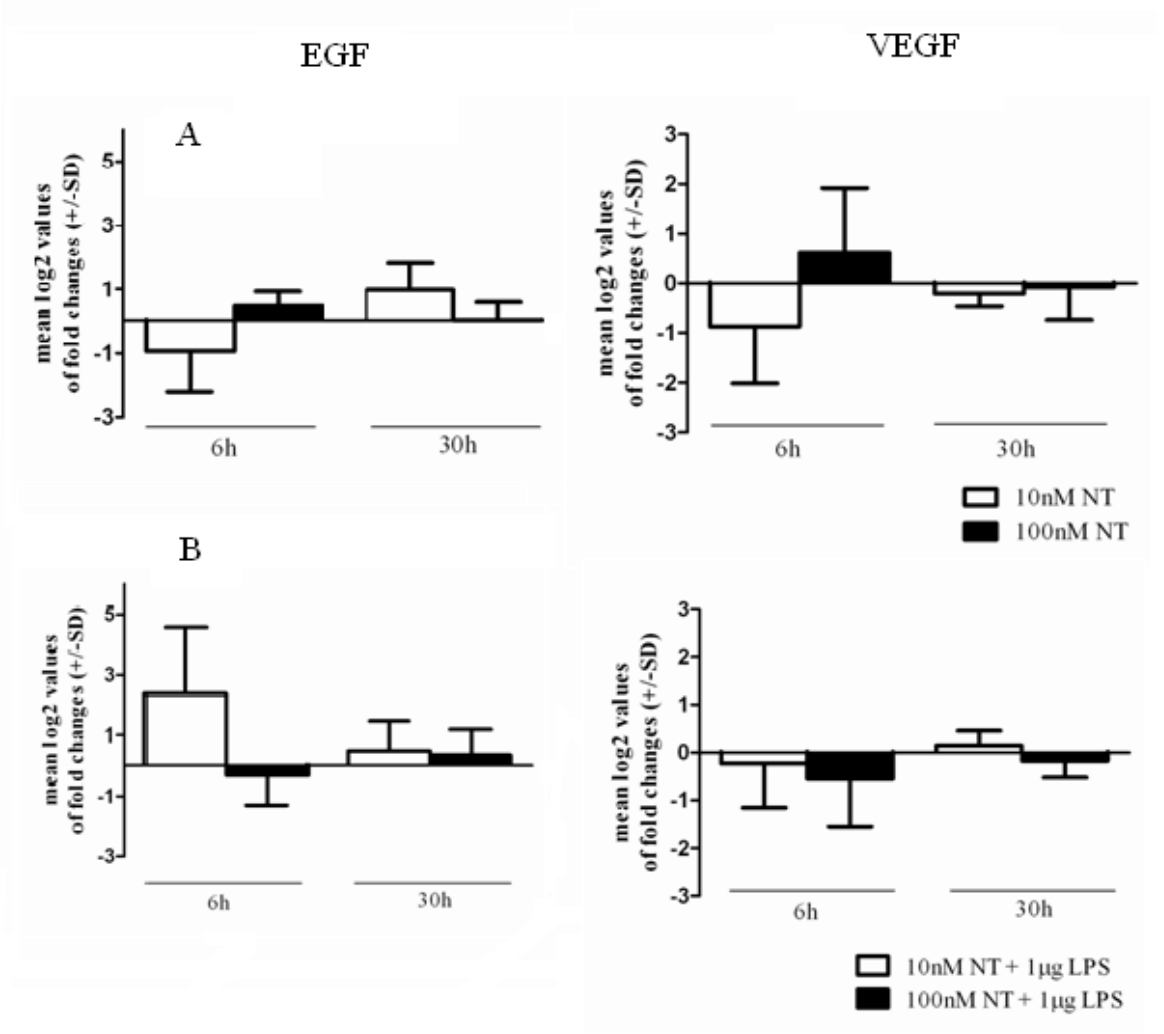


Figure 28 – The expression of the growth factors was differentially modulated by NT and LPS in FSDCs. Cells were plated at 2×10^6 cells/well in 6-well microplates in a final volume of 6 ml of medium and treated with 10 nM (white bars) or 100 nM (black bars) of NT (A) and treated with 1µg of LPS and 10 or 100 nM of NT (B), during 6 or 30h, at 37◦C, with 5% CO^2. Total RNA was isolated and retrotranscribed as indicated in experimental procedures. The mRNA levels were assessed by quantitative real-time PCR. Gene expression is indicated as mean log2 values of fold changes relatively to the control. Each value represents the mean±S.D. from at least three independent experiments (one way ANOVA multiple comparison Dunnett's test between 10 and 100 nM of NT stimuli and corresponding control).

9. FSDCs morphology

9.1. Cell morphology

FSDCs were incubated with 10, 50 and 100 nM of NT, with LPS or with LPS in the presence of different concentrations of NT, from 1 to 7 days of incubation, to analyze the morphology of the cells by transmission microscopy. NT (10, 50 and 100 nM) did not change FSDCs morphology, either in the presence or in the absence of LPS during the 7 days, as observed in Figure 29. However, LPS alone or in the presence of NT, provoked morphologic alterations in the cells at day 7, as evidenced by the heterogeneity of the cells, characterized by irregular morphology with rounded and fusiform cells.

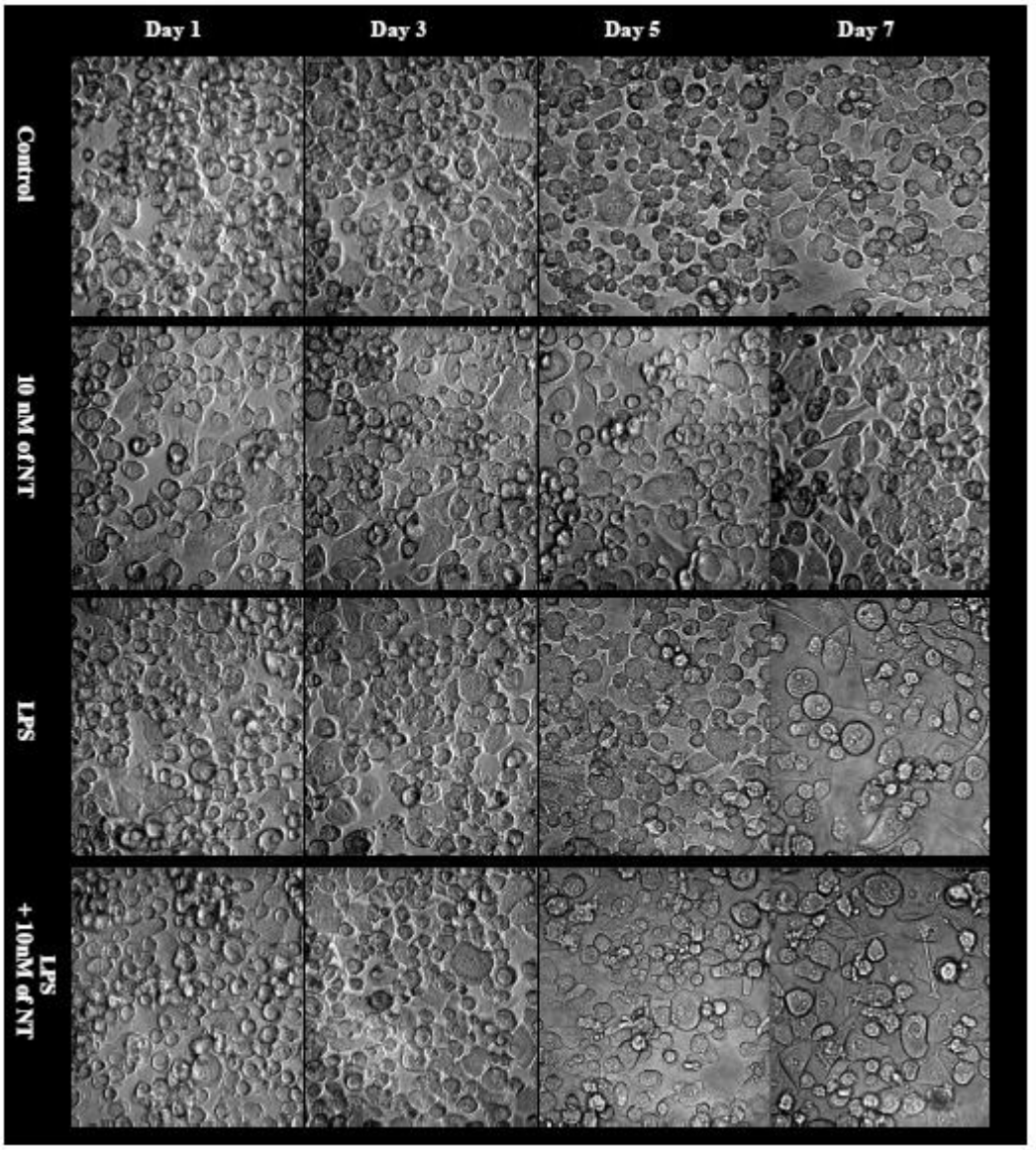


Figure 29 – FSDCs morphology.
FSDCs were maintained in IMDM medium (control), or were incubated with 1 µg/ml of LPS, or with LPS in the presence of 10, 50 or 100 nM of NT, during 7 days. The images were acquired by transmission microscopy and photographs were taken with 200X magnification.

9.2. Cytoskeleton and nuclei morphology

FSDCs were incubated with either 10 or 100 nM of NT, 1 μg/mL of LPS, or LPS and NT (10 or 100 nM), during 24h. FSDCs morphology was analyzed by fluorescence microscopy, in order to evaluate actin and nuclei staining (Figure 30). In control conditions, FSDCs had actin arrangements dispersed through the entire cell, but with sutured actin filaments in the periphery of the cytoplasm, evidencing numerous vesicles related to the high endocytic activity of these immature cells. Some cells also had dendritic-like projections (Figure 30, E-G). When cells were stimulated with LPS or NT, the actin in the periphery of the cytoplasm was more sutured in some regions than in others and the actin cortical network appeared to be disassembly, probably demonstrating FSDCs adjustments for migration. Some dendritic-like projections were also seen in LPS or NT stimulated cells (Figure 30, H-J). Generally, the nuclei of FSDCs in control conditions showed normal morphology (Figure 30 – A and B). However, in some cases, the chromatin appeared condensed and the DNA and nuclei fragmented (Figure 30 – C and D), revealing a programmed cell death (apoptosis). In inflammatory conditions, this apoptotic nuclei morphology was enhanced.

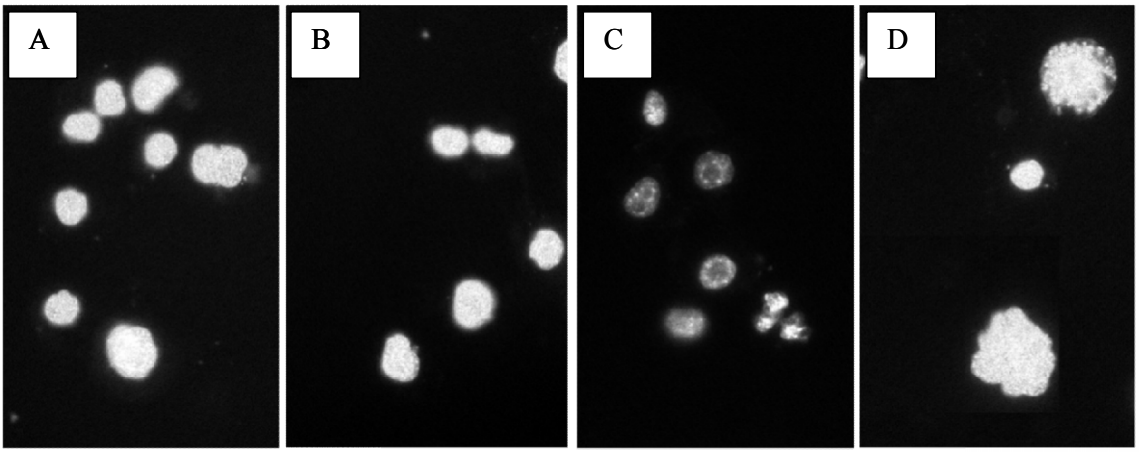


Figure 30 – FDSC cytoskeleton and nuclei morphology.

