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Discrete adrenergic influence on the skin response to pathogen-associated molecular pattern (PAMPs)

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Neuchâtel, le 30 juin 2008

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Discrete adrenergic influence on the skin response to pathogen-associated molecular pattern (PAMPs)

Thesis presented at the Faculty of Science
Institute of Biology
University of Neuchâtel

For the achievement of the degree of Doctor in Science

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Presented the 26th May 2008

University of Neuchâtel
2008

Mots clés en français: Système nerveux sympathique; Peau; Cellules dendritiques; TLRs; Récepteur NOD2; Récepteurs β-adrénergiques, Auto-immunité.

Mots clés en anglais: Sympathetic nervous system; Skin; Dendritic cells; TLRs, NOD2 receptor; β-adrenoceptors, Autoimmunity.

Résumé:

La neuroimmunologie est la science qui étudie les interactions entre le système nerveux central et le système immunitaire. Différentes études morphologiques ont démontré que le système nerveux sympathique innerve tous les organes lymphoïdes primaires et secondaires et que la plus grande partie des cellules immunitaires expriment à leur surface les récepteurs adrénergiques nécessaires à la transduction du signal de ce système.

Dans ce travail, nous avons montré *in vivo* que le blocage des récepteurs β–adrénergiques influence la réponse immunitaire innée de la peau au peptidoglycanne (PGN), mais pas au lipopolysaccharide (LPS), en induisant l'expression génique des cytokines IL-23, IL-12 et IFN-γ. Le blocage de ces récepteurs influence aussi la réponse immunitaire adaptative qui est polarisée vers une réponse de type Th1. Celle-ci a été évaluée par la mesure de la réponse d'hypersensibilité retardée(DTH) et par la production de cytokines dans le ganglion drainant. Cette réponse est aussi caractérisée par une augmentation de la migration de cellules dendritiques plasmacytoides au ganglion drainant le site d'injection. La migration de ces cellules est aussi augmentée par l'utilisation de PGN en combinaison avec de l'extrait de glandes salivaires (EGS) des tiques *Ixodes ricinus*. Cette observation suggère que l'EGS contient des agonistes aux PRRs (pour Pattern Recognition Receptors) ou des chémokines qui pourraient agire en synérgie avec le PGN. Nous avons aussi démontré que le blocage simultané des récepteurs β1 et β2 adrénergiques est nécessaire pour influencer la réponse immunitaire *in vivo*.

L'influence de l'activation des récepteurs β–adrénergiques par la norépinephrine (NE) sur la production des cytokines induites a aussi été analysée *in vitro* par l'activation de différents PRRs chez les cellules dendritiques (CDs). Nos résultats ont montré que, en accord avec les résultats obtenus *in vivo*, la NE possède des propriétés anti-inflammatoires et inhibe la production des cytokines IL-12, IL-23 et IL-6 chez les CDs incubées avec le PGN. La production de la cytokine anti-inflammatoire IL-10 est quant-à-elle induite. En outre, l'utilisation d'autres agonistes spécifiques pour les récepteurs TLR-2 et NOD2 ont montré que l'augmentation de la production d'IL-10 induite par la NE chez les CDs incubée avec du PGN est due à la modulation de la signalisation du récepteur TLR-2 mais pas à celle de

NOD2. Cette production augmentée d'IL-10 a aussi un effet inhibiteur sur la production des cytokines caractéristiques des Th17, à savoir IL-6 et IL-23.

Nous avons aussi démontré que la NE peut induire *in vitro* via les récepteurs β2-adrénergiques la production d'IL-6 chez le CDs dont le récepteur NOD2 est activé. Ce résultat est important pour la compréhension des mécanismes régulant la production de cette cytokine et de la pathogenèse de maladies où une augmentation de la concentration d'IL-6 est observée. Des résultats préliminaires ont aussi montré que l'injection chez les souris de CDs préalablement incubées avec les agonistes spécifiques des récepteurs β2-adrénergiques, TLR-2 et NOD2 en présence d'une protéine soluble, favorise le développement d'une réponse de type Th17 au détriment de celle de type Th1. Ceci a été évalué par l'analyse de la production des cytokines IL-17 et IFN-γ. Ces résultats aident à la compréhension du développement de la réponse Th17 et des maladies auto-immunitaires et des cancers qui en dépendent.

Acknowledgements

I would like to thank everybody at the Istituto Cantonale di Patologia in Locarno for their support and help during my thesis. In particular, my thesis director in Ticino Dr. Georges J. Maestroni for the motivation, advises and for his availability. Elisabeth Hertens and Paola Galli for their assistance and contribution in performing the experiments and Dr. Francesca Botta for her support and the pleasant coffee break.

I would like also to thanks my family and all my friends, who have always support me during all these years.

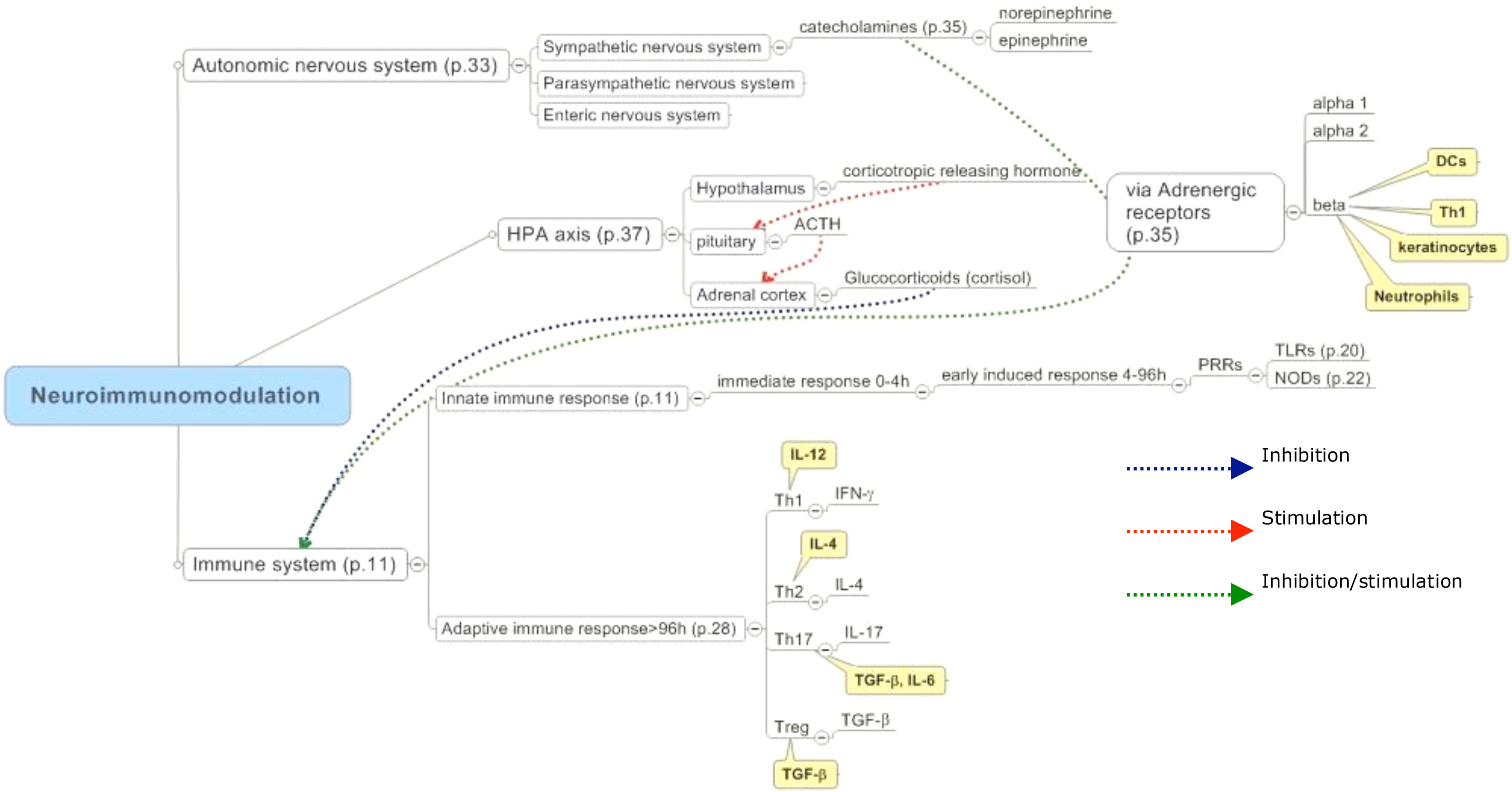
ABBREVIATIONS

2-ME	2-mercaptoethanol
ACh	Acetylcholine
ACTH	Adrenocorticotropic hormone
ANS	Autonomic nervous system
APCs	Antigen presenting cells
ARs	Adrenergic receptors
BM	Bone marrow
CAs	Catecholamines
CARD	Caspase recruitment domain
CLA	Cutaneous lymphocytes antigen
CNS	Central nervous system
CRH	Corticotropin releasing hormone
CTLs	Cytotoxic T lymphocytes
iE-DAP	γ-D-glutamyl-meso-diaminopimelic acid
DCs	Dendritic cells
DTH	Delayed type hypersensitivity response
E	Epinephrine
FITC	Fluorescein isothiocyanate
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GRs	Glucocorticoid receptors
HPA	Hypothalamic pituitary adrenal axis
i.d.	intradermally
IFN	Interferon
IL	Interleukin
KLH	Keyhole limpet hemocyanin
LCs	Langerhans cells
LN	Lymph node
LPS	Lipopolysaccharide
LRRs	Leucin rich repeats domain
MACS	Magnetic activated cell separation
MDP	Muramyl dipeptide

MHC	Major histocompatibility complex
NE	Norepinephrine
NKs	Natural killer cells
NKTs	Natural killer T lymphocytes
NOD	Nucleotide binding oligomerization domain
PAM	N-Palmitoyl-S-[2,3-bis(palmitoyloxy)-(2RS)-propyl]-[R]cyteine
PAMPs	Pathogen associated molecular pattern
PANS	Parasympathetic nervous system
PBS	Phosphate buffered saline
PCR	Polymerase-chain reaction
pDCs	Plasmacytoid dendritic cells
PE	Phycoerythrine
PGN	Peptidoglycan
PRO	Propranolol
PRRs	Pattern recognition receptor
SALT	Skin-associated lymphoid tissue
SNS	Sympathetic nervous system
STATs	Signal transducers and activators of transcription
TCR	T cell receptor
TGF	Transforming growth factor
Th 1/2/17	T-helper cell type 1/2/17
TLRs	Toll like receptors

I. Introduction

Introduction plan



1. The immune system

1.1 Introduction to the immune response

The immune system is not a well defined organ, as the kidney or the liver, but is composed of many different cell types, tissues and soluble products disseminated all around the body and which are engaged in the continuous fight against external attacks by infectious agents, but also against injuries and host generated threats, such as cancer. It is divided in two different parts: the innate and the adaptive, or acquired, immune system.

The innate immune system is the ancestral system present in all multicellular organisms and it is the first host defense that is activated immediately after exposure to an external pathogen. As long as the pathogen is stopped by the innate response, the adaptive response will not be elicited. The innate immune system uses physical and chemical barriers to exclude antigens and a small number of pre-existing non specific receptors, called pattern recognition receptor (PRRs), to recognize conserved molecular motifs present on a broad range of pathogens (pathogen associated molecular pattern; PAMPs). These receptors induce a rapid, but not specific response to practically all infectious agents. However, no immune memory is generated, meaning that a second exposure to a specific antigen causes an identical response in magnitude and type than the first. In conclusion, the aim of the innate immune system is not to discriminate between pathogens but to provide an early warning system able to rapidly limit the infection and initiate and direct the specific adaptive immune response[1].

The adaptive immune response appeared in vertebrates 450 millions years ago and is complementary to the innate immune system, but differs from it in some important aspects. First of all, in contrast with the innate response, the adaptive response is highly specific to each pathogen. In fact, it is potentially able to recognize an infinite number of antigens using specific receptors expressed on B and T lymphocytes, the main effectors cells of the acquired immune system. This recognition leads to the activation of specific mechanisms for the production of the effector response. Compared to the innate response, the adaptive response is delayed but necessary for the elimination of a particular antigen. The adaptive immune response is also characterized by the ability to develop a memory concerning a specific antigen. This ability enables the organism to mount a stronger and faster response during a second exposure to a pathogen and allow its elimination preventing the development of diseases.

The innate and the adaptive immune response do not operate separately but by a tight crosstalk. For instance, elements of the innate immunity are able to enhance and direct the adaptive response, while cells of the acquired immune system resident nearby the surface barriers improve the effectiveness of the innate reaction.