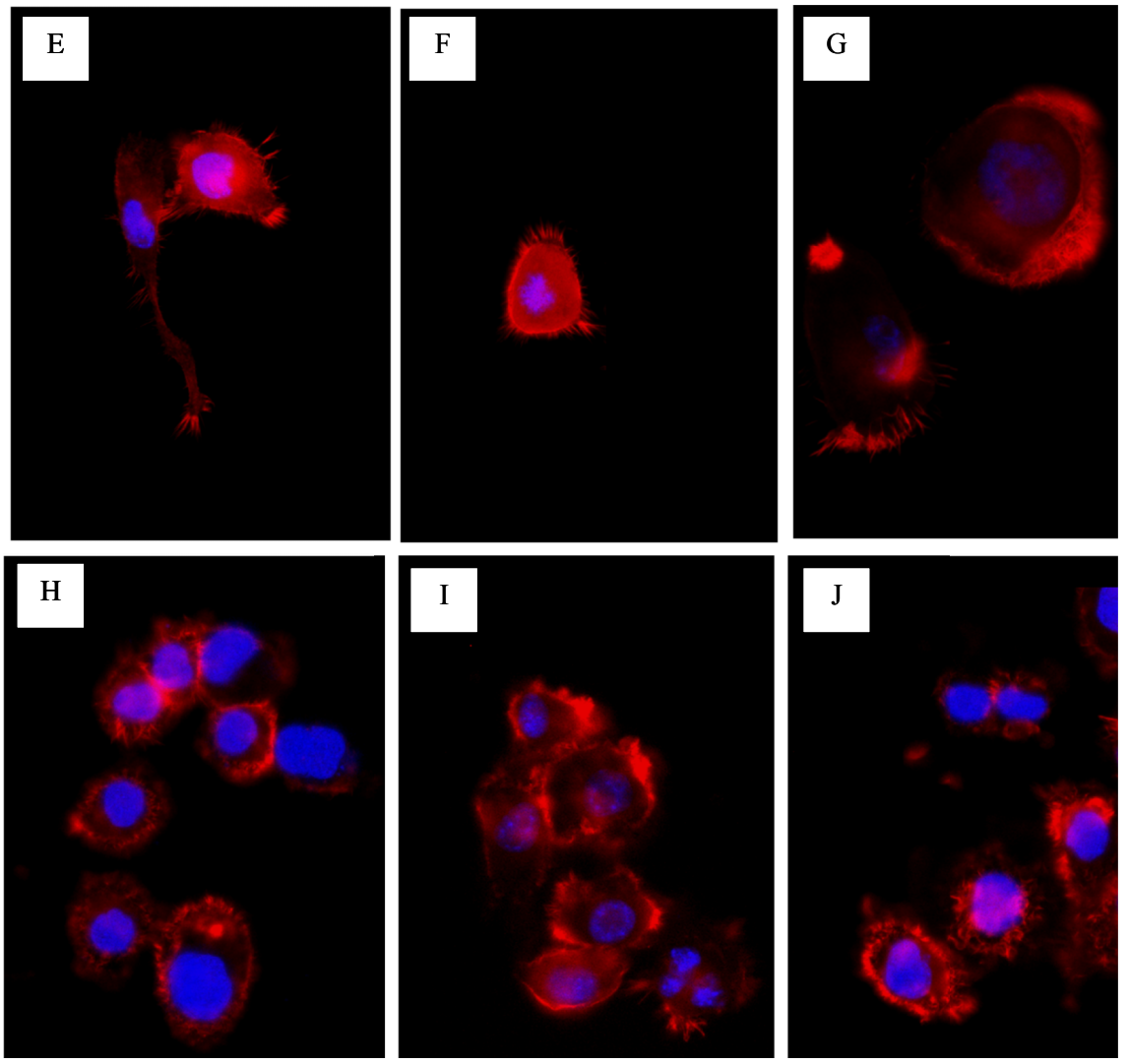


Figure 30 (continued) FSDCs were imunostained with Alexa Fluor 555 phalloidin (red) antibody for actin and stained with DAPI (blue) for the nuclei. Images A to D show nuclear morphology; E, F and G represent FSDCs in normal conditions; H, I and J are representative of LPS or LPS and NT stimulated cells. Immunostaining was performed as described in "Material and Methods". The images were acquired by fluorescence microscopy and photographs were taken with 400X magnification.

10. Summary

Table III –The effect of neurotensin on FSDCs

	10 nM of NT		100 nM of NT		LPS + 10 nM of NT		LPS + 100 nM of NT	
Time incubations	Short-time incubation	Long-time incubation	Short-time incubation	Long-time incubation	Short-time incubation	Long-time incubation/ NT pre-treatment	Short-time incubation	Long-time incubation/ NT pre-treatment
Viability	=	1^{st} ↑ 2^{nd} ↓	=	↓1^{st} ↓2^{nd} ↑	=	↓	=	↑
Nitrite production	___N/A	↓Day 1 and 3 ↑Day 7	___N/A	↓Day 1 and 3 ↑Day 7*	___N/A	↑Day 1 ↓Day3,5,7	___N/A	↑Day 1,3,5,7
Signaling	↑Akt, p38 ↓NF-κB	___N/A	↑Akt, p38 ↓NF-κB	___N/A	↑p38, ERK ↓JNK, NF-κB	___N/A	↑p38, ERK ↓JNK, NF-κB	___N/A
Cytokines	↓IL-6, TNF-α*, IL-10* ↑IL-1β	↓ TNF-α , IL-1β ↑ CCL5	↑IL-6, TNF-α, IL-10 ↓ IL-1β	↑IL-10, IL-1β, TNF-α*	↓IL-6, TNF-α ↑CCL5	↑IL-1β*, IL-6, CCL5	↓TNF-α, CCL5 ↑IL-6	↑TNF-α, CCL5
Growth Factors	↓EGF, VEGF	↑EGF	↑EGF, VEGF	=	↑EGF	↓EGF, VEGF	=	=

Time incubations: Short-time incubations: Viability, Nitrite production and signaling: 5-60min; Cytokines and Growth Factors: 6h
Long-time incubations: Viability, Nitrite production: 1-7days; Cytokines and Growth Factors: 30h
Legend: =No alteration observed; ___N/A – Not available; ↑Upregulation; ↓Downregulation;
IL – Interleukin; TNF – Tumor necrosis factor; EGF – Epidermal growth factor; VEGF – Vascular endothelial growth factor
Statistical differences: $p < 0.05$

Table IV – The effect of LPS on FSDCs

	Control	LPS
Viability	=	↓
Nitrite	=	↑
Signaling	=	↑NF-κB, JNK, ERK, p38
Cytokines, Chemokines and GFs	IL-1β, IL-6, TNF-α, G-CSF, IL-10, CCL5, EGF, VEGF and PDGF.	↑TNF-α, IL-1β, IL-6, G-CSF, CCL5 and IL-10
NT	=	↑***
NTRs	↑NTR1, NTR3*	↓NTRs (NTR2*)
IRS-1	+25 mM +30 mM	+25 mM +30 mM
GLUT4	+25 mM +30 mM	+25 mM +30 mM
Morphology	=	Heterogeneity: 1)↑size, rounded 2) Thin, directional dendrite-like projections

Legend: =No alteration observed; ↑Upregulation; ↓Downregulation
IL – Interleukin; NTR – Neurotensin receptor; GLUT4 – Glucose transporter 4; IRS-1 – Insulin receptor substrate-1
Statistical differences: *$p < 0.05$; ***$p < 0.001$

Chapter IV. Results on BJ cells

1. Neurotensin and neurotensin receptors

NTR1, NTR2 and NTR3 were all expressed in BJ cells, whereas the NTR2 was the most abundant (Figure 31A), as determined by real-time RT-PCR analysis. An inflammatory stimulus of LPS during 6h on BJ cells revealed an increased expression

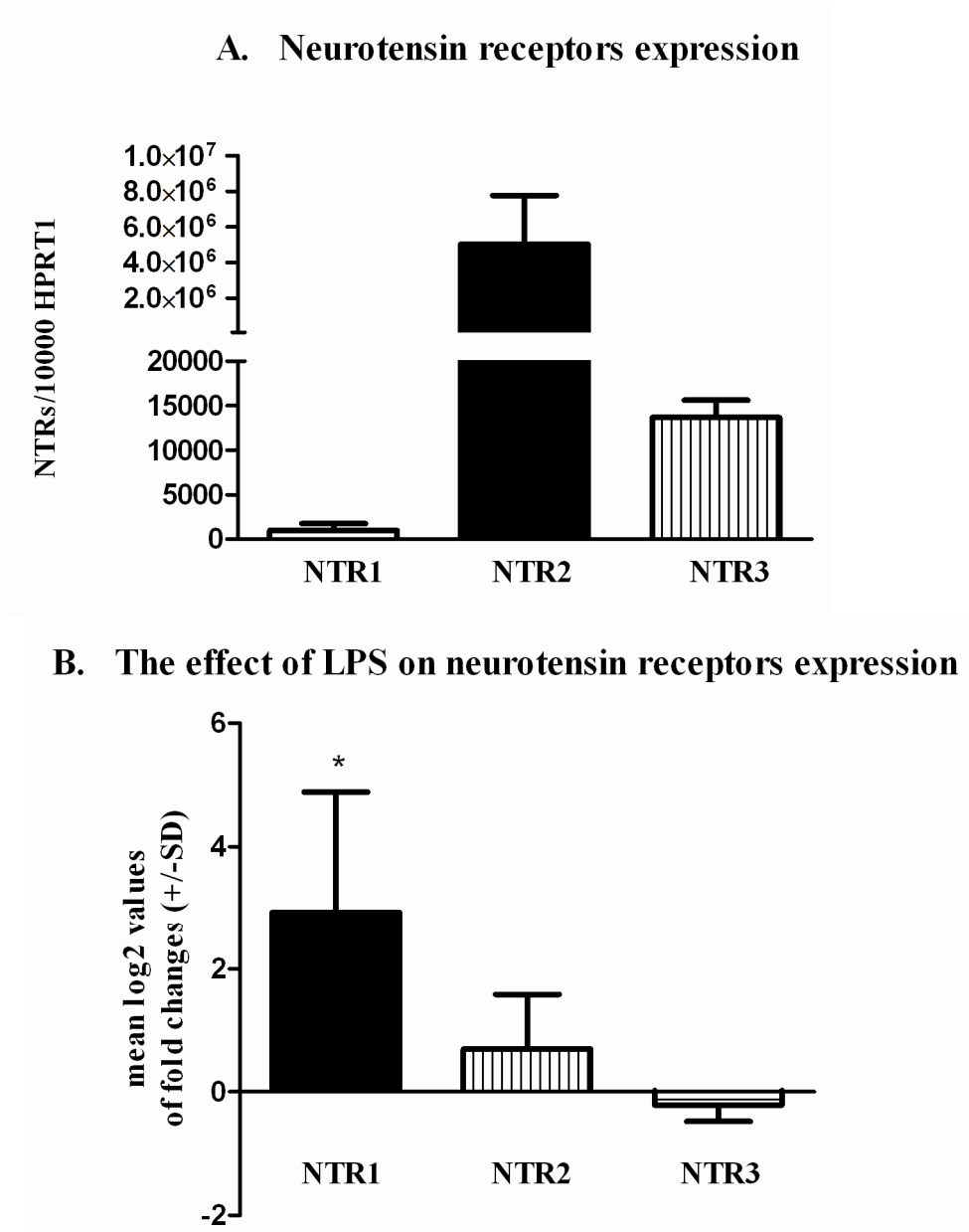


Figure 31 – Expression of NTRs (NTR1, NTR2 and NTR3) in BJ cells. Cells were plated at 8×10^5 cells/dish in 60mm dishes in a final volume of 6ml of medium, at 37◦C, with 5% CO^2. Total RNA was isolated and retrotranscribed as indicated in experimental procedures. The mRNA levels were assessed by quantitative real-time RT-PCR. Gene expression is indicated as genes studied/10 000 molecules of the reference gene HPRT1 (A) and mean log2 values of fold changes (B) relatively to the control. Each value represents the mean±S.D. from three independent experiments (*p < 0.05 unpaired two-tailed t-student test in Figure 31B).

of the NTR1 by 2.9 ± 1.9 (*$p < 0.05$, n=3) and 0.7 ± 0.9 (n=3) fold, relatively to the control, while the expression of NTR3 slightly diminished -0.2 ± 0.3 (n=3) fold relatively to the control (Figure 31B). NT was not constitutively expressed in BJ cells, although barely expressed when BJ cells were stimulated with LPS during 6h (data not shown).

2. The effect of glucose and LPS on BJ cells

To determine if high glucose levels disturbed insulin signals and GLUT4 trafficking in BJ cells, cells were cultured in DMEM medium with 30 mM of glucose to reproduce the hyperglycemic conditions observed in diabetic patients and with lower concentrations of glucose (25 mM). Additionally, the response of BJ cells to different glucose concentrations was analyzed in an inflammatory environment, by stimulating the cells with LPS for 6h.

The expression of IRS-1 and GLUT4 was studied in BJ cells cultured with 25 or 30 mM of glucose. Similar to FSDCs, BJ cells expressed both IRS-1 and GLUT4, but to a large extent than FSDCs. In addition, cells cultured at higher glucose levels (30 mM) tended to induce higher IRS-1 and GLUT4 expression per reference gene, by $4.27x10^6$ ± $4.09x10^5$ and $5.66x10^3$ ± $3.3\ x10^3$ (n=3) relatively to cells cultured at 25 mM of glucose with the expression values per reference gene of $2.44x10^3$ ± $1.46x10^3$ and 7.79 ± 4.07 (n=3) (Figure 32A). This increase was also observed in FSDCs. The expression of IRS-1 and GLUT4 was also analyzed at 25 or 30 mM of glucose in inflammatory conditions (6h of LPS). BJ cells cultured in 25 mM of glucose showed a decrease of -0.28 ± 0.40 (n=3) fold in IRS-1 expression and an increase of 0.64 ± 0.14 (n=3) fold in GLUT4 expression, relatively to the control. In contrast, when cells were cultured with 30 mM of glucose, both IRS-1 and GLUT4 expression increased by 1.28 ± 0.86 and 1.59 ± 1.15 fold, relatively to the control (an event not observed for IRS-1 expression in

FSDCs) (Figure 32B). Indeed, glucose and LPS modulated IRS-1 and GLUT4 expression in BJ cells: LPS and glucose increased the expression of these genes, not only in normal but also in inflammatory conditions.

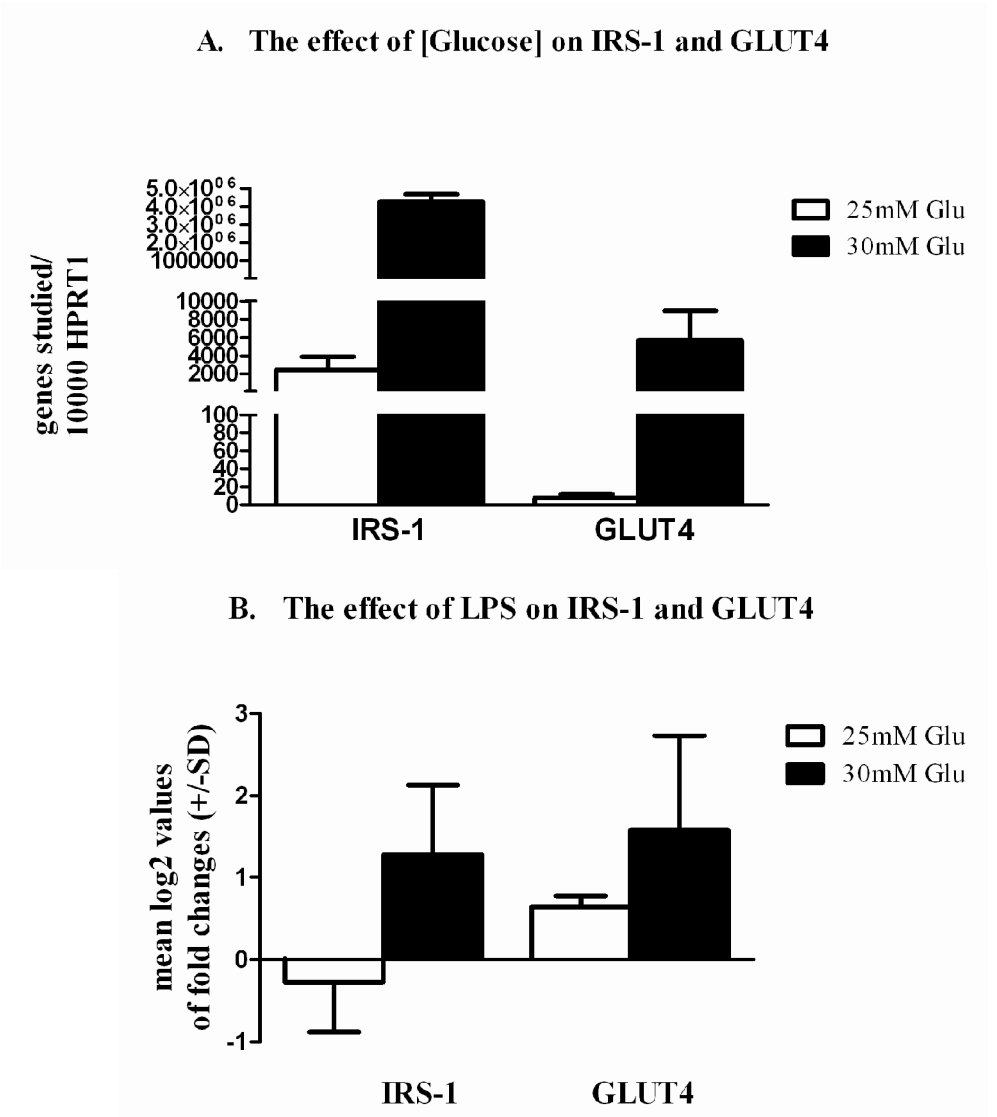


Figure 32- The expression of IRS-1 and GLUT4 was differentially modulated by glucose and LPS, in BJ cells.

Cells were cultured in IMDM medium in a final concentration of 25 mM of glucose (white bars) and supplemented with 5 mM of glucose (black bars) of glucose, during at least 1 weak; with (B) or without (A) a stimulus of LPS during 6h. Cells were plated at 8×10^5 cells/dish in 60mm dishes in a final volume of 6ml of medium, at 37◦C, with 5% CO^2. Total RNA was isolated and retrotranscribed as indicated in experimental procedures. The mRNA levels were assessed by quantitative real-ime RT-PCR. Gene expression is indicated as genes studied/10 000 molecules of the reference gene HPRT1 (A) and mean log2 values of fold changes (B), relatively to the control. Each value represents the mean±S.D. from three independent experiments (one way ANOVA multiple comparison Dunnett's test between 25 and 30 mM of glucose with corresponding control, in Figure 32B).

3. Cytokine, chemokine and growth factor's profile.

BJ cells were maintained in DMEM medium or incubated with 1 μg/mL of LPS during 6h. After incubation, total RNA was isolated from cells, quantified and reverse transcribed to cDNA, to finally perform real-time PCR.

Non-stimulated BJ cells expressed abundantly various chemokines, like CCL5, CCL11 and IL-8, while CCL4 was only slightly expressed. The cytokines IL-1β and IL-6 were also expressed by BJ cells, being IL-6 the most abundant. EGF was also highly expressed. When stimulated with LPS, the expression levels of all genes were upregulated and the chemokine CCL4 started to be expressed in large amounts, by 8.14 ± 0.53 (***p< 0.001, n=6) fold, relatively to the control (Table V).

Table V – Expression of cytokines, chemokines and growth factors in BJ cells.

Gene	Ctrl (Genes studied/10 000 HPRT1)	LPS (Mean log2 values of fold changes)
CCL4	6.44	8.14 ± 0.53 (***p< 0.001, n=6)
CCL5	4427.68	6.23 ± 0.88 (***p< 0.001, n=4)
CCL11	25317.38	4.42 ± 0.16 (***p< 0.001, n=3)
IL-8	27505.50	5.84 ± 0.97 (***p< 0.001, n=5)
IL-1β	262.44	6.02 ± 0,66 (***p< 0.001, n=5)
IL-6	15834.42	6.08 ± 0,54 (***p< 0.001, n=5)
EGF	1007.31	2.13 ± 0.42 (***p< 0.001, n=3)

Cells were plated at 8×10^5 cells/dish in 60mm dishes in a final volume of 6 ml of medium and treated with 1μg of LPS during 6h (LPS), or left untreated (Ctrl), at 37◦C, with 5% CO^2. Total RNA was isolated and retrotranscribed as indicated in experimental procedures. The mRNA levels were assessed by quantitative real-time RT-PCR. Gene expression is indicated as Genes studied/10 000 molecules of the reference gene HPRT1 (left column) or mean log2 values of fold changes (right column), relatively to the control, as indicated in Table V. Values from right column represent the mean±S.D. from at least three independent experiments (***p< 0.001 unpaired two-tailed t-student test with corresponding contro). Legend: IL – Interleukin; EGF – Epidermal growth factor.

4. Modulation of cytokine, chemokine and growth factor's profile by neurotensin

Cells were incubated with 10 or 100 nM of NT during 30h; 1 μg/mL of LPS during 6h; and pre-treated with 10 or 100 nM of NT during 24h before an additional stimulus of LPS during 6h. After incubation, total RNA was isolated from cells, quantified and reverse transcribed to cDNA, to finally perform real-time RT-PCR.

NT (10 nM) tended to decrease the expression of the chemokines CCL4 and CCL11 by -0.31 ± 0.41 and -0.66 ± 0.35 (n=3) fold, while increase the expression of IL-8 by 0.88 ± 0.28 (n=3) fold, relatively to the control. The cytokines IL1-β and IL-6 tended to augment by 0.83 ± 0.54 and 0.53 ± 0.35 (n=3) fold and EGF expression significantly increased 1.07 ± 0.45 (*$p<0.05$, n=3) fold, relatively to the control. Moreover, 100 nM of NT induced a similar cytokine and chemokine profile in BJ cells, although the chemokines CCL5 and IL-8 tended to decrease, potentially inducing a smaller inflammatory behavior in these cells (Figure 33A). All the results shown were referenced to 30h of NT. As the cytokine/chemokine response of FSDCs to NT during 6h was more prominent than that at 30h, perhaps, BJ cells incubation with NT during 6h would give even more pronounced responses. When cells were pre-treated with NT during 24h and then stimulated with LPS for 6h, the behavior of these cells was different. NT at 10 nM essentially decreased chemokines expression, namely CCL4, CCL11 and IL-8 by -0.59 ± 0.16 (*$p < 0.05$, n=3), -0.37 ± 0.12 (n=3) and -0.43 ± 0.10 (n=3) fold, relatively to the control. Cytokines were mostly unaltered while the expression of EGF increased by 0.90 ± 0.01 (*$p < 0.05$, n=3) fold, relatively to the control. NT at 100 nM also diminished the majority of the chemokines, namely CCL4 and IL-8, by -0.57 ± 0.19 (n=3) and -1.59 ± 1.00 (*$p < 0.05$, n=3) fold, while enhanced CCL11 by 0.65 ± 0.25 (*$p<0.05$, n=3) fold, relatively to the control.

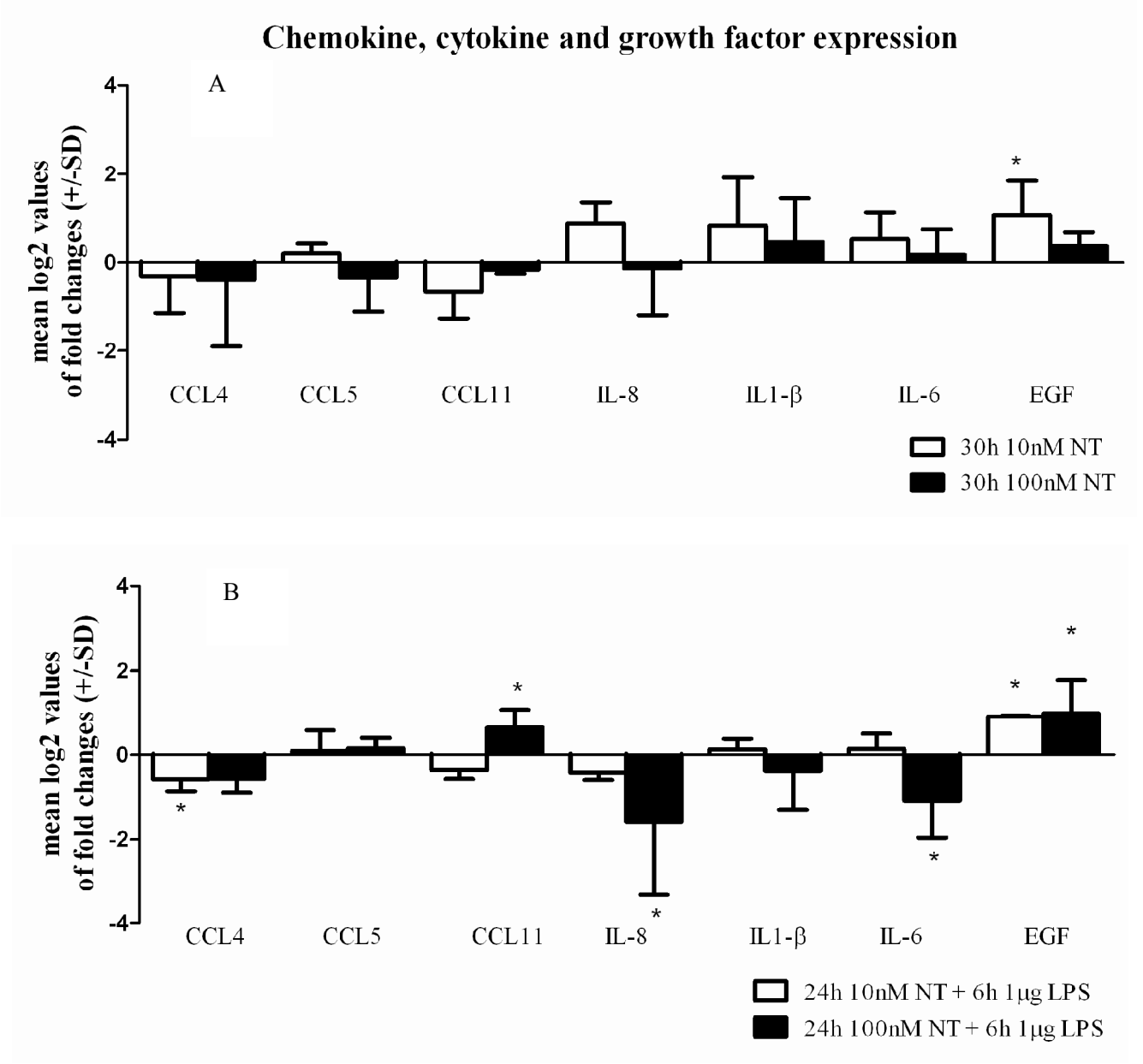


Figure 33 – The expression of cytokines, chemokines and growth factors was differentially modulated by LPS (1µg) and NT (10 and 100 nM) after 30h of incubation in BJ cells.

Cells were plated at 8×10^5 cells/dish in 60mm dishes in a final volume of 6 ml of medium and treated with 10 nM (white bars) and 100 nM (black bars) of NT during 30h (A) or pre-treated with 10 nM (white bars) and 100 nM (black bars) of NT during 24h and stimulated with1µg of LPS during 6h (B), at 37◦C, with 5% CO^2. Total RNA was isolated and retrotranscribed as indicated in experimental procedures. The mRNA levels were assessed by quantitative real-time RT-PCR. Gene expression is indicated as mean log2 values of fold changes relatively to the control (A) and LPS (B). Each value represents the mean±S.D. from three independent experiments (*$p < 0.05$ one way ANOVA Dunnett's test between 10 and 100 nM of NT with corresponding control).

The cytokine IL1-β was also downregulated by -0.39 ± 0.41 fold, relatively to the control. Again, EGF expression was significantly increased by 0.99 ± 0.45 (*$p < 0.05$, n=3) fold, relatively to the control (Figure 33B). The expression of CCL5 was unaffected by 10 or 100 nM of NT, as shown in Figure 33.