The immune response is divided in three phases, which take place at different times (Fig.1).

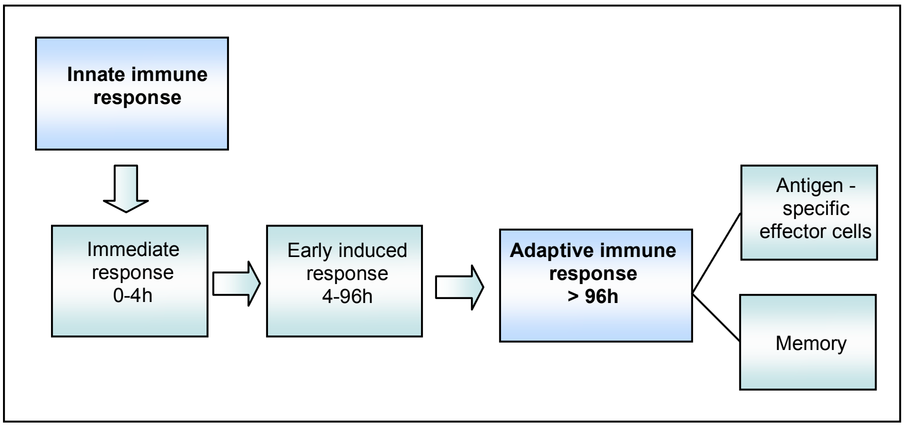


Figure 1: The three phases of the immune response to an initial infection.

Following the attack by a pathogen the first line of defense are the non-specific, non-inducible elements of the innate immune system, such as the enzymes present in the mucosa or the acidity of the skin. The second phase takes place 4-96 hours following the attack and involves the recognition of the pathogen by the PRRs. This recognition permits the induction of the innate host immune mechanisms, such as phagocytosis of the antigen by leukocytes or production of cytokines. If the antigen is completely removed by these mechanisms, the adaptive immune response is not activated. In contrast, if the innate immune response does not completely clear the pathogen, the acquired response is fully activated.

In the following chapters the cellular and humoral mechanisms of the innate and adaptive immune response will be discussed.

1.2 The skin-associated lymphoid tissue (SALT)

The skin is the largest and most exposed interface of the body in direct contact with the environment and consequently with an enormous quantity of pathogens potentially able to colonize our body and threaten our health. To cope with this danger, a complex defense system including physical barriers, chemical factors and cells of the innate and adaptive immune system developed in the skin. The immune system elements implicated in this defense are collectively called skin-associated lymphoid tissue (SALT).

The skin is divided in three distinct compartments: the epidermis, the derma and the hypoderma (Fig. 2).

a) The epidermis

The epidermis is composed by an outer matrix of keratin fibers, which provide a physical barrier against external pathogen, and an inner stratified layer of keratinocytes, specialized squamous epithelial cells producing the keratin. In the deepest layer of the epidermis new, less differentiated keratinocytes are constantly produced in organized waves of cells physically connected by specialized intracellular tight junctions called desmosomes. The role of the desmosomes is to ensure the formation of horizontal layers of keratinocytes that migrate trough the epidermis as a unit to reach the outer keratin matrix where they die. This mechanism ensures the regular renewal of the skin and prevents the penetration of pathogens in the organism. In addition to these physical and mechanical barriers, billions of commensal bacteria normally colonize the skin and actively contribute to its defense by competing for space and nutriments with others pathogens and by secreting antimicrobial peptides. Moreover, Gram-positive bacteria of the normal flora secrete lipases that digest the lipids present on the cutaneous surface in fatty acids. These acids, together with the sebum produced by the sebaceous glands, contribute to the maintenance of the pH of the skin at a low level, which discourages the microbial replication and helps in maintaining the microbial population on the skin at a manageable level[1].

Till some years ago, keratinocytes were thought to be involved in the skin immune surveillance only as a physical barrier by maintaining the structure of the epidermis and by secreting keratin. However, it is now clear that keratinocytes are involved actively in the epidermal immune response. Several studies have in fact demonstrated the ability of the keratinocytes in producing important molecules necessary for the innate and adaptive immune response[2]. For instance, it has been demonstrated that human and murine keratinocytes express constitutively some members of the Toll-like receptor (TLRs) family[3,4]. This important receptor family is a PRR implicated in the recognition of the microbes by binding the PAMPs derived from the pathogen and by this way triggering the innate immune response by the production of proinflammatory cytokines, chemokines and antimicrobial peptides. The keratinocytes derived cytokines, such as TNF-α, and chemokines, such as IL-8/CXCL-8, play an important role in the initiation of the inflammatory response and in the recruitment of various immune cells at the site of infection. Keratinocytes derived antimicrobial peptides, such as defensins and cathelicidines, are able to kill or inactivate a wide spectrum of microorganisms mainly by forming pores and permeabilizing the microbe membrane.

Although keratinocytes are the major cell type of the epidermis, making up about 90% of epidermal cells, some T lymphocytes and epidermal dendritic cells, called Langerhans cells (LCs), are also found at the level of the basement membrane. In humans the majority of T cells found in the epidermis are $\alpha\beta$ T cells, while in mice the $\gamma\delta$ T cells are predominant. LCs present in the epidermis express CD1 as well as high levels of major histocompatibility complex class I and II (MHC class I and II), allowing them to effectively present peptide and glycolipide antigens to the T cells and triggering by this way the adaptive immune response.

Finally, many nerve terminations are also present in the epidermis and there is growing evidence of a crosstalk between the nervous and the immune system in the skin[5]. The latest development of this interesting growing research will be discussed more extensively in the following chapters.

b) The basement membrane

The basement membrane is an additional protective layer that separates the derma from the epidermis. It is composed of collagen, laminin, heparan sulphate and glycosaminoglycan produced by keratinocytes and of fibronectin produced by dermal fibroblasts. The epidermis lacks blood vessels, thus the leukocytes and the important nutrients necessary for the maintenance of keratinocytes must exit in the dermal compartment and diffuse through the basement membrane. Leukocytes possess specific enzymes able to dissolve partially the basement membrane and allowing them to rapidly migrate from the derma to their final destination in the epidermis[1].

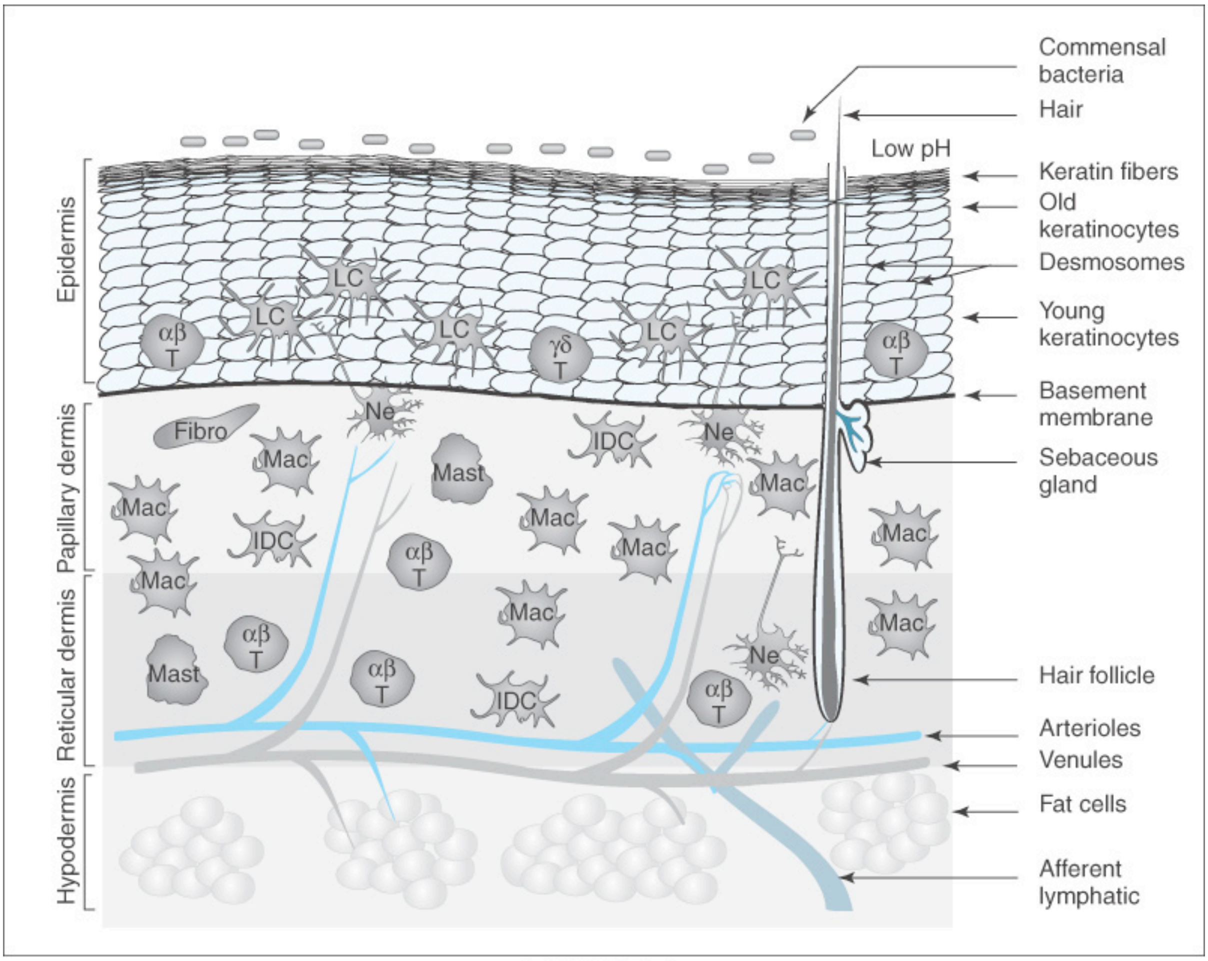


Figure 2: The SALT structure. The skin is composed of three compartments: the epidermis, two dermal layers and the hypoderma. In each compartment important immune cells are found and implicated in the innate and adaptive immune response of the skin. LC: Langerhans cells; T: T lymphocytes; Fibro: fibroblasts; Ne: neurons; Mac: Macrophages; Mast: Mast cells; IDC: immature dendritic cells. (Mak, T.W., Saunders, M.E., The Immune Response, Basic and Clinical Principles, Academic Press, 2006.)

c) The derma

The derma is composed of two layers: the papillary derma, placed just under the basement membrane, and the reticular derma. Both layers are composed of collagen, which provides structural support to the skin, elastin fibers, which give elasticity, and hyaluronic acid, which is in charge of trapping the water and moisturize the skin. In the reticular derma the connective tissue is denser than in the papillary derma. Dermal fibroblasts are mostly responsible for the production of collagen and elastin but they are also implicated in the innate immune response by secreting important chemokines, cytokines and growth factors, necessary respectively for leukocytes migration, differentiation and survival[6]. In addition to fibroblasts, the derma is also composed by macrophages, dermal dendritic cells, mast cells, T lymphocytes but not B lymphocytes. The leukocytes reach the derma, and later the epidermis, via the extravasation process in the dermal post-capillary venules. At this site the endothelial cells express important adhesion molecules, such as selectin, which may interact with the cellular adhesion molecules expressed on leukocytes and allows the binding of the immune cells to the endothelial wall and their passage into the tissues. The cellular adhesion molecules expressed both on endothelial cells and leukocytes are upregulated during the inflammatory process, increasing by this way the number of leukocytes entering the tissue. This permits to improve the effectiveness of the immune response in the skin and is an important mechanism regulating the passage of immune cells from the blood to the tissues.

Dermal dendritic cells express, as LCs, high level of CD1 as well as MHC class I and II molecules. Even if their morphology is different, the role of these cells is similar both in the epidermis and in the derma. They are professional antigen presenting cells (APCs) able to capture, process and present antigens to T lymphocytes and activate them.

Mast cells are also important cells implicated in the immune response in the skin. They express important PRRs and are able to produce cytokines and chemokines. In particular, mast cells possess presynthetized tumor necrosis factor (TNF), a potent inflammatory mediator, which can be released immediately after the exposure to a pathogen[7]. Moreover, mast cells possess granules containing histamine that can be released after specific stimulation and are known to be implicated in IgE hypersensitivity.

Finally, dermal T lymphocytes represent 98% of all T cells in the skin. They are primarily memory T cells expressing the memory marker CD45RO and high levels of CD25. There is no difference in the amount of $CD8^+$ and $CD4^+$ T cells in the derma[1].