Recapitulating, NT essentially diminished chemokines expression, except CCL11 which was significantly augmented when stimulated by LPS and 100 nM of NT. The expression of cytokines was slightly enhanced by the addition of NT but diminished when NT was added before LPS. The expression of CCL5 was not affected by NT while EGF expression was significantly enhanced.

5. Cytoskeleton and nuclei morphology

BJ cells were incubated with 10 or 100 nM of NT, 1μg/ml of LPS, or simultaneously with LPS and NT (10 or 100 nM) for 24h. The morphology of BJ cells was visualized by fluorescence microscopy, in order to evaluate actin and nuclei staining (Figure 34). Cells were organized with actin microfilaments, displaying distinct arrays of F-actin, namely stress fibers (green arrows in Figure 34) and fillopodia (white arrows in Figure 34). Actin microfilaments provide forces for cellular and intracellular movement, giving dynamic and polarization to the cells. Indeed, several BJ cells were found with migration-like structure. In contrast to FSDCs, the nuclei of BJ cells were not modified by NT or LPS, displaying normal and non-apoptotic nuclei. Additionally, migrating cells displayed flattened nuclei, as shown in Figure 34D.

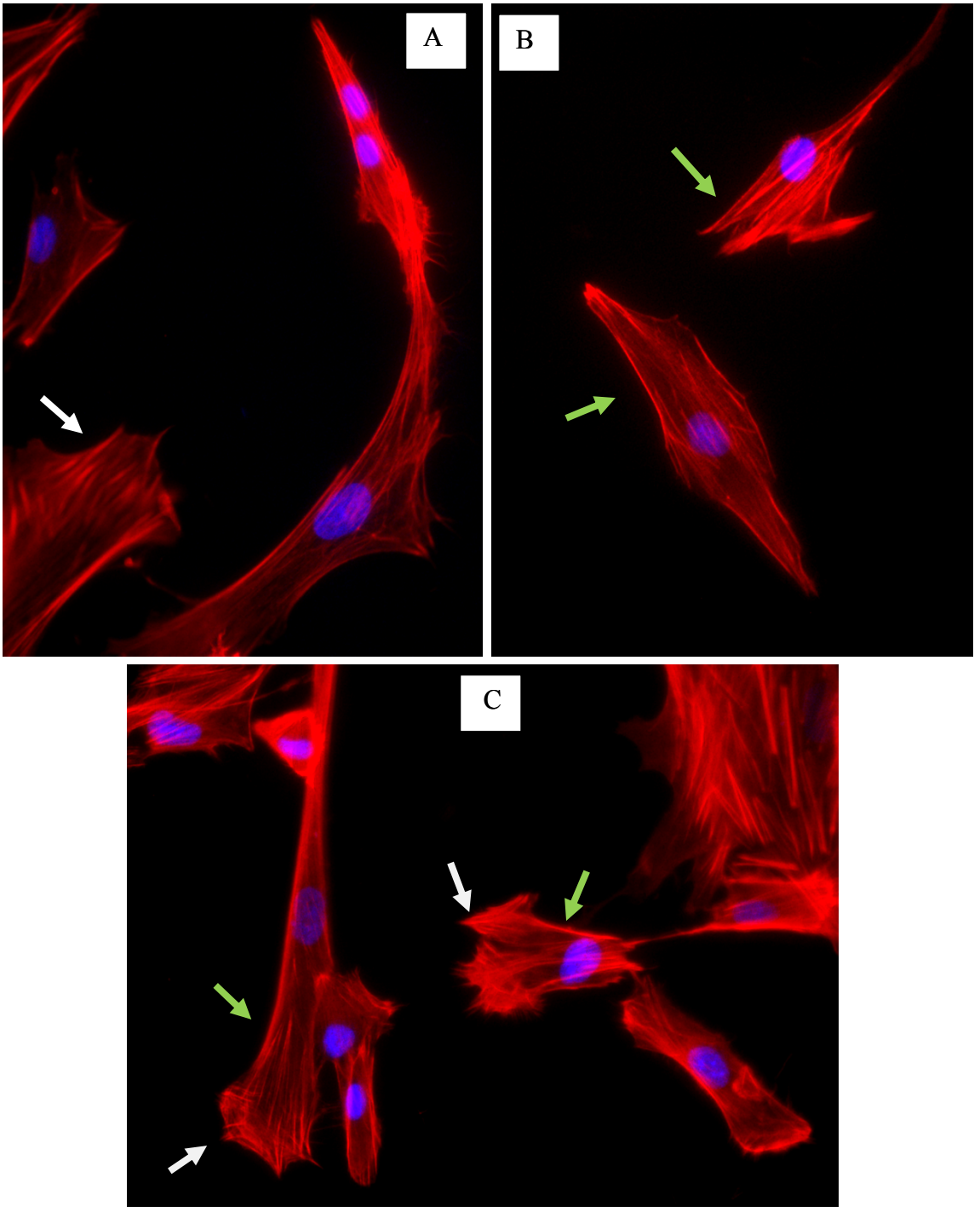


Figure 34 - Actin arrangements in BJ cells.
BJ cells were imunostained with Alexa Fluor 555 phalloidin antibody (red) for actin and stained with DAPI (blue) for nuclei. Immunostaining was performed as described in "Material and Methods". The images were acquired by fluorescence microscopy and photographs were taken with 200X (D-E) and 400X (A-C) magnification. Legend: Green arrows: Stress fibers; White arrows: Fillopodia

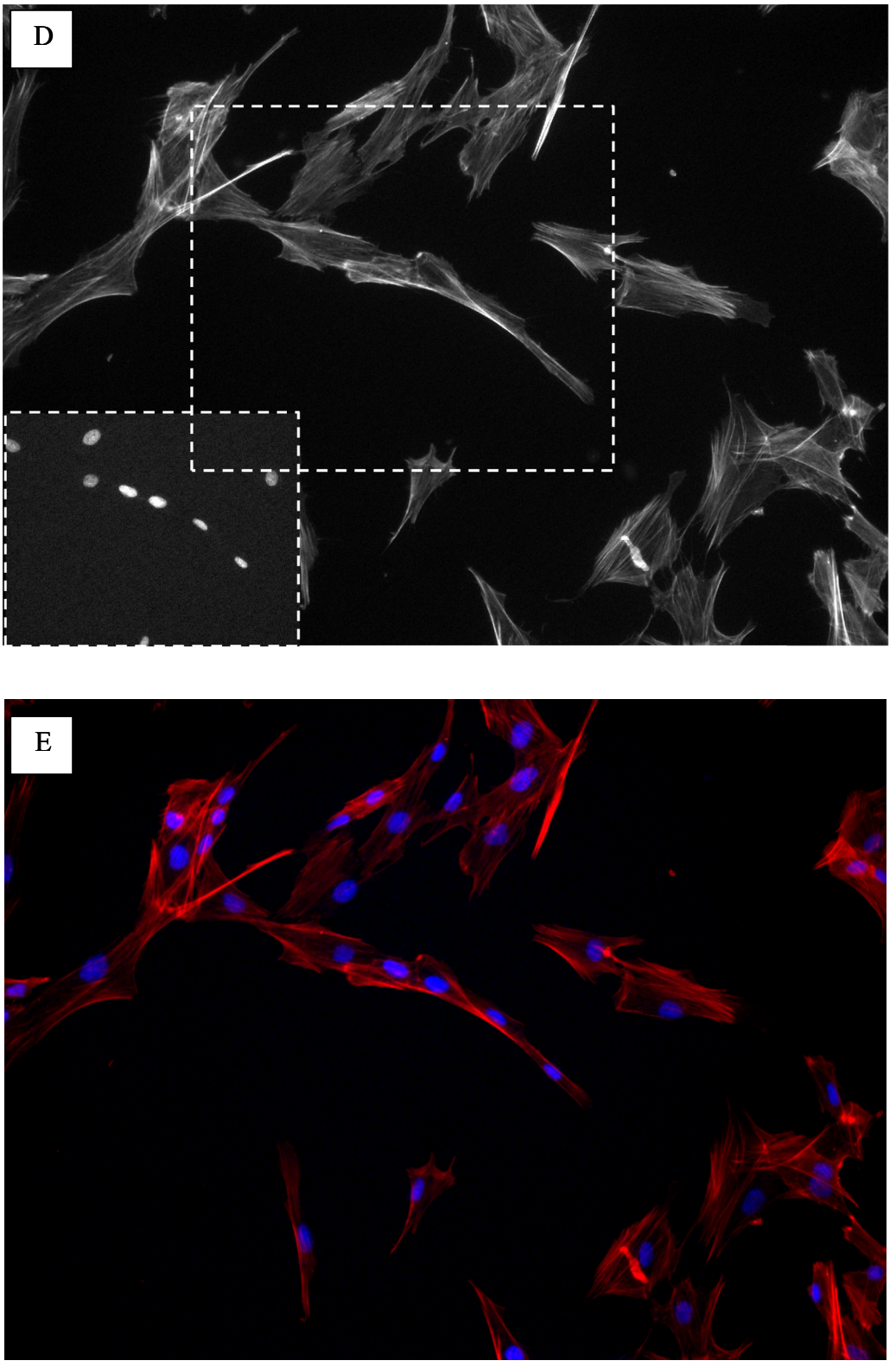


Figure 34 - Actin arrangements in BJ cells (continued).

6. Modulation of BJ cell migration by neurotensin

BJ cells were incubated with 10 or 100 nM of NT, 1μg/ml of LPS, and simultaneously with LPS and NT (10 or 100 nM) for 24h, after making the scratch. Before the incubation of NT and LPS, and after incubation, photographs were taken to evaluate BJ cell migration. NT appeared to modulate BJ migration: NT (10 nM) increased the number of migrated cells in the scratched area, in BJ cells treated or non-treated with LPS; 100 nM of NT did not modify BJ migration in non-treated LPS cells, although it increased cell migration in LPS treated cells. These results so far are representative of only one experiment, but may indicate that NT can possibly modulate BJ migration, conceivably upregulating cell migration.

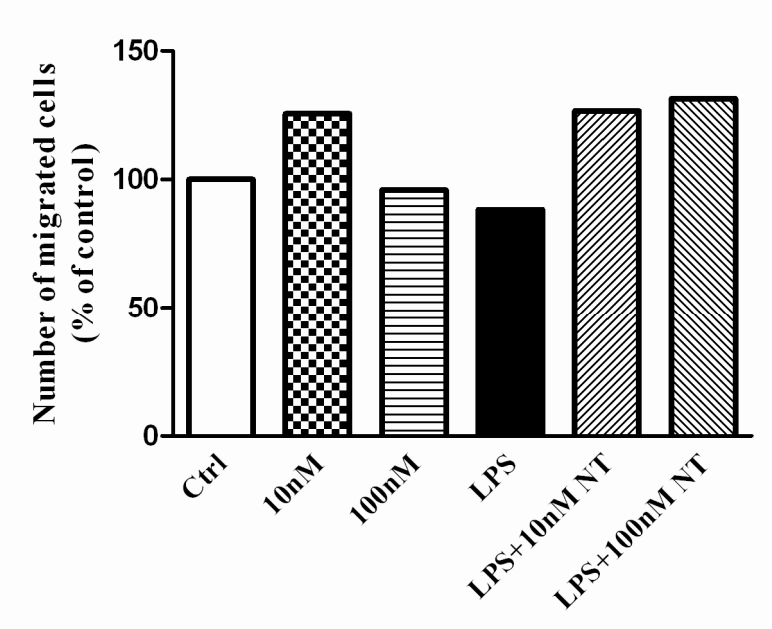


Figure 35 – NT modulated BJ migration.
A scratch assay was performed as described in "Material and Methods" and the number of migrating cells was counted in the scratched area. Results are representative of one experiment.

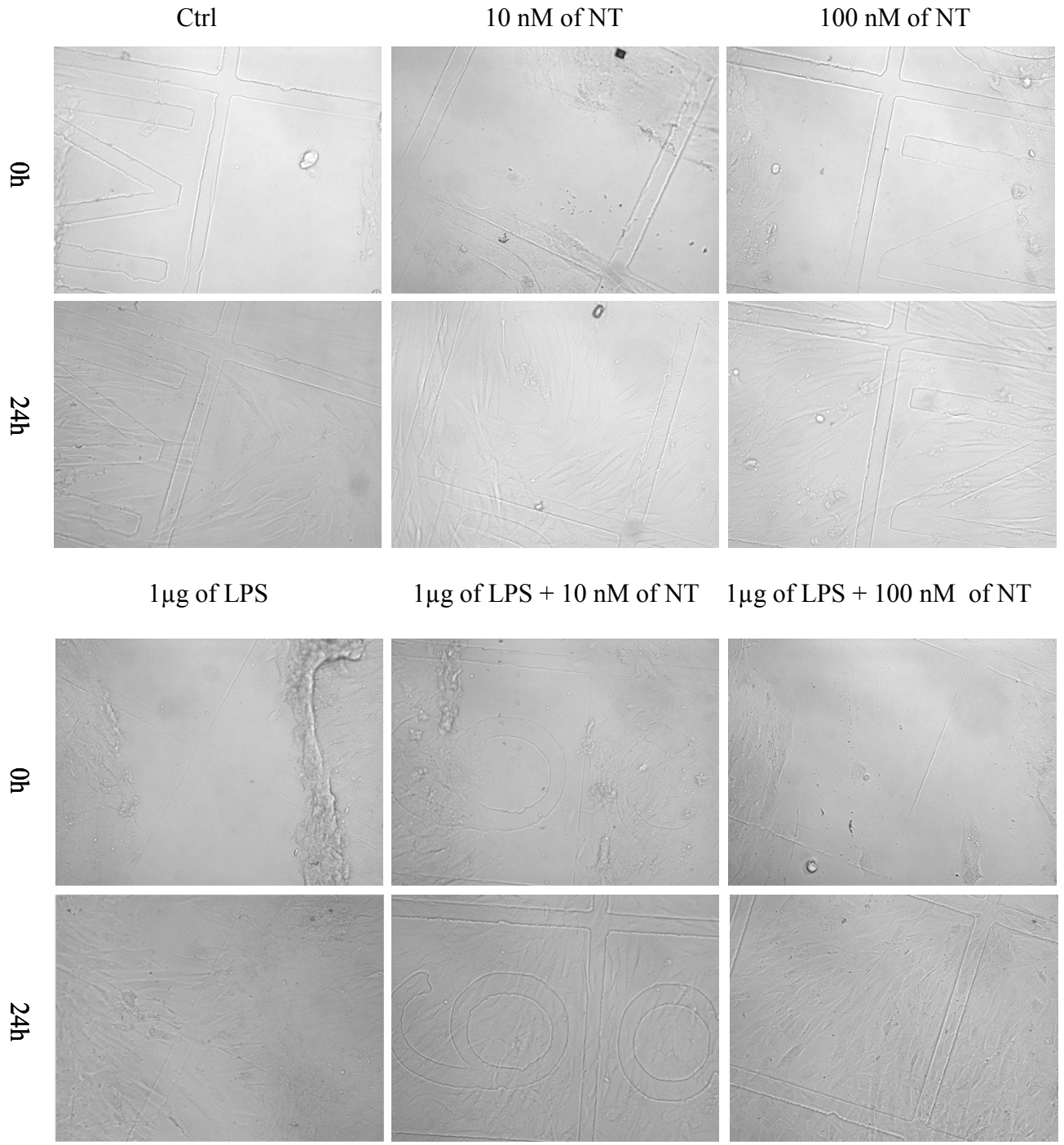


Figure 36 - Analysis of BJ cell migration by the *in vitro* scratch assay, during 24h of incubation.
Cells were treated with LPS or NT (10 and 100 nM) or left untreated (Ctrl) and a scratch assay was performed as described in "Material and Methods". The images were acquired by transmission microscopy and photographs were taken before cell treatment (0h) and after 24h of LPS or NT incubation (24h) with 200X magnification. Results are representative of one experiment.

7. Summary

Table VI – The effect of neurotensin on BJ cells

Incubations Times	10 nM of NT	100 nM of NT	10 nM of NT (24h) + LPS (6h)	10 nM of NT (24h) + LPS (6h)
Cytokines, chemokines and growth factors	↓ CCL4, CCL11 ↑IL-8,IL-1β, IL-6,EGF*	↓ CCL4, CCL5, CCL11 ↑IL-1β, EGF	↓CCL4*, CCL11, IL-8 ↑EGF*	↓CCL4, IL-8*, IL-1β, IL-6* ↑CCL11*, EGF*
Cell Migration	↑	=	↑	↑

Time incubations: Cytokines, chemokines and growth factors: 30h; Cell Migration: 24h
Legend: =No alteration observed; ↑Upregulation; ↓Downregulation; IL – Interleukin, EGF – Epidermal growth factor
Statistical differences: *$p < 0.05$

Table VII – The effect of LPS on BJ cells

	Control	LPS
Viability	=	=
Cytokines, chemokines and growth factors	CCL4, CCL5, CCL11, IL-8, IL-1β, IL-6, EGF	↑CCL4, CCL5, CCL11, IL-8, IL-1β, IL-6, EGF
NT	Not expressed	Not expressed
NTRs	NTR1, NTR2, NTR3	↑NTRs (NTR1* and NTR3)
IRS-1	+25 mM ++30 mM	-25 mM ++30 mM
GLUT4	+25 mM ++30 mM	+25 mM +30 mM
Morphology	With stress fibers and fillopodia	

Legend: =No alteration observed; ↑Upregulation; ↓Downregulation; NTR – Neurotensin receptor, IRS-1 – Insulin receptor substrate 1, GLUT4 – Glucose Transporter 4, IL – Interleukin, EGF – Epidermal growth factor
Statistical differences: *$p < 0.05$

Chapter V. Discussion

The main focus of this work was to evaluate the role of NT in the expression of inflammatory markers by FSDCs or BJ cells, as well as, its potential repercussions in different phases of WH. Changes in NT levels can be associated with pathological conditions, like diabetic wound healing, and this effect will also be discussed.

The results demonstrated that NT (10 nM) downregulated the immune function of FSDCs in homeostasis[#] and *in vitro* inflammatory* (induced by LPS) conditions. Indeed, NT decreased nitrite production, reduced the activation of the inflammatory signaling pathways NF-kB[#]* and JNK*, and reduced the expression of pro-inflammatory cytokines[#*]. NT also induced the expression of the promoter of the last phases of WH – EGF[#*] – and the activation of the survival signaling pathway ERK*. On the other hand, NT also modulated chemokines expression, inducing the leukocyte chemoattractant mediator – CCL5[#]*, and diminished the expression of the vascular hyperpermeability and angiogenic inducer – VEGF[#]. In contrast to 10 nM of NT cell treatment, that appears to signal through NTR1 binding, higher doses of NT (100 nM) that seem to bind to NTR2 and NTR3, upregulated the previous pro-inflammatory cytokines. This NT pro-inflammatory profile was also observed when cells were pre-reated with NT and stimulated with LPS. It can be speculated that this *in vitro* effect mimics a WH treatment with NT, in inflammatory conditions.

NT (10 or 100 nM) binding to NTR1, NTR2 or NTR3 diminished the expression of pro-inflammatory cytokines and chemokines in BJ cells, induced the expression of EGF*[#] and CCL11[#], and finally, promoted BJ cell migration. Following, these results will be discussed.

1. The effect of LPS and NT on FSDCs viability

The MTT assay was used to determine if NT provoked toxicity to FSDCs, in either short or long-time periods of incubation. As dehydrogenases activity remained similar after NT exposures, and their activity is proportional to mitochondria activity and therefore to the number of viable cells, it can be considered that no significant changes in cell viability was observed in cells treated with NT. This result was concordant with unchanged cell morphology, revealing their normal morphologic characteristics through 7days of NT incubation, as well as, concordant with unaltered nuclei morphology. However, some cells displayed chromatin condensation, as well as, nuclei and DNA fragmentation, representative of cell death – apoptosis. Furthermore, when cells were stimulated with LPS, more cells displayed apoptotic nuclei after 24h of LPS incubation, as well as, altered cell morphology after seven days of LPS incubation. Accordingly, inflammation signals cell death if cells cannot respond efficiently to inflammation, which might have occurred for some cells population. Observing BJ cells, these rarely presented apoptotic nucleus, evidencing that LPS may not activate death receptors nor induce mitochondrial dysfunction in these cells.

The MTT assay gives information about the cells metabolism, since cells more viable/active consume more ATP, which enhances mitochondrial and dehydrogenases activity. Although with no statistical difference, 10 nM seemed to augment cell viability on the first day of NT incubation, 50nM of NT on the third day, and 100 nM of NT on the fifth and seventh days. Indeed, it appears that 10 nM NT was sufficient to increase cell metabolism in the first day, although in long NT treatments (during 7days), which mimics the period of WH resolution, large amounts of NT might be needed to maintain NT effects.

2. Cell and cytoskeleton morphology

2.1. FSDCs and cell maturation

FSDCs presented a rounded but irregular cell structure in normal conditions. Cells also comprised size heterogeneity, although bigger cells rather than smaller cells were preferentially present in culture. The actin cytoskeleton of these cells was essentially disposed in the periphery of the cytoplasm, displaying sutured actin filaments and evidencing numerous coated pits and vesicles, responsible for a high endocytic uptake capacity of these immature non-stimulated cells. Some cells also had dendritic-like projections. After stimulation with LPS, cells underwent morphologic changes, increasing their size and becoming rounded, or becoming fusiform and displaying a migratory morphology. This morphology is concordant with DCs maturation, which is characterized by the loss of the endocytic capacity and the acquisition of migratory properties. In addition, some cells appeared to have the actin cortical network disassembly, probably demonstrating cytoskeleton adjustment for migration, by losing the actin organization needed for endocytosis. Additionally, the peripheral actin in the cytoplasm was more sutured in some regions than in others, showing the arrangement of coated pits and vesicles in specific points, probably due to cell interaction with LPS plus subsequent endocytosis.

2.2. BJ cells and its migratory capacity

BJ cells displayed huge actin microfilaments in all their structure. Cytoskeleton organization gives shape and size to cells, as well as, distributes organelles inside of the cell, contributing for their size and shape. Additionally, cytoskeleton arrangement gives important information about significant cell functions, like cell cycle, transcription, migration and others. Besides normal actin filaments arrangements that BJ cells

possessed, they also displayed different arrays of F-actin, stress fibers and fillopodia. This arrangement is characteristic of migrating FBs, as cells need to adjust their cytoskeleton and induce forces for cellular and intracellular movement, giving dynamism and polarization to the cells. BJ migration was observed in cells treated or non-treated with LPS or NT. Taking this knowledge in consideration, the effect of NT in the migratory capacity of BJ cells was studied and, in fact, their migratory capacity was enhanced after cell treatment with 10 and 100 nM of NT. FBs migration is crucial in the proliferation and remodeling phases of WH, as FBs migrate to the injured site and induce fibrosis, producing ECM proteins which are important in the structure of new granulation tissue. Indeed, NT may be involved in FBs migration in WH, when NT is released in the skin by skin cells or nerve fibers.

3. Modulation of neurotensin receptors by LPS and low/high NT doses in FSDCs

The current data showed an altered immune response of FSDCs after treatment with 10 or 100 nM of NT. While 10 nM appeared to promote a decrease in FSDCs immune function, 100 nM of NT appeared to slightly activate FSDCs-mediated production of pro-inflammatory cytokines.

3.1. Expression of NTRs under control and inflammatory conditions

FSDCs expressed both NTR1 and NTR3 receptors, being NTR3 expressed at a large extent, as shown in Figure 13. NTR1 and NTR3 gene expression was concordant with NTRs protein levels. In addition, NT signaling in FSDCs occurred through NTR1, as the signaling pathways modulated by NT, after NTR1 inhibition by the selective antagonist SR48692, returned to normal (Figures 20 to 24).

The expression of NTRs with low (10 nM) and high (100 nM) doses of NT has been controversial. With high doses of NT, some studies infer that NT exerts a chronic

negative regulation on NTR1 expression (Azzi *et al*., 1996), while others infer that high doses of NT promote NTR1 gene activation (Najimi *et al*., 1998). Considering the cell treatment with low doses of NT, the literature is concordant and states that NT induces NTRs expression, as this is the only source of NTRs reposition into cell membranes, even though, with limiting number of *de novo* synthesized receptors (post-transcription mRNA destabilization) (Najimi *et al*., 1998). The present data showed that low doses of NT during 6h increased NTR3 expression, in accordance with the literature. The expression of NTRs with high doses of NT was not determined in this work.

NTRs expression declined in LPS-stimulated cells (Figure 14) probably explaining the non-prominent NT effects observed in these studies. Furthermore, these results demonstrate that NT levels and consequent effects are downregulated in FSDCs, under inflammatory conditions.

3.2. Endogenous NT expression

In inflammatory conditions, endogenous NT expression was augmented in FSDCs, although these elevated NT levels would not act on FSDCs, as NTRs were downregulated by inflammatory conditions (Figure 14). Furthermore, dendritic cells expressed this neuropeptide, which may be released and further exert its effects in other surrounding skin cells, like KCs, FBs, and others. Additionally, in inflammatory conditions, the downregulation of NTRs might be important to suppress the effects of high NT concentrations that are pro-inflammatory (discussed further in detail). In fact, some pathologies are characterized by the upregulation of NTRs expression which induce an excessive pro-inflammatory environment (Castagliuolo *et al*., 1999; Wang *et al*., 2000). Concluding, 10nM of NT induced an anti-inflammatory character in FSDCs, under normal or inflammatory conditions.

Although the increase of endogenous NT expression guided us to its importance in these cells, this augment may omit the effect of the external NT added to the cells in inflammatory conditions.

3.3. NTRs sensitization

In accordance to the literature and previous results, NTRs expression is augmented with low doses of NT and might be diminished or augmented with high doses of NT exposures. At low doses of NT, the expression of NTRs is effectively important, as this is the only source of receptors replacement in the cell surface, in contrast to high doses of NT. Indeed, at low doses of NT, NTR1 is targeted to lysossomal degradation after NT-NTR1 binding, however, at high doses of NT, NTR1 is targeted to other compartment (juxta-nuclear compartment identified as the Trans Golgi Network/PNRC) being recycled to the membrane, without the agonist (Sorrell J. & A.I. Caplan, 2006). It is also known that NTR3 is essentially located in the Trans Golgi Network (TGN) compartment and NTR1 may form heterodimers with NTR3. Indeed, with high doses of NT, NTRs increase at the cell surface and not only NTR1 can be recycled to the membrane, but also NTR3, while with low doses of NT, only *de novo* synthesis introduces NTRs in cell membranes. In summary, low doses of NT induce cell desensitization by NTR1 degradation and high doses of NT induce permanent cell sensitivity to NT, probably analogous to the previous results observed with 10 nM and 100 nM of NT. Indeed, the results observed with 10 nM of NT may indicate that NTRs undergo the process of desensitization but increase the expression of NTRs (later) that contradicts the decrease of NTRs in the cell membrane after internalization. Independently of NTRs expression, results with 100 nM of NT may show that FSDCs undergo the process of NTRs sensitization, targeting NTR1 and NTR3 to the cell surface and rapidly augmenting NT effects. In this work this process

was not clarified. To clarify these results, immunocitochemistry of the NTRs at the cell surfaces, in cells treated with low and high doses, should be performed.