1.3 The immune response in the skin

As previously explained, the immune response in the skin is the result of the interaction of many different mechanisms and cell types which cooperate in the defense of the skin to prevent infections (Fig. 3).

When a pathogen succeeds in penetrating the skin by passing the protective keratin layer, it injures the underlining keratinocytes which are consequently stimulated in producing potent inflammatory mediators, notably IL-1α and TNF-α. These cytokines may act in a paracrine manner on the surrounding keratinocytes inducing them to synthesize and release additional inflammatory cytokines (IL-6, TNF) chemokines (IL-8) and growth factors (GM-CSF). These molecules not only stimulate cells within the epidermis but are also able to diffuse through the basement membrane forming a chemokines gradient from the epidermis to the derma and the post capillaries blood vessels[1].

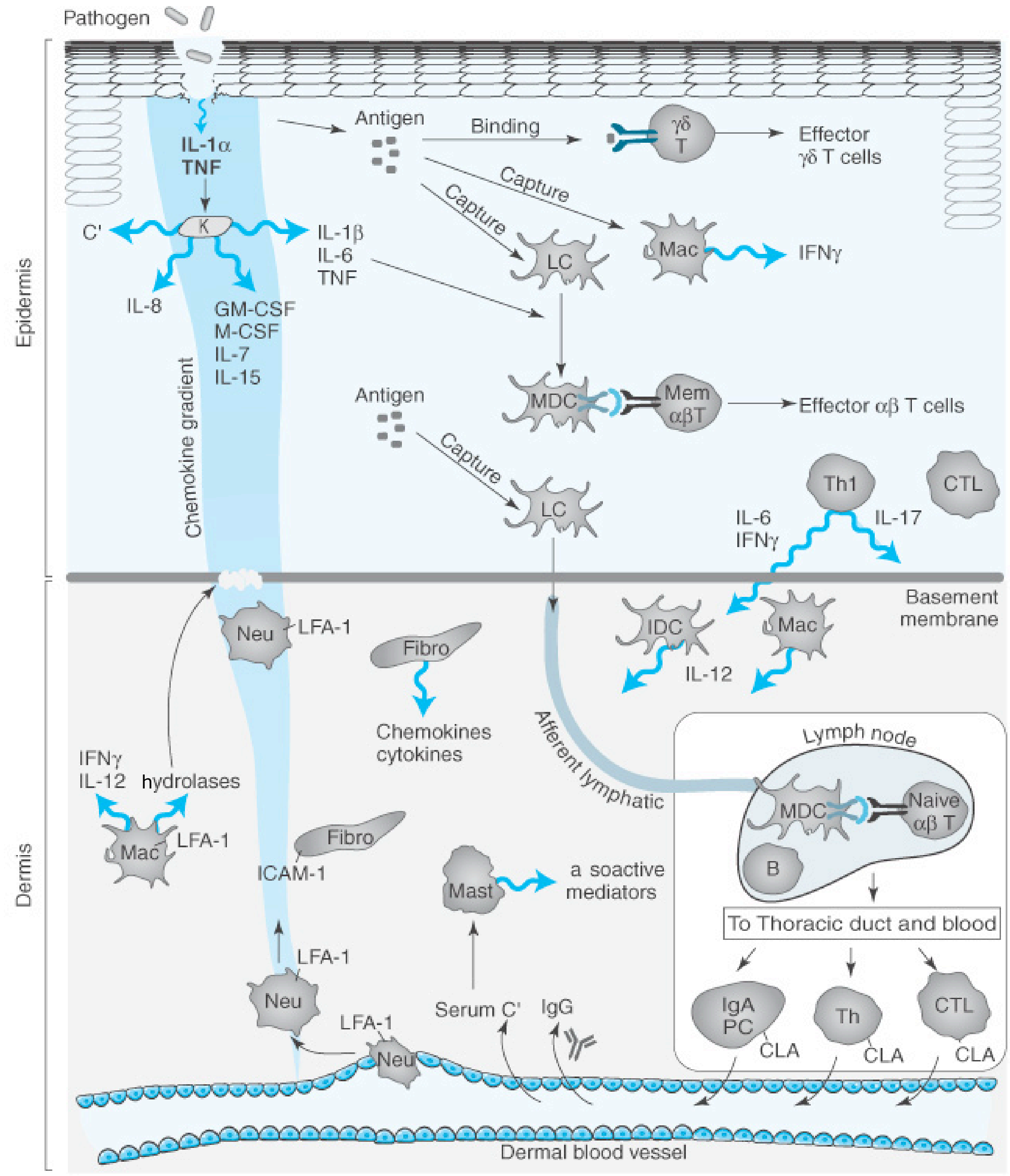


Figure 3: The immune response in the skin. . LC: Langerhans cells; T: T lymphocytes; B: B lymphocytes; Fibro: fibroblasts; Ne: neurons; Mac: Macrophages; Mast: Mast cells; IDC: immature dendritic cells; MDC: mature dendritic cells; Neu: Neutrophiles; CTL: Cytotoxic T lymphocytes; CLA: Cutaneous lymphocyte antigen. (Mak, T.W., Saunders, M.E., The Immune Response, Basic and Clinical Principles, Academic Press, 2006.)

There, cytokines and chemokines may promote vasodilation and upregulation of the adhesion molecules on endothelial cells, improving consequently the extravasation of immune cells into the inflamed skin. Once in the tissue, leukocytes may follow the chemokines gradient and move from the derma into the epidermis. During this process, the essential adhesion molecules necessary for migration, such as the lymphocyte function-associated antigen 1 (LFA-1), are upregulated and interact with the dermal matrix and the intercellular adhesion molecules (ICAM-1) expressed by fibroblasts.

Once leukocytes reach the basement membrane, they are also induced to produce specialized enzymes, in particular hydrolases, able to partial digest the basement membrane and allowing them to migrate into the epidermis and to the site of infection, where they are fully activated by the presence of proinflammatory cytokines produced by injured keratinocytes. The complement system is also activated during the inflammatory response. Complement components are synthetized by keratinocytes or they can derive from the serum complement proteins. These complement elements may help the pathogen clearance by complement mediated opsonozation and phagocytosis. Mast cells are also activated by the complement system, which induce their degranulation and the release in the tissue of molecules responsible of sustaining the local inflammation.

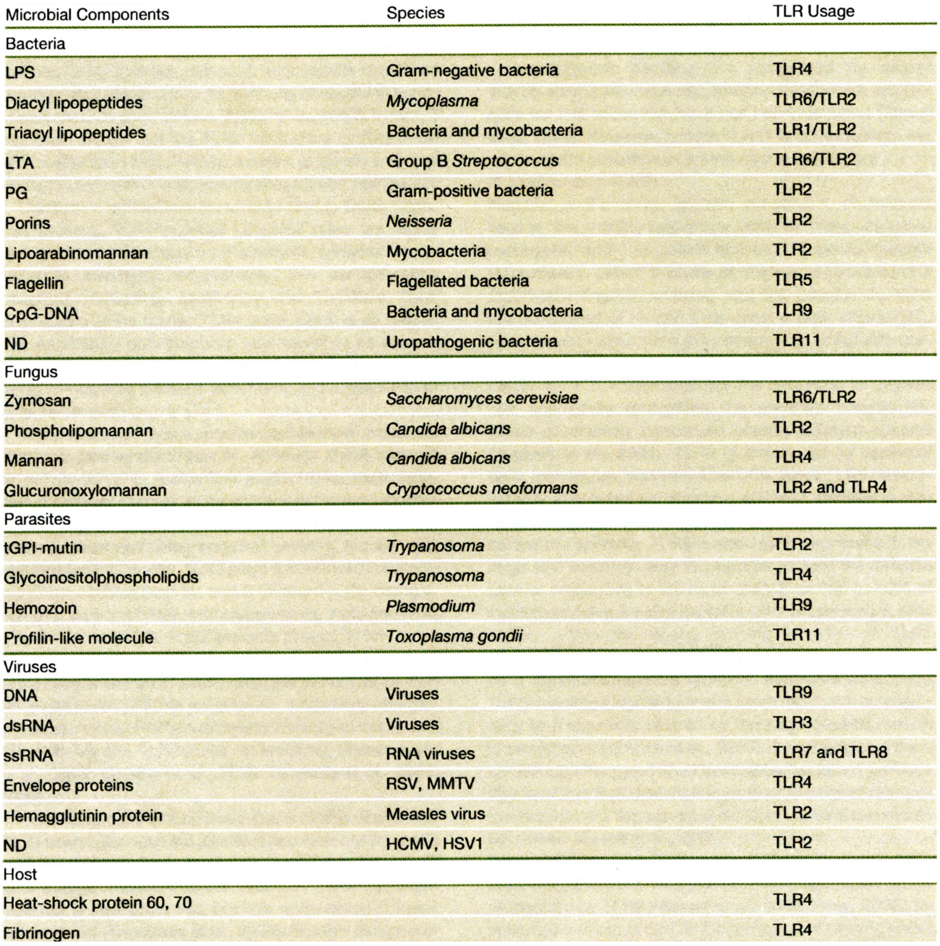
While the inflammatory response takes place, antigens resulting from the pathogen may be recognized by the PRRs of macrophages, which begin the phagocytosis process in order to fight against the infection. Moreover, activated macrophages are also a relevant source of IFN-γ that is an important Th1-cell promoter. LCs also capture and recognize antigens nearby the site of infection by their PRRs and, in presence of the correct cytokine microenvironment, they become fully mature and directly present the antigen to the epidermal memory αβ T cells. In contrast, epidermal γδ T cells can recognize the antigens using their TCRs and differentiate in effector γδ T cells without the intervention of DCs. Activated memory αβ T lymphocytes differentiate predominantly in Th1 effector T cells, due to the high concentration of IFN-γ at the site of infection. Th1 effector cells are also an important source of IFN-γ and other proinflammatory cytokines, which may diffuse through the basement membrane and activate dermal DCs and macrophages. The activation of these cells induces the production of high amounts of IL-12, responsible for the enhancement of the production of IFN-γ and of the inflammatory response. In addition, activated macrophage express important enzymes, which helps the permeabilization of the basement membrane. About 24-36 hours after the initiation of the inflammatory response, LCs cells and immature dermal DCs, which have captured antigens and have been activated by cytokines produced by Th1 effector cells, may leave the skin via the afferent lymphatic vessels and reach the draining lymph node, where they may initiate the systemic adaptive immune response by presenting the antigens to naïve T cells. LCs and dermal DCs complete their maturation in the draining lymph node upregulating the expression of MHC class II and of B7 molecules necessary for the activation of T cells. Naïve T lymphocytes expressing the TCRs recognizing the antigen presented by DCs, become fully activated and begin the differentiation in effector T cells. The effector T cells originating from the activation of naïve T cells by LCs and dermal DCs in the lymph node express the cutaneous lymphocytes antigen (CLA), a skin lymphocyte homing receptor, which allow them to specifically bind to the endothelial cells of the post-capillary venules of inflamed derma. If the infection is stopped within 36 hours, the anti-inflammatory cytokines secreted by phagocytes and keratinocytes, such as IL-10, and by macrophages and lymphocytes, such as TGF-β, replace the proinflammatory cytokines. Thus, effector T cells arriving at the site of infection from the draining lymph node find an anti-inflammatory environment that promotes the Th2 differentiation. Effector Th2 cells secrete IL-4 and IL-10, two potent anti-inflammatory mediators, further enhancing the production of TGF-β by the surrounding cells and therefore increasing the anti-inflammatory milieu counteracting the inflammation process. Eventually, homeostasis is reached and fibroblasts are stimulated to produce new collagen to repair the damaged skin.

When the infection is not completely stopped within 36 hours, high amount of IFN-γ are still produced in the epidermis when effector T lymphocytes arrive. The chemokines secreted switch to the CC class, in particular CCL5 and CCL27, which promote the influx of Th1 lymphocytes and macrophages in the inflamed tissue. Th1 cells and macrophages become hyperactivated, increasing their microbicidal power and leading to a delayed type hypersensitivity reaction (DTH), which is characterized by the swelling of the site of infection. The response is further boosted by the activation of antigen-specific cytotoxic T lymphocytes (CTLs) and natural killers (NKs). If these mechanisms fail to clear the pathogen, a switch to a Th2 response is induced by the production of IL-10 by macrophages and by high amounts of IL-4 secreted by mast cells. The Th2 lymphocytes may help B cells in the fight against the infection and they may also recirculate to the draining lymph node, where they interact directly with the antigen producing B cells. In case that the pathogen is not completely removed from the body, the infection persists and eventually generates a disease.