3.4. Differences between the biological functions of NTR1 and NTR3

The interpretation of this dual NT character guided us to reflect about the effects of low and high levels of NTRs at the cell surface, as well as, the possible increase of NTR3 at the cell surface, with high doses of NT. As demonstrated in this work, FSDCs expressed and produced both NTR1 and NTR3. However, NT signaling occurred mainly through NTR1 (demonstrated by NTR1 inhibition, Figures 20 to 24) and possibly because the NTR3 might be essentially inside of the cell and not present at the cell surface. In addition, low (10 nM) NT concentrations bound to NTR1 and mediated the inactivation of inflammatory pathways, although at high concentrations (100 nM), NT might have caused permanent NTRs sensitization, inducing an excessive presence of NTRs at the cell surface, namely NTR1 and NTR3. As a consequence, 100 nM of NT may bind to NTR1 or NTR3 in an unspecific way, inducing an opposite response to 10 nM of NT, a pro-inflammatory response. This pro-inflammatory response may be mediated by the binding of NT to NTR3 or to NTR1 and NTR3.

While all NTRs have affinity for NT binding, the consequent cell response after NT binding to a NTR may be different. Indeed, the difference between NTR1 and NTR3 support this affirmation: NTR1 is a G protein-coupled receptor, whereas NTR3 is an intracellular receptor with a single transmembrane domain. In addition, these receptors have different intracellular domains which are responsible for intracellular activation. Not only NTRs differences may alter cell response, but the activation of GPCR and the host cell of the receptor. To illustrate the previous statements, a more documented NT function in the nervous system and the gastrointestinal tract was analyzed. Indeed, in the nervous system, NT has a pro-inflammatory role, inducing the

expression of several cytokines/chemokines, including IL-1 and MIP-2 in microglia cells, mediated by NT binding to NTR3 (Dicou *et al*., 2004). In a recent study performed in the gastrointestinal tract, NT mediates an anti-inflammatory response, characterized by the decrease of IL-6 and TNF-α (the receptor by which this response was mediated was not clarified) (Assimakopoulos *et al*., 2008). In conclusion, different receptors in different host cells mediate different molecular responses. Accordingly, 10 nM of NT may bind to NTR1 and mediate inflammation downregulation, while 100 nM of NT may highly increase NTR1 and NTR3 at cell surfaces, where NTR3 binding may be responsible for the opposite immune response, a pro-inflammatory response. The effects mediated by NT binding to NTR3 could be elucidated by the inhibition of NTR3 with an antagonist. However, until the present date it was not developed an antagonist to NTR3.

4. Modulation of neurotensin receptors by LPS and low/high doses of NT in BJ cells

In contrast to FSDCs, as shown in the Figures 21, 23, 26 and 27 in the results, BJ cells did not seem to mediate different NT responses when NT was added in low and high doses. In fact, BJ cells responded to NT in a dose-dependent manner.

4.1. NTRs expression under homeostatic and inflammatory conditions

In control conditions, BJ cells highly expressed the NTR2, being the NTR3 expressed to a less extent. FSDCs highly expressed the NTR3 although FSDCs mediated immune functions probably occurred through NTR1, as shown in Figure 12 and 13. Indeed, the same neuropeptide may bind different NT receptors, in different cells of the same tissue, like the skin. Attending that the NTR3 is present essentially in

the cytoplasm, it can be speculated that NT signaling and consequent immune responses might have occurred through the NTR2.

After cell exposure to LPS, instead of observing a decrease in NTRs expression, as it occurred in the case of FSDCs (Figure 14), LPS on the other hand induced the expression of NTR1 and the NTR2, and a modest decrease of the NTR3 expression. Accordingly to these results, cells do not respond equally to inflammatory conditions and may readjust their transcription machinery in opposite ways. While FSDCs repressed the expression of NTRs and diminished excessive NT binding, BJ cells activated NTRs expression to augment NT effects. In addition, as BJ cells express NTR1, NTR2 and NTR3 under inflammatory conditions, NT effects may occur through one of the three receptors, in preference by the NTR1, as the NTR3 gene expression is slightly decreased and the NTR2 has less affinity for NT in comparison to the other two receptors (Chalon *et al*., 1996).

4.2. Expression of endogenous neurotensin

In contrast to FSDCs (Figure 13 and 14), BJ cells did not express NT and it was barely induced by LPS. In inflammatory conditions, the expression of NTRs increased, indicating that NT may be important for BJ cells in these conditions. Indeed, NT produced by other cells, like FSDCs, or released by peripheral nerves in inflammatory conditions, may bind NTRs and activate consequent signaling in BJ cells. In normal and inflammatory conditions, NT downregulated the expression of chemokines and upregulated the expression of EGF, reducing inflammation and possibly inducing the last phases of WH (discussed further in this chapter).

4.3. NTRs sensitization

FSDCs expressed NTR1 and NTR3, and even in the presence of NTR3, the signaling occurred essentially through NTR1, at short periods of incubation. In normal conditions, BJ cells expressed the NTR2 and the NTR3, whereas the NTR2 was highly expressed although with low affinity for NT (Chalon *et al*., 1996), and NTR3 was possibly present inside of the cell, considering the literature. With high concentrations of NT, NT signaling through NTR3 should augment, as these receptors are targeted to cell membrane and since this receptor has high affinity for NT. In summary, with low and high doses of NT, the effects of NT occur through the NTR2 and the NTR3, explaining similar biological effects, although to a further extent with high doses of NT that signal through NTR3.

4.4. Biological functions of NTR2 and NTR3

In this case, with low and high doses of NT, signaling may occur simultaneously through two receptors (NTR2 and NTR3) and through the three receptors (NTR1, NTR2 and NTR3) in LPS-stimulated cells, instead of one (in contrast to NT signaling in FSDCs). As the NTR responsible for the NT effects was not confirmed by NTR inhibition in these cells, since neither an antagonist for NTR2 or for NTR3 exists, the function of NTR2 and NTR3 alone was not determined. Even though, NT signaling in normal and in inflammatory conditions induced a similar outcome, decreasing immune cells function. This similar function may be related with a similar intracellular domain but is discondart with NTRs structure, as NTR1 and NTR2 are GPCRs, whereas NTR3 is an intracellular receptor with a single transmembrane domain. Also, this response can also be mediated simply by the augment of NTRs at the cell surface and NT signaling.

Table VIII – Evaluation of the NTRs signaling in FSDCs and BJ cells surfaces.

	NTRs in FSDCs	NTRs in BJ cells
Low doses of NT	**NTR1**, (NTR3)	NTR2, (NTR3)
High doses of NT	NTR3, NTR1	NTR2, NTR3
Inflammatory exposure	NTR1, (NTR3) ↓NTRs	NTR1, NTR2, (NTR3) ↑NTRs

()-Expressed but essentially existing inside of the cell; ↓↑ Diminishment/Augment of expression; NT has less affinity for NTR2; **Bold** – NTR responsible for NT functions.

5. Cytokine, chemokine and growth factor's profile

5.1. FSDCs

As seen before, 10 nM of NT decreased IL-6, TNF-α, IL-10 and PDGF expression, while it increased the expression of CCL5 and EGF. IL-6 and TNF-α are acute phase cytokines known to induce the first phase of inflammation in WH. IL-6 (↓) induces C-reactive proteins, B cells stimulation to produce antibodies, influences antigen-specific immune responses, promotes T cell growth and cytotoxic T cell differentiation, enhances adhesion molecules in ECs, and improves leukocyte accumulation at the site of inflammation (Jones *et al*., 2005). TNF-α (↓) induces adhesion molecules and chemokines in the skin, leading to attachment of inflammatory cells to vessels, rolling, emigration, and eventually chemotaxis into the skin (Briscoe *et al*., 1992; Swerlick *et al*., 1992). All these functions are crucial to the onset of inflammation in the initial phases of WH. IL-1β, an important cytokine in innate immunity essential to kill pathogens and produce inflammatory mediators, like NO, have their expression levels whether increased (short-time incubations) or decreased (long-time incubations) in NT treated cells, as shown in Figure 26 and 27A. Indeed, NT may not be responsible for the modulation of the expression of this cytokine. All the

cytokines mentioned before are important molecules in the acute phase of inflammation, imperative for the initial steps of WH. However, all these cytokines have their expression levels decreased, evidencing that FSDCs may repress this phase of WH.

IL-10 (↓) is the most studied anti-inflammatory cytokine, mediating the end of the inflammatory phase of WH (opposite to the previous cytokines). Indeed, IL-10 is known to suppress antigen presentation by LCs, dermal DCs, MΦs and monocytes; to repress cytokine and chemokine production, such as TNF-α, IL-1, IL-6, IL-8, IL-12 from monocytes, MΦs, eosinophils, T cells, MCs and neutrophils; to induce the expression of MHC class II, CD86, CD54 and CD40 in monocytes and enhance MΦs antigen presentation; to induce IL-1RA production and soluble TNF receptors in monocytes, MΦs and neutrophils; to induce antigen-induced histamine liberation in MCs; to promote NK cells cytotoxicity and B cells growth; and finally, to induce E-selectin in ECs (Asadullah *et al*., 2003). Although FSDCs are not producers of high amounts of IL-10, NT downregulated the modest IL-10 expression of FSDCs, as shown in Figure 26 and 27A.

CCL5 or RANTES (↑) expression was upregulated by NT. RANTES induces leukocyte migration by binding to specific receptors, namely CCR1, CCR3, CCR4 and CCR5. It mediates the activation, traffic and homing of classical lymphoid cells such as T cells and monocytes, as well as, T cells, DCs, eosinophils, NK cells, MCs and basophils to and from the site of inflammation (Rossi D. & A. Zlotnik, 2000). After 30h of incubation, NT increased CCL5 expression in control and inflammatory conditions. As CCL5 is an unspecific chemokine, it can be modulated by different signaling pathways (described later), and so, after long-time NT incubations, this unspecific chemokine can be activated. Indeed, the immunosuppressive function of NT did not seem to modulate this cytokine.

Epidermal growth factor (↑) expression was increased by NT and, according to its denomination, it induces epidermal growth. EGF-R is strongly, although not exclusively, expressed in the basal layer of the epidermis and EGF binding to EGF-R induces KCs proliferation, differentiation and migration, promoting epidermal thickness (Jost *et al*., 2000). Furthermore, it is known that EGF treatment of the wound accelerates wound healing (Grzybowski *et al*., 1999) and also that EGF is important in the last phases of WH. Indeed, the upregulation of EGF expression by NT may promote the migration-remodeling phases of WH.

VEGF-A (↓) was decreased by NT at short-time incubation periods. VEGF is an important regulator of endothelial cell proliferation, migration and cell survival, inducing vascular hyperpermeability and angiogenesis, as well as, inducing monocyte/macrophage recruitment (Chalon *et al*., 1996). All these processes are involved in the first phases of WH, to create blood vessels which are important in the migration of APCs to lymph nodes and leukocytes to the injury site. However, in the last phases of WH, this growth factor is no longer important and may be decreased.

Indeed, low doses of NT may repress the inflammatory phase of WH and enhance the proliferating and remodeling phase of WH, by diminishing the expression of cytokines and VEGF, and increasing EGF. However, high doses of NT promoted the opposite response, a pro-inflammatory response of FSDCs that may be responsible for the constant inflammatory state of wound healing observed in skin wound diseases.

5.1.1. Signaling pathways and cytokine, chemokine and growth factor's profile

The association between the signaling pathways and cytokine profile will be discussed in cells which were incubated with 10 nM of NT. The activation of signaling pathways with 100 nM of NT was also tested and the results were very similar to those obtained with 10 nM. As NT incubations were performed for the period of 1h, the

observation of different signaling pathways' activation with 100 nM of NT incubations should conceivably be done during longer incubation periods, to permit NTRs sensitization.

The activation of signaling pathways was determined in two different conditions, with and without an inflammatory LPS stimulus. LPS (a component of bacteria) bound to toll-like receptor (TLR)-4 in the cells, activate inflammatory signaling pathways, like NF-κB and MAPKs (ERK, p38 and JNK). This activation is mediated by the TLR cytoplasmic domains resemble that of the IL-1 receptor, and hence called Toll-IL-1-Receptor (TIR) domains. After LPS binding to TLR, dimerization occurs and TLR recruits the adaptor protein MyD88 (Medzhitov *et al*., 1998) and induces consequent downstream signaling. In accordance, the interpretation of the activation of signaling pathways by LPS and NT interference was easily determined, while with no LPS stimulus, the inflammatory pathways were only slightly activated and so, NT effects on signaling were difficult to observe in these studies.

Although these pathways have a distinct inflammatory profile, inside of the cell, the phosphorylation of a single protein involved in one signaling pathways may have repercussions on other signaling pathways. NT downregulated the activation of signaling pathways involved in inflammation, namely NF-κB and JNK, upregulated a signaling pathway involved in cell growth and survival – ERK – and upregulated a pro-inflammatory pathway – p38, as shown in Figure 18 to 24. In order to establish a connection between NT (in)activated signaling pathways and NT induced cytokine profile, a deep literature research about the involvement of these signaling pathways and cytokine expression was done and considered when analyzing the present data.