1.4 The Toll-like receptor family

The Toll receptor family was originally discovered in Drosophila and found to play an important role in embryonic development. However, in 1996, it has been demonstrated that mutant flies for Toll receptor family members were highly susceptible to fungal infection. These findings placed the Toll receptor family between the molecular mechanisms essential in the innate immune system. Later, homologues to the Toll family in the Drosophila were discovered in mammals and named Toll-like receptors. Functional analysis of mammalian TLRs has demonstrated that they belong to the PRRs family, which recognize conserved microbial molecular pattern, the PAMPs[8].

Table 1: TLR recognition of microbial components.



Microbial Components	Species	TLR Usage
Bacteria		
LPS	Gram-negative bacteria	TLR4
Diacyl lipopeptides	*Mycoplasma*	TLR6/TLR2
Triacyl lipopeptides	Bacteria and mycobacteria	TLR1/TLR2
LTA	Group B *Streptococcus*	TLR6/TLR2
PG	Gram-positive bacteria	TLR2
Porins	*Neisseria*	TLR2
Lipoarabinomannan	Mycobacteria	TLR2
Flagellin	Flagellated bacteria	TLR5
CpG-DNA	Bacteria and mycobacteria	TLR9
ND	Uropathogenic bacteria	TLR11
Fungus		
Zymosan	*Saccharomyces cerevisiae*	TLR6/TLR2
Phospholipomannan	*Candida albicans*	TLR2
Mannan	*Candida albicans*	TLR4
Glucuronoxylomannan	*Cryptococcus neoformans*	TLR2 and TLR4
Parasites		
tGPI-mutin	*Trypanosoma*	TLR2
Glycoinositolphospholipids	*Trypanosoma*	TLR4
Hemozoin	*Plasmodium*	TLR9
Profilin-like molecule	*Toxoplasma gondii*	TLR11
Viruses		
DNA	Viruses	TLR9
dsRNA	Viruses	TLR3
ssRNA	RNA viruses	TLR7 and TLR8
Envelope proteins	RSV, MMTV	TLR4
Hemagglutinin protein	Measles virus	TLR2
ND	HCMV, HSV1	TLR2
Host		
Heat-shock protein 60, 70		TLR4
Fibrinogen		TLR4

ND: not determined. (Akira, S., Uematsu, S., Takeuchi, O., 2006, Pathogen Recognition and Innate Immunity, Cell 124, 783-801)

TLRs are broadly expressed on different immune and non immune cell types, such as macrophages, dendritic cells, keratinocytes or fibroblasts. Their expression on these cells is modulated by a variety of stimuli, in particular cytokines, environmental stressors or pathogens. TLRs may be expressed extracellularly (TLRs 1, 2, 4, 5 and 6) or in intracellular compartments (TLRs 3, 7, 8 and 9). Intracellular TLRs recognize mainly nucleic acids that require internalization in the cell, notably in the endosome. In contrast, extracellular TLRs are able to bind a broad range of PAMPs, from lipopeptides to mannan and fibrinogen (Table 1). To date, 11 members of this family have been described in humans.

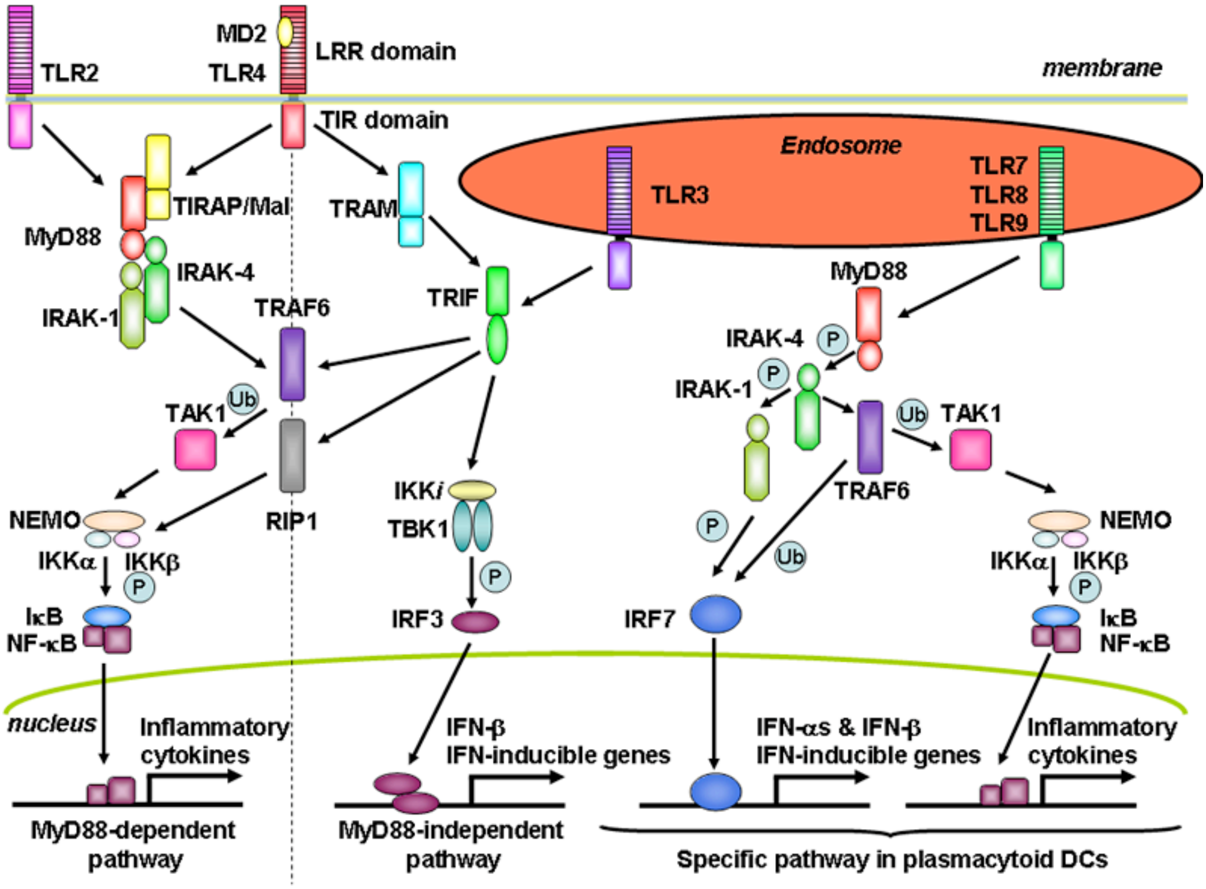


Figure 4: TLRs signaling pathway. Each TLR family member has its specific signaling pathway. MyD88-dependent pathway is characteristic of all the TLR family members except for TLR3. It is a common pathway to induce inflammatory cytokines. In addition, TLR3 and TLR4 possess TRIF-dependent pathway. TLR7, TLR8 and TLR9 also have a specific pathway to induce IFN-α in plasmacytoid DCs. (http://www.biken.osaka-u.ac.jp/act/act_akira_e.php)

As previously explained, TLRs are important in the initiation of the inflammation process in the skin, but also in others tissues. In fact, recognition of PAMPs by TLRs induces the production of proinflammatory cytokines and chemokines, which may increase the influx of leukocytes in the inflamed tissue. Moreover, in APCs, TLRs induce phagocytosis of antigens and their presentation to T lymphocytes.

TLRs are transmembrane receptors, with an extracellular leucin rich repeats (LRRs) domain and a cytoplasmic Toll/IL-1 receptor (TIR) domain, homologue to the IL-1 receptor. The TLRs signaling pathway consists in a myeloid differentiation factor 88 (MyD88) dependent pathway, common to all the TLRs, and a MyD88-independent pathway peculiar of TLR3 and TLR4 (Fig. 4). After ligand binding, TLRs undergo a conformational change required for the association with the MyD88 adaptor molecule. Next, MyD88 recruits IL-1 receptor-associated kinase (IRAK), which is activated by phosphorylation and then associates with TNF receptor-associated factor 6 (TRAF6). This leads to the activation of the nuclear factor-κB (NF-κB) pathway that eventually activates the proinflammatory cytokine gene transcription.

In addition to the NF-κB pathway, the JNK pathway and the interferon regulatory factor 5 (IRF-5) may also be activated by the MyD88 dependent pathway and modulate the proinflammatory cytokine gene expression (Not shown on Figure 4)[8].

The TIR domain-containing adaptor inducing IFN-beta (TRIF) dependent pathway is particular of TLR-3 and TLR-4 signaling. TRIF interacts with the receptor-interacting protein 1 (RIP 1) which is responsible of the activation of the NF-κB pathway[9]. On the other hand, TRIF may also activate TANK-binding kinase 1 (TBK1) and direct phosphorylate IRF-3 and IRF-7[10], which may be translocated in the nucleus and induce the expression of the type I IFNs genes. In plasmacytoid dendritic cells, it exists a specific pathway for TLR-7, TLR-8 and TLR-9. This signaling is MyD88 dependent and leads to the induction of IFN-α gene. In contrast with the induction of IFN-inducible genes by TLR-3 and TLR-4, this pathway is TRIF and TBK1 independent.

1.5 The nucleotide-binding oligomerization domain family

As previously discussed, microbial PAMPs are recognized by specific host PRRs, such as TLRs. The nucleotide-binding oligomerization domain receptors (NODs) are also members of the PRRs family. In contrast with TLRs, NOD family members are cytosolic soluble receptors in charge of the recognition of peptidoglycan (PGN) derived molecules not recognized by TLRs. NOD1 (which is encoded by the caspase recruitment domain 4 gene, *CARD4*) and

NOD2 (which is encoded by the caspase recruitment domain 15 gene, *CARD15*) are mainly expressed by APCs and mucosal epithelial cells. These cell types are exposed under physiological conditions to bacterial PGN and involved in the response to commensal organisms. Recent studies have also highlighted the involvement of *CARD15* in the pathogenesis of Crohn's disease, while *CARD4* was associated with inflammatory bowel disease[11].

NOD1 and NOD2 are composed of three main domains. The LRR domain, similar to the LRR of TLRs, which is involved in the ligand recognition, a central NOD domain, responsible of the self oligomerization and possessing an ATPase activity, and an amino-terminal domain composed of caspase recruitment domains (CARD), in charge of the initiation of the signaling (Fig 5)[12]. Although initial studies have reported lipopolysaccharide (LPS) as a NOD2 ligand, it is now clear that NOD1 and NOD2 detect respectively PGN derived γ-D-glutamyl-meso-diaminopimelic acid (iE-DAP) and PGN derived muramyl dipeptide (MDP). MDP is found both in Gram-positive and Gram-negative PGN. This designates NOD2 as a general sensor of most bacteria. In contrast, iE-DAP is a specific component of Gram-negative PGN, meaning that NOD1 mainly sense Gram-negative bacteria. The recognition of NOD1 and NOD2 ligands is done by the LRR domain, which consequently activates the NOD and CARD domain. Then, a CARD containing serine/threonine kinase (the receptor-interacting serine/threonine kinase, RICK) is recruited and physically associated with the CARD domain by a CARD-CARD interaction. In the case of NOD2, RICK activation leads to the polyubiquitylation of IKKγ, followed by phosphorylation of IKKβ and of IκB, resulting in the release of NF-κB. Finally, NF-κB may translocate to the nucleus, where it may act on the inflammatory gene transcription (Fig 5). The signaling pathway of NOD1 is not well studied. However, the activation of RICK by NOD1 also leads to the activation of NF-κB and to the modulation of the inflammatory genes transcription.

In addition to this signaling pathway, NOD1 and NOD2 may also activate the mitogen-activated protein kinases (MAPK) pathway by an as yet unknown mechanism.