The activation of the signaling pathway NF-κB is involved in diverse immune responses mediated by cells, increasing genes transcription like chemokines (IL-8,

CCL5, MIP-1 and MCP-1), cytokines (IL-6, IL-1β, TNF-α, GM-CSF, IL-4 and IL-5), adhesion molecules (VCAM-1, ICAM-1 and E-selectin), enzymes that produce secondary inflammatory mediators (iNOS, COX-2 and PLA2) and inhibitors of apoptosis (Ghosh *et al*., 1998). Indeed, NF-κB downregulation observed in FSDCs may have caused the decrease of NO production observed in FSDCs stimulated with 10 nM of NT in homeostatic and inflammatory conditions (Figure 17 and 21), probably by the decrease of iNOS expression (discussed further in this chapter). Additionally, the downregulated expression of the cytokines IL-6 and TNF-α might have been caused by decreased NF-κB activation, as the association between NF-κB activation and IL-6 and TNF-α expression was already described in our lab, in FSDCs (Neves *et al*., 2009). As this signaling pathway is activated in the initiation of acute phase response and consequently induces the expression and production of acute phase-related genes, NF-κB downregulation may diminish this phase and so, diminish the immune responses of FSDCs.

The JNK signaling pathway is also involved in diverse inflammatory functions. In addition to NF-κB, JNK can be activated by LPS or inflammatory cytokines such as TNF and IL-1 (Davis R.J., 2000). After activation, JNK may induce the production of multiple cytokines, including type I interferon and IL-6 (Chu *et al*., 1999). JNK also activates different transcription factors, such as c-JUN (component of the transcription factor AP-1), ATF2 and SP-1, involved in the transcription of different cytokines. Indeed, the inactivation of this signaling pathway can also be involved in the downregulation of IL-6 expression. Furthermore, considering that NT downregulated this signaling pathway (as it also occurred with NF-κB), it still remains to be elucidated how NT downregulation occurs. Accordingly to the literature, one possible way of JNK downregulation, may involve the activation of MAPK phosphatases. MKPs may

dephosphorylate different substrates of different signaling pathways with different affinities. For instance, MKP-1 has great affinity for p38 and pJNK (but less for ERK) (Tanoue *et al*., 2000) and MKP-5 for JNK, rather than p38 (Zhang *et al*., 2004), and these phosphatases are involved in the downregulation of the cell's immune functions, triggered by these pathways. In accordance, MKP-1 knockout mice are involved in enhanced innate immune responses, as this phosphatase regulates cytokine production in MΦs and DCs. In MΦs, MKP-1 negatively regulates IL-10 and in MKP-1-deficient DCs the levels of pro-inflammatory cytokines (including TNF-α and IL-6) and the anti-inflammatory cytokine IL-10 increased (Dong *et al*., 2002; Nimah *et al*., 2005; Zhao *et al*., 2006). In MKP-5-deficient mice, in response to various TLRs ligands, cytokines increased and DCs displayed increased CD4+ and CD8+ Tcell priming capability (Zhang *et al*., 2004). Considering the role of MKPs, and specifically the function of MKP-1 and MKP-5 and their specificity for JNK, MKP-1 or/and MPK-5 might be involved in JNK downregulation in FSDCs, and consequently, IL-6, TNF-α and IL-10 downregulation, presented in Figure 23 and 26A.

In contrast to the previous signaling pathways, the signaling pathway p38 is upregulated in FSDCs (Figure 24). The signaling pathway p38 is also an inflammatory pathway. Studies in mice deficient in the p38 kinase MKK3 showed decreased LPS-induced IL-12 production in MΦs and p38-specific inhibitors decreased the production of others cytokines, like TNF-α, IL-6, IL-1α and IL-1β (Lu *et al*., 1999; Wysk *et al*., 1999). Although this signaling pathway modulate the expression of different cytokines, in FSDCs, the upregulation of the p38 signaling pathway may be related with IL-1β outcome, at 6h of NT exposure, while the inactivation of the others signaling pathways (NF-κB and JNK) may have a larger effect in the other cytokines (IL-6 and TNF-α), decreasing their expression. The expression of the chemokine CCL5 increased after 30h

of NT incubation and after 6h of LPS and NT incubation. Accordingly with the literature, the inflammatory signaling pathways previously described induce the expression of chemokines, like CCL5. In a focused literature research in diverse cell types, CCL5 production is primarily induced by two signaling pathways, the NF-κB and the p38, as well as, by the Interferon regulatory factor (IRF)-8, an important factor in IFN-δ-mediated signaling and in the development and function of DCs (Liu J. & X. Ma, 2006). Taking this information into consideration, and largely supported by previous results from our lab that shows that LPS-induced CCL5 expression is upregulated by p38 (Neves *et al*., 2009), it can be considered that p38 phosphorylation can also be involved in the increase of CCL5 observed in LPS stimulated and non-stimulated FSDCs.

ERK can directly phosphorylate many transcription factors including Ets-1, c-Jun and c-Myc, as well as, phosphorylate and activate the 90kDa ribosomal S6 kinase (p90Rsk), which then leads to the activation of the transcription factor CREB. Moreover, through an indirect mechanism, ERK can also lead to the activation of the NF-κB transcription factor. These transcription factors induce the expression of important genes involved in: cell cycle progression, like Cdks and cyclins (regulated by CREB, Ets, Ap1 and c-Myc transcription factors) and GFs, such as PDGF and heparin-binding (HB)-EGF (regulated by the transcription factor Ets); apoptosis prevention, such as the antiapoptotic genes Bcl-2 and p53 (regulated by CREB, Ets and AP-1 transcription factors); and cytokines, like GM-CSF, IL-2 and IL-3 (regulated by Ets and Ap1 transcription factors) (Chang *et al*., 2003). Considering the NT-induced increase of EGF expression in FSDCs, the activation of ERK may be involved in this increase. Also, considering previous results from our lab (Neves *et al*., 2009), the augment of IL-1β observed at 6h, can also be upregulated by this pathway.

Akt is a multifunctional protein kinase involved in the regulation of multiple cellular processes such as glucose metabolism, transcription, apoptosis, cell proliferation, angiogenesis and cell motility (Brazil *et al*., 2002). Akt was slightly activated by NT alone, although none of these functions were determined in these cells. However, in previous results from our lab (Neves *et al*., 2009), Akt was involved in the negative regulation of IL-10. Although WB results did not show an increase of Akt, in short-time incubations of LPS with cells, probably at later times, this signaling pathway may be activated and induce the downregulation of this cytokine.

The association between the (in)activation of signaling pathways and the cytokine expression profile observed in this work is in accordance with previous results performed in FSDCs and with the results from the literature. However, to confirm these results, the signaling pathways should be inhibited with specific inhibitors before cell stimulation with LPS and NT. Afterwards, the increase/decrease of cytokines should be measured, determining which signaling pathways is responsible for a specific increase/decrease on cytokines expression.

5.2. BJ cells

In agreement with the results on FSDCs, NT also decreased BJ pro-inflammatory action, in homeostasis$^{\#}$ and inflammatory* conditions. Indeed, 10 nM decreased the expression of the chemokines CCL4$^{\#}$*, CCL11*$^{\#}$ and IL-8*, whereas the expression of the cytokines IL-1β, IL-6 and the chemokine IL-8 were slightly increased only in homeostatic conditions. High doses of NT induced a major downregulated immune profile, decreasing CCL5$^{\#}$ and IL-8* expression, inhibiting the increase the expression of cytokines$^{\#}$ and even downregulating* it, and increasing the expression of CCL11*, as shown in Figure 22.

NT downregulated chemokines expression, namely CCL4, CCL5, CCL11 and IL-8, that mediate immune cells recruitment to the site of inflammation (Esche *et al*., 2005). Indeed, IL-8 is a neutrophil-activating cytokine, inducing chemotaxis and the release of granule enzymes (Baggiolini *et al*., 1989) and CCL11 (or eotaxin) is an important eosinophil chemoattractant which also recruits basophils, Th2 lymphocytes and tryptase-chymase MCs (De Paulis *et al*., 2001; Forssmann *et al*., 1997; Garcia-Zepeda *et al*., 1996; Sallusto *et al*., 1997). Being fibroblasts sentinels of inflammation and having their chemokines expression downregulated when treated with NT, leukocytes are not attracted to the injured skin and consequently, inflammation decreases.

NT also promoted the expression of EGF in homeostatic and inflammatory conditions and CCL11 expression in inflammatory conditions (Figure 22). Beyond CCL11 chemoattractant and physiological actions, eotaxin may induce eosinophil degranulation (Fujisaw *et al*., 2000) and IgE-independent degranulation of basophils (Uguccioni *et al*., 1997), promoting adaptive immune responses, by the selectively recruitment of Th2 lymphocytes, which is dependent on the expression of CCR4 and CCR8 on Th2 (Ono *et al*., 2003). Indeed, the increased expression of CCL11 mediated by NT can be implicated in a Th2 mediated response. Moreover, the enhancement of EGF may induce an autocrine effect in BJ cells, inducing their proliferation and differentiation, and promoting a contractile BJ phenotype, important in ECM production and wound closure in WH. Additionally, BJ induced-EGF expression may also modulate epidermal proliferation and differentiation, by a paracrine function in KCs.

6. The role of NT in homeostasis, inflammatory and pathological conditions

The dual NT (10 and 100 nM) role in cells, as well as, the variation of levels and types of NTRs expression at cell surfaces may characterize a pro- or anti-inflammatory environment needed in tissues, in different conditions.

6.1. Low NT concentrations (10 nM of NT)

In FSDCs, low NT levels released in normal conditions modulated cell's immune responses, downregulating inflammation during short and long-time incubation periods. Cells maintained in an *in vitro* inflammatory environment (LPS during 6h) with a simultaneous treatment with NT may mimic the release of NT by skin cells or nerves, in an *in vivo* inflammatory condition. The results obtained indicated that, in this inflammatory state, the expression of endogenous NT in FSDCs increased although the expression of NTRs was repressed. Indeed, what high NT levels would promote in FSDCs – pro-inflammatory cell behavior – as NTRs expression was downregulated, not all NT bind to NTRs and so, NT act as if its concentration was still low.

In BJ cells, NT also induced the downregulation of cell's chemotactic function and, consequently, their capacity to migrate, while increased EGF expression, a crucial GF involved in cell growth and WH. In an inflammatory environment during 6h, BJ cells still expressed NTRs and even increased their expression. Indeed, the release of NT by nerves or expressed by surrounding cells may maximize the NT effect observed in non-inflammatory conditions, as the NT effects are dose-dependent. Indeed, chemokines and cytokines are significantly downregulated and the expression of EGF is significantly increased.

6.2. High NT concentrations (100 nM of NT)

High NT levels may occur through an alteration in cell transcription, or by an extra NT release by the sensory nerves, which may occur under a chronic stress or inflammation state, for instance in diabetic wound healing. In normal conditions, high NT levels induced a pro-inflammatory character in FSDCs, determined by an increase of pro-inflammatory cytokines and chemokines, and the production of NO. Moreover, in inflammatory conditions, although there was slight and unclear decrease in the expression of TNF-α and CCL5, a clear increase of IL-6 may determine a possible promotion of FSDCs pro-inflammatory functions.

In BJ cells, high NT levels in inflammatory and non-inflammatory conditions mediated the downregulation of chemokines and cytokines, and increased the expression of EGF. These cells are influenced by NT in a dose-dependent manner, and so, both with low or high NT concentrations, BJ cells respond in the same way, decreasing their immune capacity.

6.3. The effects of neurotensin on wound healing and diabetic wound healing

In summary, low doses of NT may induce the repression of FSDCs inflammatory action; although at high concentrations it may induce a pro-inflammatory profile in FSDCs. Addressing FSDCs modulation by NT into WH, if low levels of NT are released in the beginning of the inflammatory processes, NT may induce pathological effects, diminishing the capacity of cells to respond to inflammation and probably promoting infections; however, if low doses of NT are released in the middle or at the end of inflammation, as well as, in the migration-remodeling phases of WH, NT may modulate de degree of inflammation, end inflammation and induce the progression to migration-remodeling phases of WH. Indeed, in this last case, NT acts as a WH promoter. Concerning high doses of NT into WH, if NT is released either in

inflammatory or migration-remodeling phases, it can activate pathological FSDCs functions, increasing FSDCs pro-inflammatory activity, which may inhibit the progression of inflammation to migration-remodeling phases; if released in the begging phases, high NT levels may promote and enhance inflammation, which can be important in the initial phases of wound healing.

Concerning BJ cells, low or high doses of NT may not be pathological if NT exposure occurs in the middle and end of inflammation and in the migration-remodeling phase, as in these conditions the diminishment of inflammation is needed; if NT exposures occurs in the beginning of the inflammatory phase, as discussed earlier, the decreased inflammation can be pathological as it may inhibit BJs sentinel function and may promote infection.

NT is inexistent in the skin of human and rats (Seethalakshmi *et al.*, 1997; Pincelli *et al.*, 1990) in homeostatic conditions, however, NT levels might increase in inflammatory conditions or in different phases of wound healing (Figure 36). In fact, this work still requires *in vivo* studies to determine when and in which amounts NT exists in the skin.