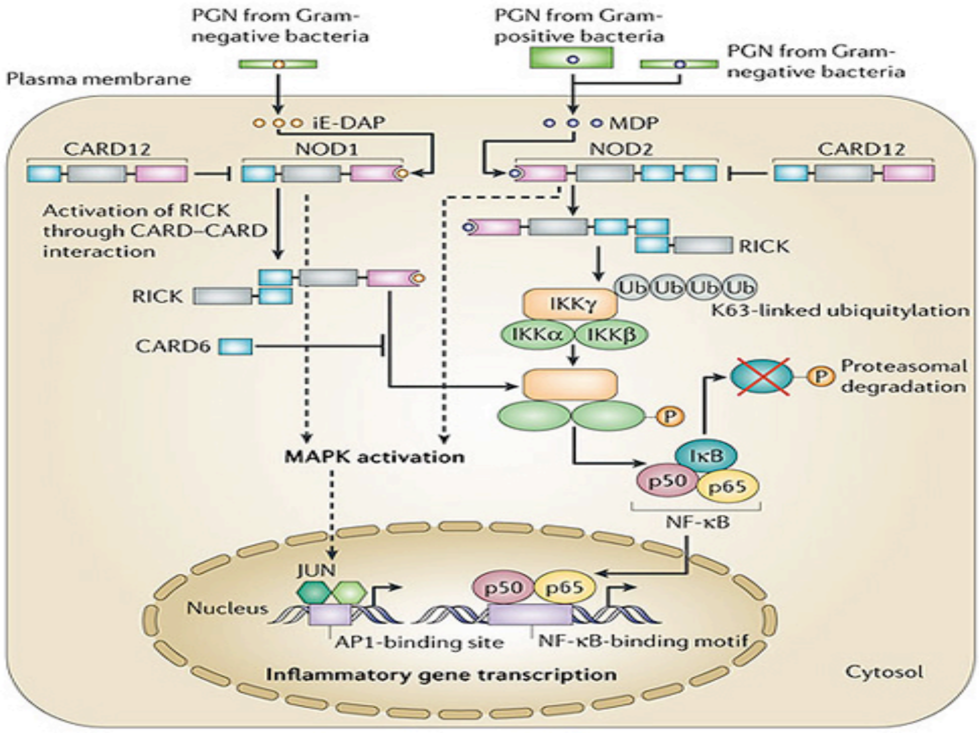


Figure 5: Signaling pathway of NOD1 and NOD2. PGN: Peptidoglycan; iE-DAP: γ-D-glutamyl-meso-diaminopimelic acid; MDP: muramyl dipeptide (Strober, W., Murray, P.J., Kitani, A., Watanabe, T., 2006, Signaling pathway and molecular interactions of NOD1 and NOD2, Nature Reviews Immunology, 6 (1), 9-20).

1.6 Cytokines

Cytokines are small water-soluble proteins and glycoproteins released mainly by leukocytes and which have an important role in the innate and adaptive immune response. In general, cytokines are involved in the regulation of three major homeostatic mechanisms: the innate immune response, the adaptive immune response and the growth and differentiation of hematopoietic cells. The structure of cytokines is varied and members similar in structure may have completely different effects. For instance, interleukin 10 (IL-10) and interleukin 12 (IL-12) possess the same structural motif, a long chain 4 α-helix, but their effects are opposite. In fact, IL-10 has anti-inflammatory activity, whereas IL-12 is a proinflammatory cytokine.

In general, cytokines and chemokines are not expressed under physiological conditions, but they are synthesized by activated leukocytes in response to a microbial or viral product. Moreover, the half-life of cytokines mRNA and proteins is very short. Therefore, after stimulation by pathogens a wave of cytokines is secreted, followed by the return to the resting condition in case of absence of a new stimulus. Cytokines generally exert their effects in a paracrine or autocrine manner and their effects depend on the expression of cytokine receptors on target cells. Frequently, cytokines possess pleiotropic or redundant effects. Furthermore, the expression of a cytokine may also induce or repress the expression of other cytokines and receptors, resulting in an enhanced or inhibited response to a stimulus. Hereunder, the properties of eight important cytokines, namely IL-4, IL-6, IL-10, IL-12, IL-17, IL-23, IFN-γ and TGF-β are discussed in detail.

a) IL-4

IL-4 is a pleiotropic cytokine mainly secreted by Th2 cells, but also by mast cells, basophils and Natural Killers T (NKT) cells. The IL-4 receptor is widely expressed and consequently IL-4 influences all cell types. This cytokine possesses antagonistic effects to IFN-γ on Th cells[13]. In fact, it promotes Th2 cells differentiation and growth, while inhibiting the Th1 development. Moreover, IL-4 (formerly know as B cell-growth-factor) supports also B cell growth and differentiation and it induces the upregulation of MHC class II and CD23. This promotes the development of the humoral immune response in charge of fighting against extracellular pathogens. IL-4 is also important for the antibody isotype switching to IgG1 and IgE while it inhibits the production of IgG2a. Consequently, IL-4 is involved in the allergic response by supporting the IgE-mediated mast cells degranulation. In general, IL-4 possesses anti-inflammatory properties and inhibits the production of proinflammatory cytokines, chemokines and adhesion molecules by macrophages. However, IL-4 may also activate a negative feedback mechanism that induces production of IL-12 in DCs and macrophages.

b) IL-6

Similarly to IL-4, IL-6 is a multifunctional cytokine, which is primarily secreted by mononuclear phagocytes, fibroblasts, vascular endothelial cells and some activated T cells in response to IL-1 and TNF. IL-6 plays a role in inflammation, adaptive immune response, hematopoiesis, nervous and endocrine mechanisms. IL-6 is also crucial for the acute phase response by inducing the production of acute phase proteins and stimulating pathogen clearance by neutrophils. Moreover, IL-6 supports the differentiation and maturation of B and T cells, macrophages, megakaryocytes, osteoclasts and some neurons. IL-6 receptor (IL-6R) consists in two subunits: IL-6Rα and gp130. IL-6Rα is in charge of the binding of IL-6 while the gp130 chain mediates signal transduction via Jak1, Jak2 and Tyk2. These kinases may recruit and activate various signal transducers and activators of transcription (STATs), which may translocate to the nucleus and activate additional IL-6-inducible genes implicated in the acute phase response. IL-6 has been reported to be implicated in many different diseases. For instance in chronic inflammatory bowel diseases, IL-6 is known to activate the STAT3 transcription factor, which results in a T cell resistance against apoptosis[14]. Moreover, in synovial fluid from rheumatoid arthritis patients IL-6 activity is significantly increased compared to control patients[15].

c) IL-10

IL-10 is an anti-inflammatory cytokine induced not only by pathogen invasion but also by the presence of others proinflammatory cytokines, such as IL-6, IL-12, TNF-α and type I IFNs. IL-10 is mainly secreted by activated monocytes, macrophages and Th2 cells but also by non-immune cells, such as keratinocytes and hepatocytes. The primary function of IL-10 is to counteract proinflammatory cytokines by inhibiting NF-κB-activated transcription of proinflammatory genes, notably of IL-1, IL-6, IL-8 and IL-12. Moreover, IL-10 promotes Th2 differentiation while inhibiting IFN-γ and IL-2 secretion. In addition, IL-10 inhibits the expression of MHC class II, B7-1 and B7-2 molecules on APCs cells further reducing the CD4+T cell response[1].

d) IL-12

IL-12 is the main proinflammatory cytokine secreted by activated macrophages and in a minor amount by neutrophiles, DCs, monocytes and B cells. IL-12 is composed of two subunits, p35 and p40, which is shared with IL-23. The main effects of IL-12 are: a) Promotion of the differentiation of naïve T cells in IFN-γ producing Th1 cells; b) Induction in synergy with IL-18 of the secretion of IFN-γ by activated Th1 cells and NK cells; c) Stimulation of the cytolytic activity of activated CD8+ and NK cells; d) Enhancement of the production of Ig2a associated with Th1 response while inhibiting the other Th2 isotypes. A deregulated production of IL-12 has also been associated with the development of Th1-mediated diseases, such as Crohn's disease and multiple sclerosis[16,17].

e) IL-17

IL-17 is exclusively produced by a subpopulation of activated memory CD4+ T cells and its expression is regulated by IL-23, IL-6 and TGF-β. IL-17 is well known for its proinflammatory effects, in particular it is involved in the induction of IL-6, IL-8 and G-CSF by keratinocytes, fibroblasts and epithelial cells[18]. Moreover, IL-17 mediated production of IL-8 has been associated with the recruitment of immune cells to peripheral tissues during inflammation. However, IL-17 does not seem to regulate T cells function. Recent studies have correlated overexpression of IL-17 with several proinflammatory mediated diseases in humans, such as rheumatoid arthritis, multiple sclerosis and asthma[19,20].

f) IL-23

IL-23 is a heterodimeric cytokine composed of a p19 subunit and a p40 subunit shared with IL-12. The majority of IL-23 is produced by activated DCs. However, there is also evidence of production of IL-23 by keratinocytes, which may contribute to the cutaneous inflammatory response[21]. Recent studies have also highlighted the implication of IL-23 in the differentiation of the newly discovered Th17 cells. In fact, it seems that IL-23, in combination with IL-6 and transforming growth factor beta (TGF-β), may enhance the development of IL-17 producing T cells, which are implicated in the clearance of extracellular pathogen and autoimmunity[22].

g) IFN-γ

IFN-γ is mainly produced by activated Th1 cells and in minor amounts by CTLs and NK cells. As the type I IFNs, IFN-γ possesses antiviral and antiproliferative activities. In fact, it is able to induce in target cells an antiviral state, which is characterized by the production of proteins able to interfere with virus replication. Moreover, IFN-γ may inhibit cell growth, restraining by this way the expansion of infected cells. In addition to these effects, IFN-γ is also able to activate other immune cells, such as macrophages, which increase their inflammatory cytokines production and pathogen elimination. Furthermore, IFN-γ may enhance MHC class I and II and B7 molecules expression on APCs promoting T cell activation. Moreover, it inhibits the production of IL-4 and IL-17 triggering Th1 development[13].

h) TGF-β

TGF-β exists in three distinct isoforms; TGF-β1, TGF-β2 and TGF-β3, derived from three distinct genes. TGF-β molecules are synthesized mainly by hematopoietic cells, such as T and B cells, DCs and macrophages, but also by some non-hematopoietic cells. TGF-β1 is the main product of these cells, but all three cytokines signal through the same receptor and possess practically an identical function. TGF-β molecules are pleiotropic and play a role in adhesion, proliferation, differentiation, transformation, chemotaxis and immunoregulation. In particular, TGF-β1 inhibits the proliferation of activated T cells and promotes the development of the regulatory T cells lineage (Treg), which is immunosuppressive. TGF-β1 inhibits also macrophages activation, Ig production, MHC class II expression and cytotoxic activities. Moreover, it may block the effects of proinflammatory cytokines and downregulates the production of IL-1, IL-2, IL-6, IFN-γ and TNF[23].

1.7 T-helper-cell lineage development

As previously mentioned, as soon as a pathogen succeeds in entering the body, an immune response is triggered. Antigens derived from the pathogen may be recognized by PRRs expressed on different cells types, which may be activated and increase their production of proinflammatory cytokines boosting the inflammatory reaction. Moreover, APCs, notably DCs, may also capture antigens and present them in association with MHC class I and II complexes to naïve T cells after migration to the draining lymph node. During migration, DCs become fully mature and increase the expression of adhesion molecules and their ability in presenting antigens. The upregulation of adhesion molecules is essential to establish strong interactions with naïve T cells and consequently to promote recognition of the antigen presented on the MHC complex[1].

Full activation of naïve T cells in lymph nodes by APCs requires three different signals[1]. The first signal is the binding of the antigen presented on MHC class I or II to the specific T cell receptor (TCR). A minimum threshold of TCR-MHC contacts has to be reached for activation of naïve T cells. These interactions induce an internal rearrangement of the T cell cytoskeleton, allowing the redistribution of the surface receptors nearby the interface between the two cells. This increases the probability of T cell and DCs contact. The second signal necessary for the full activation of naïve T cells is the binding of co-stimulatory molecules. These molecules are expressed on APCs and usually may decrease the threshold required for T cells activation or enhance the TCR-MHC signal. The most important co-stimulatory interaction is the CD28-B7 couple. B7 ligands are expressed on APCs and recognized by CD28 receptors expressed on T cells. The binding of B7 on the CD28 leads to the production of important cytokines, such as IFN-γ, IL-10 and IL-6. In addition, this binding induces certain signaling pathways, such as the MAP kinases pathway.

The third signal required is the binding of cytokines on the upregulated receptors on the now activated T cells. These cytokines are responsible for T cell survival, proliferation and finally differentiation in effector T cells.

The differentiation of T helper cells in Th1, Th2, Th17 or T regulatory (Treg) cells depend in fact on the cytokines milieu (Fig 6)[22,24].