6.4. Treatment of wound healing with NT

In vitro pre-treatment (24h) of cells with NT mimics an exogenous and previous (to inflammation) treatment with NT. In BJ cells this pre-treatment would not evoke a different cell response, as NTRs were not downregulated at 6h of incubation. However, in FSDCs, as these cells have their receptors downregulated after 6h of LPS treatment, NT treatment before inflammation (24h of pre-treatment) could promote a different response. In these incubations, in addition to the pro-inflammatory stimulus of LPS, NT enhanced the inflammatory response evoked by LPS, probably provoked by the cumulative effects of LPS and NT that can be related to an increase in NTR3 at cells

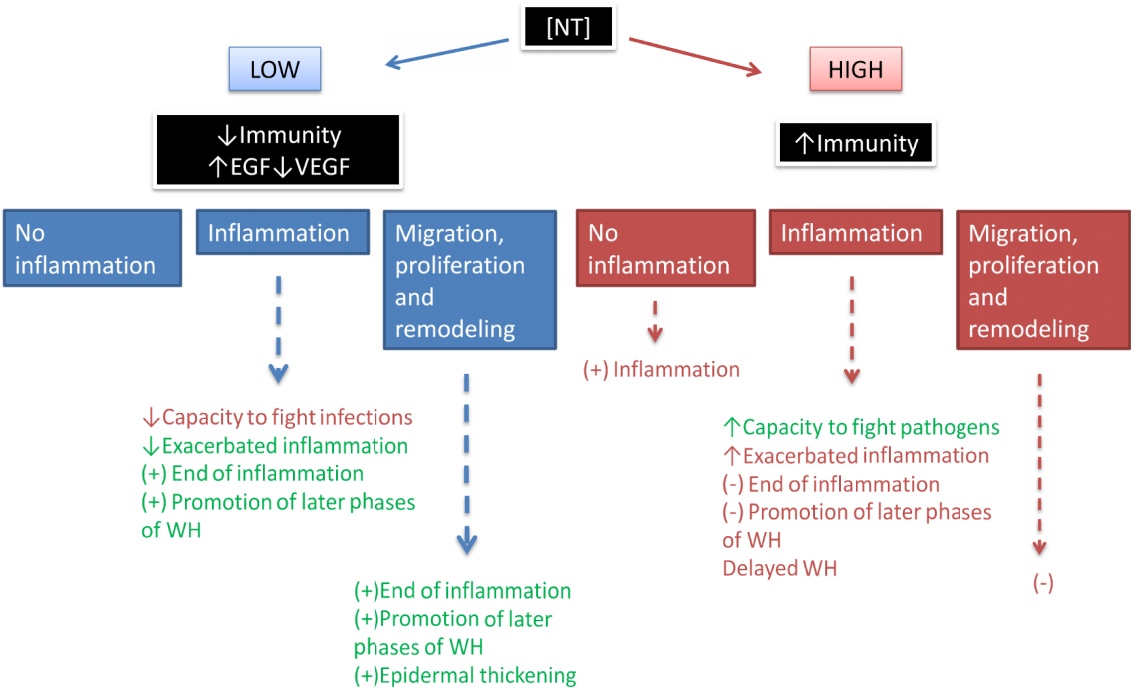


Figure 37 – Different NT concentrations promote physiological and pathological effects in different phases of WH.
Legend: Green-Physiological effects; Red-Pathological effects; -► Point in WH where [NT] (↓or↑) may act.

surfaces, inducing this pro-inflammatory state. Indeed, this pro-inflammatory profile in FSDCs may be important in the first phases of WH. Addressing these *in vitro* studies to an *in vivo* situation, if NT is applied immediately after injury in the wounds, previously to the start of the inflammatory process, NT can in fact upregulate cell immune's function in the inflammatory phase of WH, being promissory in the treatment of normal WH. On the other hand, if low doses of NT are applied in the wounds after injury, when the inflammatory process had already started (*in vitro* cell stimulation with LPS and NT during 6h), NT can negatively modulate the cells' immune responses. In these circumstances, delayed wounds known to have higher levels of NT, could possibly be treated with antibodies for NT and decrease the NT concentration in the wounds,

promoting WH. Moreover, once NT levels are determined in skin pathologies and if NT is then defective, a treatment with low doses of NT may promote WH.

7. The production of NO is decreased by neurotensin, in FSDCs

In the skin, iNOS expression can be upregulated by external pathogens which induce the production of high amounts of NO. In the human skin, NO is involved in cell growth and cell death, in cell-cell and cell-matrix interactions and in the regulation of inflammatory and immune responses (Bruch-Gerharz *et al*., 1998). Furthermore, NO is implicated in the promotion of innate immunity required in the skin, being part of the first barrier against external organisms. In addition, NO is an imperative upregulator of cells' phagocytic activity against pathogens, killing pathogens like bacteria, virus and parasites, as well as, is involved in the increase of Th1-mediated responses. NO can be produced by important immune cells of the skin, like LCs, and affect their function, altering its microbicial activity, antigen presenting function and cytotoxicity (Qureshi *et al*., 1996), and so, having an essential role in the immunity of the skin.

In the present studies, NT modulated NO production. Indeed, FSDCs treatment with NT decreased the release of nitrite during 7 days, as well as, cell treatment with 10 nM of NT and LPS stimulus, also downregulated nitrite production, from 3 to 7 days, as shown in Figure 17. Since NO is an important molecule in LCs, mediating its innate immune response, this NO decrease implies impairment in the innate LCs functions. The downregulation of NO is probably caused by NF-κB inhibition, as NT alone or added simultaneously with the inflammatory stimulus of LPS increased IkBα and so, decreased NF-κB activation.

8. GLUT4 and IRS-1 expression

8.1. FSDCs

Glucose is a vital source of energy in cells, generating ATP through glycolysis and oxidative phosphorylation. Meanwhile, inside or outside of the cells, glucose concentration requests a balance: a smaller amount does not support cellular energy requirements, although in greater amounts, glucose may glycosylate proteins and transform their physiological functions. This balance starts in the regulation of external glucose input (nutrition) and internal glucose environment (blood glucose and cellular glucose). Moreover, glucose amount in the blood and cells is regulated through glucose sensing cells, which determine high glucose amounts in the exterior environment and induce their entry into the cells. β and α cells of the endocrine pancreas sense external high glucose levels and induce the production of the hormones insulin and glucagon that are responsible to establish glucose homeostasis. For instance, insulin and glucose can be transported in the blood and when reaching a specific cell, insulin binds to the insulin receptor at the cell membrane. Insulin binding leads to IRS-1 phosphorylation and downstream signaling, important in glucose transporter trafficking to the membrane and finally, glucose entry into the cells. High concentrations of glucose in the blood are delivered to glucose defective cells. In this case, cells are induced by insulin to transport the glucose transporter to the cell membrane and mediate glucose entry into cells. Moreover, cells also have glucose sensing machinery to sense an internal defect in glucose, similar to β and α cells of the endocrine pancreas and induce the expression of molecules involved in insulin signaling.

Glucose-sensing in yeast is well clarified and involves two ways: one system responds to the crucial hexokinase step of glucose utilization and the other system involves direct binding of glucose to a “glucose-receptor”- like molecule. These systems

activate specific protein kinases that phosphorylate transcription factors in order to control either their activity or their cellular concentration. In mammals, β cells respond to changes of glucose rate metabolism through GLUT2 and glukokinase (hexokinase) that have low affinity for glucose determining blood-glucose levels (Towle *et al*., 2005) and after sensing glucose alterations, induce gene transcription changes. Taking this knowledge in reflection, it can be considered that in a surrounding hyperglycemic environment, sensing-cells like FSDCs, readjust their metabolism. Indeed, FSDCs treated with 30 mM of glucose, increased IRS-1 and GLUT4 expression to consent insulin signaling and GLUT4 trafficking (Figure 25A), as expected. However, high entry of glucose can also induce protein glycosylation which can affect their physiological function. On the other hand, in inflammatory conditions, IRS-1 expression was diminished, while GLUT4 was slightly increased (Figure 25B). In a LPS stimulus of 6h, several signaling pathways and gene transcription are activated increasing energy and glucose requirements in FSDCs. After the 6h of LPS stimulus and without changing the glucose medium, external cell glucose levels diminished in parallel with intracellular levels. Cells should readjust and augment the expression of the referred molecules, to prepare the cell for glucose entry, when glucose concentration increases (as it occurs *in vivo*). However, this process did not occur, justifying the IRS-1 decreased expression although a slight increase of GLUT4. Without energy, FSDCs activity might be disturbed and this might be involved in the downregulation of FSDCs' immune function.

In this work, the insulin signaling and GLUT4 trafficking was not evaluated. For that, glucose and insulin should be added to FSDCs followed by the determination of IRS-1 phosphorylation levels and GLUT4 protein levels by WB analysis. These

experiments would establish if cells adjust these proteins to different glucose and insulin inputs.

8.2. BJ cells

BJ cells also expressed IRS-1 and GLUT4. In 30 mM of glucose, BJ cells also increased the expression of IRS-1 and GLUT4, as it was observed in FSDCs, evidencing a cell readjustment to high glucose concentrations. However, when cells were stimulated with the LPS and cultured with 30 mM of glucose, instead of diminishing IRS-1 and GLUT4 expression levels, these levels significantly increased, indicating the physiological cell readjustment, not observed in FSDCs. Indeed, these cells must easily adapt to glucose concentration, in contrast to FSDCs, in accordance with Evans *et al.* 2003.

Chapter VI. Conclusion

Neuropeptides have received major attention by the scientific community in the recent years, since they are involved in the proliferation, differentiation and migration of cells, and in immunomodulation. In the skin, both the pro-inflammatory functions of VIP and CGRP, and the anti-inflammatory functions of α-MSH, have been previously described in the literature. Although largely studied in diverse tissues, like the CNS and the gastrointestinal tract, the role of neurotensin in the skin, has not been determined until the present.

The role of neurotensin in two types of skin cells was determined, achieving the main objectives of this work. NT essentially promoted the anti-inflammatory response in two important cell types of the skin, the LCs, here represented by FSDCs, and FBs represented by BJ cells. These cells are crucial in the different stages of WH: LCs play a major role in inflammation, reaching and processing the antigen to be present to other immune cells that return an inflammatory and immunological response; FBs are important remodelers of the skin, not only by synthesizing MMPs that degrade the ECM, important in the formation of the new granulation tissue, permitting angiogenesis and cell chemotaxis to the site of inflammation, but also by producing proteins that fill and arrange the ECM (after the degrading process of inflammation) – important in the migration-proliferation and remodeling phase of WH. The anti-inflammatory response of both cells to neurotensin may be significant in the closing stage of inflammation and the synthesis of EGF and BJ migration, crucial for the initiation of the last phases of WH.

Following this work, some future research should be done in these cell lines to complement the obtained results. Studies should further determine the expression of NTRs at cell surfaces (by immunocytochemistry), both at low and high doses of NT, as well as, in inflammatory conditions. In addition, the neurotensin receptor by which NT

signaling occurs in BJ cells should be determined, as it was not performed in this work due to the non existence of the NTR2 and the NTR3 selective inhibitors. Considering the modulation of NT in the synthesis of inflammatory mediators, the signaling pathway by which this modulation occurs should also be determined by specific inhibition of the pathways in question, namely the MAPK and Akt pathways, and the transcription factor NF-κB. In addition, to confirm the expression of the genes studied - the cytokines, chemokines and growth factors - the effective protein levels of these genes in the cell medium (after released by the cells) should be determined by Enzyme Linked Immuno Sorbent Assay (ELISA). Finally, as glucose might have disturbed the transcription machinery of FSDCs, a more directed investigation of the insulin signaling and the GLUT4 trafficking should be performed. For that, glucose and insulin should be added to FSDCs cells followed by the determination of IRS-1 phosphorylation levels and GLUT4 protein levels by WB analysis. These experiments would establish if cells adjust these proteins to different glucose and insulin inputs.

In vivo research to establish the function of NT in control and diabetic mouse models of wound healing is already under way in our laboratory. Some of the *in vivo* results obtained so far support the results presented in this dissertation. Indeed, *in vivo* studies have shown that NT improves WH in control and diabetic mice, in agreement with the effects that NT may have, as obtained in our results *in vitro*. Moreover, *in vivo* results demonstrated that the expression of the inflammatory enzyme COX2 is increased after wounding in control and diabetic mice, while decreased after the treatment with NT, in accordance with the *in vitro* results performed for the inflammatory mediator NO and for the expression of pro-inflammatory mediators, with low doses of NT. Additionally, the expression of NTR1 and NTR2 was decreased in the skin of diabetic mice, whereas NT treatment increased their expression, at day 10 in diabetic animals.

Considering the *in vitro* results obtained in this dissertation, NT signaling through NTR1 in FSDCs was important in the acquisition of an anti-inflammatory profile on these cells. NT signaling through NTR1 (higher affinity for NT than NTR2) may induce the anti-inflammatory LCs response and, promote WH.

Finally, after knowing the role of NT in different cells and at different concentrations, its application on skin wounds could be a potential therapy for skin pathologies, like DFUs, a problematic and prevalent skin disease that can lead to a final amputation. Moreover, the *in vivo* treatment of wounds with high doses of NT may induce a pro-inflammatory status, promoted by LCs, which can be important in the first phases of WH, being promissory in the treatment of normal WH. On the other hand, if low doses of NT are applied in the wounds after injury when the inflammatory process had already started, NT can negatively modulate the cells' immune responses. In these circumstances, delayed wounds known to have higher levels of NT, could possibly be treated with antibodies for NT and decrease the NT concentration in the wounds, promoting WH. Moreover, once NT levels are determined in skin pathologies and if NT is then defective, a treatment with low doses of NT may promote WH.

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