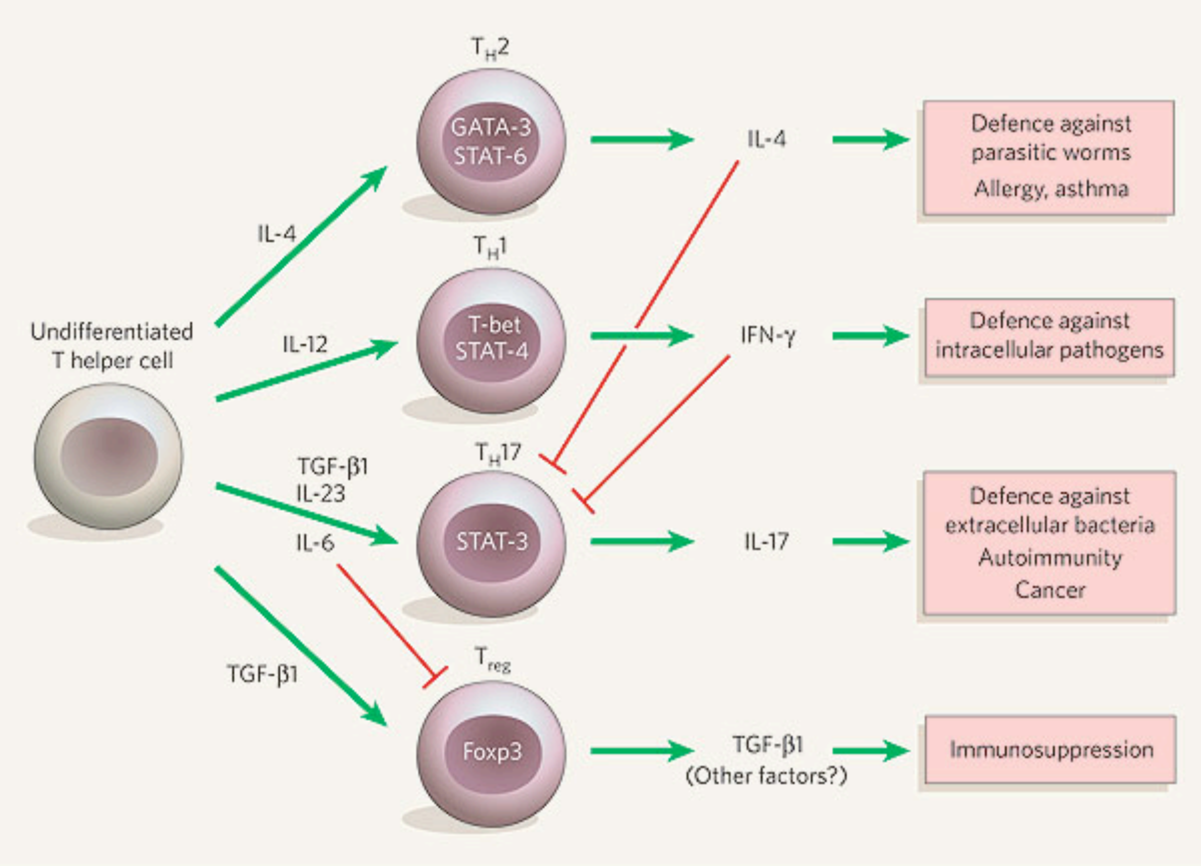


Figure 6: T helper cells differentiation. The differentiation of T helper cells in Th1, Th2, Th17 or Treg cells depend on the cytokines milieu. (Tato, C.M., O'Shea, J.J. 2006. What does it mean to be just 17? Nature 441, 166-168).

a) Th1 cells

Th1 cells develop from undifferentiated CD4+ T helper cells (Th0) in a IL-12-rich environment. The binding of IL-12 to its receptor on Th0 cells triggers intracellular signaling, which eventually induces the activation of STAT-4. This transcription factor may translocate to the nucleus, where it induces the expression of Th1 genes, including IFN-γ. Moreover, another transcription factor, “T-box expressed in T cells” (T-bet), is also induced and promotes the Th1 development inhibiting meanwhile Th2 cell differentiation by preventing the expression of GATA-3 transcription factor[25,26]. IFN-γ also contributes in the upregulation of T-bet transcription factor and in the inhibition of Th17 development. Th0 cells do not produce IL-12, therefore the cytokines milieu necessary for Th1 differentiation depends on the presence of activated APCs producing IL-12 close to the Th0 cells. Mature Th1 cells promote the defense against intracellular pathogen primarily by secreting cytokines in charge of sustaining the cellular adaptive immune response[1]. In particular, Th1 cells produce cytokines, especially IFN-γ and IL-2, essential for activation and proliferation of CD8+ T cytotoxic cells. These cells may recognize antigens, mainly presented on MHC class I complex, on target cells and induce their apoptotic death by Fas killing, cytotoxic granule release or secretion of cytotoxic cytokines. Moreover, IFN-γ and other cytokines secreted by Th1 lymphocytes improve the activation of macrophages, which increase their cytokines production and phagocytic ability. Th1 cells also supply the CD40L and IFN-γ necessary for the switch to the production of Ig isotypes implicated in the complement activation. It has also been demonstrated the involvement of Th 1 cells in the pathogenesis of organ specific autoimmune diseases such as rheumatoid arthritis and psoriasis[27].

b) Th2 cells

In contrast with Th1 cells, to differentiate Th2 cells need a IL-4 rich milieu. Binding of IL-4 to its receptor on Th0 cells induce the activation of STAT-6 via the Jak1 and Jak6 kinases. STAT-6 may upregulate the expression of another transcription factor, GATA-3, which is responsible of the inhibition of the expression of T-bet, IFN-γ and IL-12 receptor (IL-12R), consequently inhibiting the development of Th1 cells. Moreover, GATA-3 may also induce the expression of Th2 cytokines such as IL-4, IL-5, IL-10 and IL-13. It has been demonstrated that GATA3 may also be induced by CD28 signaling independently from STAT-6 and IL-4. However, it is still not clear how this signaling works[25,26]. Another transcription factor, important for Th2 differentiation, is c-maf. C-maf is expressed exclusively in maturing and in fully differentiated Th2 cells. This transcription factor works in association with the transcription factor c-jun inducing the synthesis of IL-4 meanwhile inhibiting the expression of IFN-γ and Th1 cells. IL-4 also inhibits the development of Th17 cells. The principal source of IL-4, essential for Th2 development, are activated mast cells and NKT cells present in the proximity of activated Th2 cells and Th2 cells themselves. Mature Th2 cells promote the defense against extracellular pathogens and parasites, mainly by inducing a humoral adaptive immune response. The cytokines secreted by Th2 cells sustain B cells activation and production of Th2 antibody isotypes like IgA, IgE and IgG1. These isotypes are less efficient in promoting complement activation but they may induce the neutralization of antigens without triggering the inflammation process. In addition, IL-5 produced by Th2 cells is also in charge of the activation of eosinophiles, which are crucial for parasite elimination. Others Th2 cytokines may also collaborate to regulate immune cells activation and proliferation. IL-3, IL-4 and IL-10 stimulate mast cells activation and proliferation. Moreover, IL-10 inhibits macrophage effector function, MHC II expression, IL-12 production, B7 expression, while increasing MHC class II on B cells. IL-4 may also increase the expression of MHC class II on APCs and simulate B cells proliferation while inhibiting, in collaboration with IL-13, proinflammatory cytokines production[1].

A defect in the balance of Th cells development in favor of Th2 cells has also been correlated with the pathogenesis of allergic diseases such as asthma, eczema and rhinitis[28].

c) Th17 cells

Th17 cells are a newly discovered lineage of effector CD4+ T cells characterized by the production of IL-17. Recent studies have demonstrated that TGF-β and IL-6 are crucial for the differentiation of naive T cells into Th17 cells. Moreover, IL-23 has also been involved in the proliferation of activated and memory Th17 cells but not of naïve T cells[18,29]. In fact, the IL-23 receptor is not expressed on Th0 cells but it is induced by TGF-β only on effector Th17 cells. TGF-β has also a dual function in the differentiation of Th cells. In fact, TGF-β alone induces the differentiation of Treg cells, whereas in combination with IL-6 it induces the development of Th17 cells, while inhibiting the expression of the Treg transcription factor Foxp3[30]. The differentiation of Th17 cells is independent of the transcription factors STAT-4 and STAT-6 which regulate the differentiation of Th1 and Th2 cells, but it involves the transcription factor STAT-3. STAT-3 is activated by both IL-6 and IL-23[31]. The binding of these interleukins on their receptors on naïve T cells induces phosphorylation of STAT-3, which may translocate to the nucleus and promote the expression of IL-17 gene. The action of TGF-β, IL-6 and IL-23 is antagonized by IFN-γ and IL-4 produced during the Th1 and Th2 development respectively. It has been demonstrated that Th17 cells are involved in the immune response against extracellular bacteria and parasitic worms. However, IL-17 has been associated also with autoimmune disease and cancer[29,32]. Some studies have demonstrated the ability of preventing autoimmune disease in mice by treatment with antibody anti IL-17.

d) Treg cells

Treg cells are a particular subset of Th cells in charge of controlling the immune response and establishing self-tolerance by immunosuppression. CD4+CD25+ Treg cells are a well known Treg subpopulation constitutively expressing the IL-2Rα chain (CD25). They develop from Th0 cells in case of antigen presentation by immature DCs and under a TGF-β microenvironment and are characterized by the expression of the transcription factor Foxp3. After activation, CD4+CD25+ Treg cells do not proliferate and do not secrete significant amount of cytokines. In contrast, they are able to suppress the proliferation and the IL-2 production of other effector T cells in a non specific manner by direct intercellular contact. However, the surface molecules engaged in this process have not yet been characterized. It has also been demonstrated that humans and mice lacking the Foxp3 transcription factor lack Treg cells and develop more autoimmune diseases than wild type animals[33].

2. Neuroimmunomodulation

The nervous system and the immune system are two major adaptive system of the body. For many years the immune system was thought to be autonomous and independent from the influence of other systems. However, during the last 30 years, researchers have provided strong evidence of a cross-talk between these two systems primarily via the autonomic nervous system (ANS) and the hypothalamic-pituitary-adrenal axis (HPA). The influence of adrenergic agents on lymphocytes proliferation[34] and the effect of inflammatory cytokines on plasma corticosteroids level[35] may be considered as milestones in the field. Later, other studies have provided evidence of an altered immune function following classical behavioural conditioning[36] and stressful stimuli[37,38].

2.1 The sympathetic nervous system

The ANS is independent of the conscious control and in vertebrates innervates practically all tissues and organs, except skeletal muscle fibers. It is divided in three components: the sympathetic nervous system (SNS), the parasympathetic nervous system (PANS) and the enteric nervous system, which is related to the gastrointestinal tract and regulates intestinal functions. The enteric nervous system is also modulated by the SNS and the PANS. The SNS is composed of preganglionic and postganglionic sympathetic fibers. The former originate in brain nuclei and leave the central nervous system via the thoracic and lumbar spinal nerves to terminate in para- and pre-vertebral ganglia. The latter originate in ganglia and innervate the tissues. Preganglionic fibers are cholinergic fibers, which release acetylcholine (ACh) in the ganglia. On the contrary, postganglionic fibers are noradrenergic fibers, which release catecholamines (CAs), predominantly norepinephrine (NE) (Fig. 7). Another component of the sympathetic nervous system is the adrenal medulla. As the sympathetic ganglia, the adrenal medulla originates from the neural crest and is innervated by cholinergic fibers. However, the main product released by this organ is epinephrine (E), which has systemic effects, and just to a lesser extent NE. In the parasympathetic nervous system both pre- and post-ganglionic fibers release ACh[39].

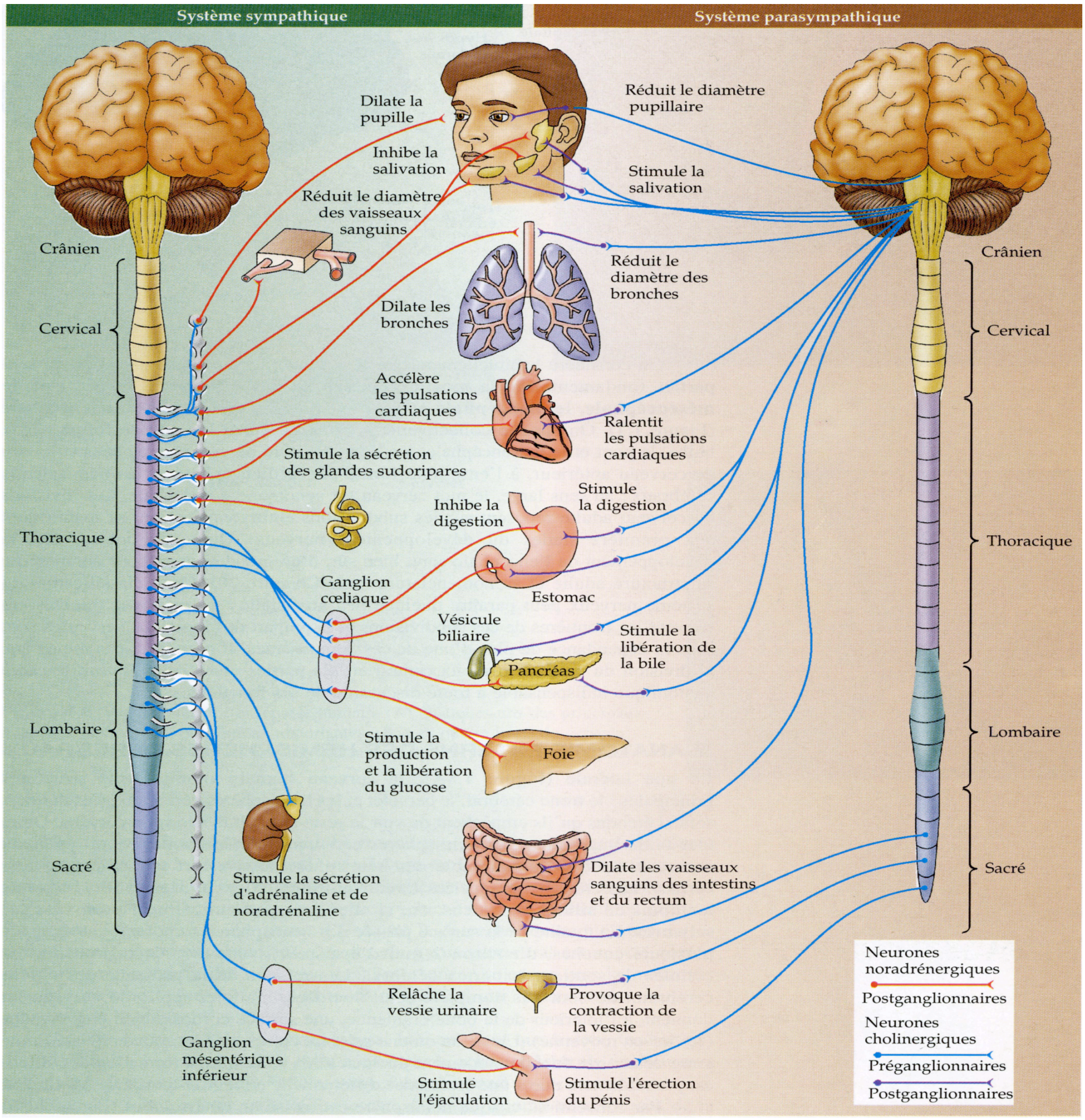


Figure 7: The autonomic nervous system. The sympathetic nervous system is composed of preganglionic cholinergic fibers and postganglionic noradrenergic fibers. Opposite, the parasympathetic nervous system is composed of pre- and post-ganglionic cholinergic fibers. The enteric nervous system is not represented. (Purves, D., Augustine, G.J., Fitzpatrick, D., Katz, L.C., LaMantia, A.S., McNamara, J.O., 1999. Neurosciences, DeBoeck Université).

The synthesis of NE takes place in the noradrenergic ending, where tyrosine is transported by a sodium dependent carrier. At that place tyrosine is converted first in dihydroxyphenylalanine and next in dopamine (DOPA) by the enzymes tyrosine hydroxylase and DOPA decarboxylase, respectively. DOPA is then transported in the synaptic vesicles, where it is converted to NE by the dopamine-β-hydroxylase. In the adrenal medulla, NE is further transformed in E by the phenylethanolamine N-methyltransferase enzyme.

NE and E mediate their effects through the adrenergic receptors (ARs). ARs are seven-chain-transmembrane receptors belonging to the class of G-protein coupled receptors. They are subdivided in three major types: α1- α2- and β-ARs. α1–ARs are further divided in the $\alpha1_A$, $\alpha1_B$, and $\alpha1_D$ subtypes; α2-ARs in the $\alpha2_A$, $\alpha2_B$ and $\alpha2_C$ subtype and finally β–ARs are subdivided in β1, β2 and β3 subtypes.

The affinity of NE and E for these receptors varies depending on the subtype considered (Table 2).

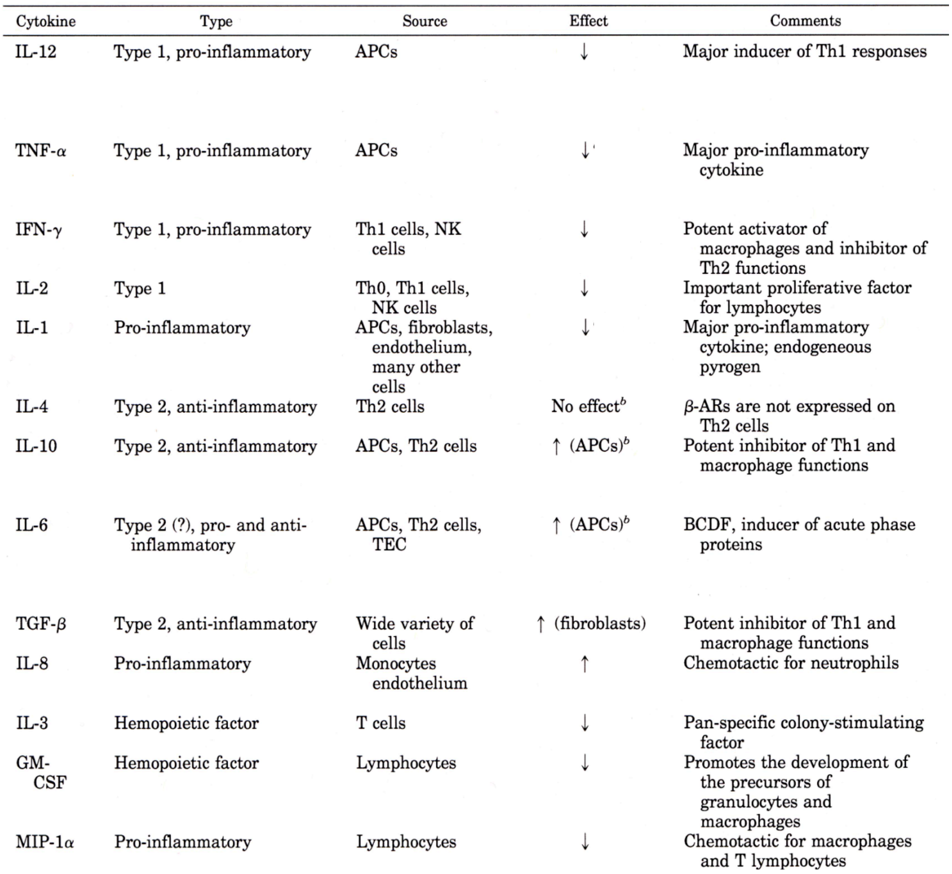
Table 2: Subdivision of adrenergic receptors and their transduction mechanism

Receptor subtype	Agonist potency order	Transduction mechanism	Agonist	Antagonist
α1	NE ≥ E	Activates $G_{p/q}$, ↑IP_3, ↑ Ca^{2+}	Phenylephrine, Metoxamine, Cirazoline	Phenoxybenzamine, Phentolamine, Prazosin, Tamsulosin, Terazosin
α2	E ≥ NE	Activates $G_{i/o}$, inhibition adenylate cyclase, ↓ cAMP	Clonidine, Lofexidine, Xylazine, Tizanidine, Guanfacine	Yohimbine
β1	NE ≥ E	Activates G_s, activation adenylate cyclase, ↑ cAMP	isoprenaline, dobutamine	Metoprolol, atenolol, betaxolol
β2	E > NE	Activates G_s, activation adenylate cyclase, ↑ cAMP	Salbutamol, Bitolterol mesylate, formoterol, isoproterenol, levalbuterol, metaproterenol, salmeterol, terbutaline, ritodrine	Butoxamine, ICI 118,551
β3	NE > E	Activates G_s, activation adenylate cyclase, ↑ cAMP	BRL-37344	bupranolol

NE: norepinephrine; E: epinephrine; $G_{s/i/p/q}$: G proteins; IP_3: inositol 1, 4, 5-triphosphate; cAMP: cyclic adenosine 5'-monophosphate. (Elenkov, I.J., Wilder, R.L., Chrousos, G.P., Vizi, E.S., 2000. The sympathetic nerve- An integrative interface between two supersystems: the brain and the immune system, Pharmacological Reviews, 52:595-638).

Primary and secondary lymphoid tissues and organs are innervated extensively by the sympathetic nervous system[40]. In general, nodular and follicular zones of developing and maturing B cells are less innervated than the zones characterized by a high concentration of T cells, macrophages and plasma cells. It has also been demonstrated that a wide range of immune and non-immune cell types express ARs. In particular, β2-ARs are expressed by Th1, DCs, neutrophils, keratinocytes and melanocytes but not Th2 cells[41,42]. The effects of CAs on lymphoid cells via β-ARs may vary depending on the dose, timing of treatment, target cells or density of ARs. Several studies have demonstrated the modulation of cytokine production and Th development by CAs through β-ARs signaling (Table 3).

Table 3: β-ARs mediated effects of CAs on cytokine and chemokine production



Cytokine	Type	Source	Effect	Comments
IL-12	Type 1, pro-inflammatory	APCs	↓	Major inducer of Th1 responses
TNF-α	Type 1, pro-inflammatory	APCs	↓	Major pro-inflammatory cytokine
IFN-γ	Type 1, pro-inflammatory	Th1 cells, NK cells	↓	Potent activator of macrophages and inhibitor of Th2 functions
IL-2	Type 1	Th0, Th1 cells, NK cells	↓	Important proliferative factor for lymphocytes
IL-1	Pro-inflammatory	APCs, fibroblasts, endothelium, many other cells	↓	Major pro-inflammatory cytokine; endogeneous pyrogen
IL-4	Type 2, anti-inflammatory	Th2 cells	No effect[b]	β-ARs are not expressed on Th2 cells
IL-10	Type 2, anti-inflammatory	APCs, Th2 cells	↑ (APCs)[b]	Potent inhibitor of Th1 and macrophage functions
IL-6	Type 2 (?), pro- and anti-inflammatory	APCs, Th2 cells, TEC	↑ (APCs)[b]	BCDF, inducer of acute phase proteins
TGF-β	Type 2, anti-inflammatory	Wide variety of cells	↑ (fibroblasts)	Potent inhibitor of Th1 and macrophage functions
IL-8	Pro-inflammatory	Monocytes endothelium	↑	Chemotactic for neutrophils
IL-3	Hemopoietic factor	T cells	↓	Pan-specific colony-stimulating factor
GM-CSF	Hemopoietic factor	Lymphocytes	↓	Promotes the development of the precursors of granulocytes and macrophages
MIP-1α	Pro-inflammatory	Lymphocytes	↓	Chemotactic for macrophages and T lymphocytes

b: CAs are probably not able to affect the production of type 2 cytokines by Th2 cells directly, simply because they do not express β-ARs. Indirectly, however, they may potentiate the cytokine production by Th2, since they remove the inhibitory restraints on these cells exerted mainly by IL-12 and IFN-γ. BCDF: B cell differentiation factor; TEC: Thymic epithelial cells (Elenkov, I.J., Wilder, R.L., Chrousos, G.P., Vizi, E.S., 2000. The sympathetic nerve- An integrative interface between two supersystems: the brain and the immune system, Pharmacological Reviews, 52:595-638).

In particular, it has been reported that NE and E inhibit the production of the proinflammatory cytokine IL-12 by monocytes and DCs via β-ARs *in vitro* and *in vivo*[43,44]. Furthermore, β-

ARs have also been reported to inhibit the development of Th1 cells, while promoting Th2 cells differentiation[42]. Others proinflammatory cytokines, notably IL-1 and TNF-α, are also inhibited by β-ARs stimulation, while the production of Th2 cytokines, such as IL-10, is upregulated[45]. This provides evidence of an anti-inflammatory effect of CAs and of their ability in switching the adaptive immune response from the Th1 to the Th2 type. Interestingly, IL-6, a cytokine of the acute phase response and responsible in collaboration with TGF-β of the Th17 development, is also upregulated by CAs[46]. This may indicate an involvement of the SNS not only in the Th1/Th2 development but also in the induction of the Th17 differentiation.

2.2 The hypothalamic-pituitary-adrenal axis

The HPA axis is composed by the hypothalamus, a brain region, and by two endocrine glands, the pituitary gland and the adrenal cortex. Exposure to proinflammatory stimuli as well as to physical or psychological stress results in activation of the HPA axis and, as a final consequence, in the release of glucocorticoids, cortisol in humans and corticosterone in mice. After stimulation, neurons originating in the paraventricular nucleus of the hypothalamus secrete the corticotropin-releasing hormone (CRH) in the hypophysial portal circulation. Next, CRH stimulates the secretion of the adrenocorticotropic hormone (ACTH) by the anterior pituitary gland. Then, ACTH acts on the adrenal cortex stimulating the release of endogenous glucocorticoids (Fig. 8).

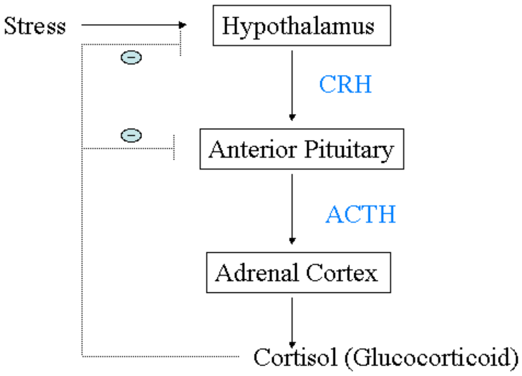


Figure 8: HPA axis. The secretion of CRH by the hypothalamus induces the release of ACTH by the pituitary anterior gland. This stimulates the secretion of glucocorticoids by the adrenal cortex. CRH: corticotropin-releasing hormone; ACTH: adrenocorticotropic hormone.

Glucocorticoid receptors (GRs) are cytosolic steroids receptors, which translocate to the nucleus upon activation by ligand binding. In the nucleus, activated GRs may modulate directly the glucocorticoid sensitive gene or interact with NFκB or AP-1 transcription factors. GRs are widely expressed throughout the body; therefore glucocorticoids have effects on practically every system. In particular, they possess modulatory properties on the immune system. It has been demonstrated that *in vivo* injection of dexamethasone, a synthetic glucocorticoid, impairs the production of proinflammatory cytokines, notably IL-1β and TNF, by thymic DCs[47]. Moreover, dexamethasone inhibits the LPS induced production of IL-12 by human monocytes[48]. Opposite, glucocorticoids have been reported to improve the production of anti-inflammatory cytokines, such as IL-10 and IL-4, by dendritic cells and macrophages[49]. Cell adhesion molecules and chemokines are also inhibited by glucocorticoids, therefore reducing the immune cell trafficking at the site of inflammation[50]. Together, these results provide evidence of anti-inflammatory and immunosuppressive properties of glucocorticoids. An altered function of the HPA axis has been associated with many different diseases. In humans, rheumatoid arthritis, irritable bowel disease and fibromyalgia are all characterized by an augmented immune response associated with an impaired HPA-axis function. On the other hand, excess in HPA-axis activity, often due to chronic stress, results in an increase susceptibility to infections and prolonged wound healing.

3. Aim of the study

Previous studies performed in our laboratory reported that NE may affect LCs migration and exert a chemotactic action on bone marrow derived DCs. This effect was mediated by the $\alpha 1$-ARs and may influence the contact hypersensitivity response (CHS)[51]. Further studies in our laboratory provided evidence of the influence of the local sympathetic nervous system on the Th differentiation in response to an antigen. In fact, these studies demonstrated that NE inhibits the LPS induced IL-12 production by DCs, while increasing the production of the anti-inflammatory cytokine IL-10. The NE effect was mediated by β- and $\alpha 2$-ARs. Moreover, adoptive transfer of NE exposed DCs promote a Th2 differentiation[52].

These results have been confirmed by other authors. Seiffert et al. have confirmed that E and NE could inhibit LCs antigen presentation during CHS. This effect was removed by the use of the $\beta 2$-ARs antagonist ICI 118,551. In addition, they showed that LCs express $\alpha 1_A$- and β-ARs mRNA[53]. Recently, other studies in our laboratory showed that LCs migration and CHS response are increased in mice treated with the $\beta 2$-ARs antagonist ICI 118,551 during FITC sensitization[54]. Moreover, this study demonstrated that maturing bone marrow DCs express $\beta 1$-, $\beta 2$-, $\alpha 2_A$-, and $\alpha 2_C$-ARs genes and that $\beta 2$- and $\alpha 2_A$-ARs are involved in the reduced production of IL-12, while only $\beta 2$-ARs stimulated IL-10. Most recently, it has also been demonstrated an involvement of $\beta 2$-ARs in dendritic cells based cancer vaccine. Adoptive transfer of immature DCs loaded with ovalbumin (OVA) after intradermal injection of PGN along with a $\beta 2$-ARs antagonist, increased the antitumor response and significantly reduced the tumor growth. This effect was mediated by an increased DCs migration to the draining lymph node, which resulted in a modulation of the OVA specific cytotoxic T lymphocyte response[55].

In summary these findings suggest a sympathetic nervous regulation of the innate and the adaptive immune response via β-ARs. The local release of catecholamines may influence DCs antigen presentation ability, cytokines production and consequently Th cell priming properties.

On the basis of the previous studies, the aims of this project are:

a) To investigate *in vivo* the possible influence of β-ARs blockade on the pro- and anti-inflammatory cytokine production upon TLRs activation in mice skin.

b) To investigate whether the possible β-ARs effects on cytokine production may affect DCs migration to the draining lymph node and the consequent Th priming during the adaptive immune response to a soluble protein.
c) To detail in *vitro* the modulation of the DC cytokines production by NE upon different PRRs activation.
d) To test the effects of salivary glands extract (EGS) from the tick *Ixodes ricinus* on pDCs migration.

II. Materials and methods

Mice

Balb/c (H-2^d) and C57/Bl6 (H-2^b) inbred mice were purchased from Harlan, Udine, Italy. β2-AR gene deficient (β2ko) mice (H-2^q) were a generous gift of Prof. Brian Kobilka, Stanford University, USA. All mice were maintained in our animal colony under specific pathogen-free conditions, food and water ad libitum and under a standard 12 hours photoperiod at a constant temperature of 21°C. Six- to ten-week-old female mice were used for all experiments. All experiments were authorized by the local veterinary authority.

Skin treatment and drugs

Mice were injected intradermally in the shaved back with a β-adrenergic antagonist. Notably, propranolol (PRO, β-adrenergic antagonist, 5μg/mouse, Sigma, St. Louis, USA) and ICI 118,551 hydrochloride (ICI, β2-adrenergic antagonist, 5μg/mouse, Tocris bioscience, Ellisville, USA) were used. Control mice were injected with phosphate buffered saline alone (PBS 1X pH 7.2, 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na2HPO4, 1.47 mM KH2PO4). The skin was collected 3 hours after the injection and stock overnight at 4°C in RNA*later* (Ambion Inc., Austin, USA).

Epidermal and dermal sheet separation

In order to evaluate a possible different TLRs gene expression between derma and epidermis, skin from Balb/c mice was cut into 3-5 mm wide ribbons and incubated in dispase 5U/ml (Roche Diagnostics, Basel, Switzerland) supplemented with 0.375% trypsin (Sigma) for 1 hour at 37°C. The dorsal part, corresponding to the epidermal sheets, was peeled away using a sterile forceps. Both, epidermal and dermal sheets, were washed with PBS 1X pH7.2 and then stocked in RNA*later* at 4°C.

Cells

Bone marrow cells from C57/Bl6 (H-2^b) mice were cultured in 10cm petri dishes at 0.25.10^{6}cells/ml in 10ml complete culture medium: RPMI 1640 (Gibco, Karlsruhe, Germany) supplemented with 10% (v/v) heat-inactivated foetal calf serum (FCS, Bioconcept, Allschwil, Switzerland), 2mM L-glutamine (L-Glut, Bioconcept), 100U/ml Penicillin (Bioconcept), 100μg/ml Streptomycin (Bioconcept), 25mM Hepes buffer (Gibco), 50μM 2-mercaptoethanol (2-ME, Sigma) and 30ng/ml GM-CSF (ReliaTech Gmbh, Braunschweig, Germany). On day

2, 10ml of fresh medium was added. On day 4 and 7, 10ml of medium was replaced with fresh culture medium. DCs were used on day 9.

XS52 cell line is a long-term Langerhans cell-like cell line established from newborn epidermis. The XS52 cells are cultured in complete culture medium: RPMI 1640 (Gibco), supplemented with 10% (v/v) heat-inactivated FCS (Bioconcept), 2mM L-Glut (Bioconcept), 100µM nonessential amino acids (Gibco), 100U/ml Penicillin (Bioconcept), 100µg/ml Streptomycin (Bioconcept), 10 µM sodium pyruvate (Gibco). Finally, 10% (v/v) culture supernatant of a NS47 stromal cell line, cultured in complete RPMI as described above, was added.

Cells sorting by MACS

Dendritic cells from *in vitro* cultures were selected by CD11c positivity using a magnetic activated cell sorting (MACS) system (Miltenyi Biotech, Bergisch Gladbach, Germany). Briefly, non-adherent cells and loosely adherent proliferating aggregate were collected and washed using the buffer MACS (PBS1X pH7.2, 2mM EDTA, 1% FCS). Cell number was determined and cells were resuspended in 80µl Buffer MACS and 20µl CD11c (N418) MicroBeads (Miltenyi Biotech) per 10.10^6 cells and incubated 15 minutes at 8°C. Cells were further washed and resuspended in 500µl Buffer MACS. Cell suspension was applied onto a LS Column (Miltenyi Biotech) placed in a magnetic field of a MACS separator. The column was washed three times with buffer MACS to collect the negative cell fraction. To collect the positive CD11c cell fraction, the column was removed from the magnetic field and placed on a collection tube. 5 ml Buffer MACS were pipetted onto the column and the magnetically labelled cells were eluted. To increase the purity of the enriched CD11c+ cell population, the magnetic separation procedure was repeated using a new column. An aliquot of purified cells was analyzed by flow cytometry to assess the percentage of CD11c+ cells. Only populations with more than 95% CD11c+ cells were used for *in vitro* experiments.

In vitro experiments

DCs, sorted by MACS system as described above, were incubated at 10^6 cells/ml in a 24-well culture plate with a TLR ligand in presence or absence of norepinephrine (NE, Fluka, Buchs, Switzerland). Notably, 10µg/ml peptidoglycan (PGN, Fluka), 2µg/ml muramyl dipeptide (MurNAc-L-Ala-D-iso-Gln, MDP, NOD2 agonist, NeoMPS, Strasbourg, France), 10µg/ml N-Palmitoyl-S-[2,3-bis(palmitoyloxy)-(2RS)-propyl]-[R]-cyteine (PAM, TLR-2 agonist, EMC microcollections Gmbh, Tuebingen, Germany), 1μg/ml lipopolysaccharide (LPS Sigma), a combination of PAM and MDP (10μg/ml PAM+ 2μg/ml MDP) and 10^{-6} M NE were used. In some experiments, 20μg/ml of the purified antibody anti-mouse IL-10 (eBioscience, San Diego, USA) were also added. Cells were collected 3 hours later for the analysis of gene expression by Real Time RT-PCR, whereas cell supernatants were collected 6 hours later for the protein quantification by ELISA.

Total RNA isolation and purification

Mice skin was frozen in liquid nitrogen and then pulverized using a pre-chilled mortar and pestle. The powder was collected in Lysis/Binding Solution (RNAqueous^{TM} Kit, Ambion Inc.) and homogenized by an electronic rotor-stator homogenizer (Polytron, Kinematica AG, Luzern, Switzerland). Alternatively, cells were collected in Lysis/Binding solution and homogenized by passing the lysate through a 25-gauge syringe needle. RNA extraction was performed following the kit instruction. Total RNA was further purified using the Turbo DNA-free kit (Ambion Inc.). The RNA concentration was quantified by ND-1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, USA).

cDNA Synthesis

0.5μg of total RNA from tissue samples or 1μg total RNA from cell samples, were reverse transcribed in a final volume of 100μl in the following reaction mixture: 5x First-Strand Buffer (Invitrogen, Carlsbad, USA), 10μM DTT (Invitrogen), 40 units RNase Inhibitor (Perkin Elmer, Waltham, USA), 500μM dNTP (Sigma), 300pM Random Hexamer pd$(N)_6$ (Pharmacia Biotech., Uppsala, Sweden), 400 units SuperScript II RT(Invitrogen). The tubes were incubated in the Peltier thermal cycler PTC-200 (MJ Research, Waltham, USA) at the following thermal conditions: 25°C for 10 minutes, 37°C for 60 minutes, 99°C for 5 minutes. An aliquot of each cDNA sample was used to perform a control PCR.

PCR (polymerase chain reaction)

5μl of cDNA was added to the following reaction mixture: 10X PCR Buffer II (Applied Biosystem, Foster City, USA), 3mM $MgCl_2$ (Applied Biosystem), 320μM dNTP, 500nM primer forward and reverse, 1.25 units AmpliTaq Gold (Applied Biosystem) in a final volume of 50μl. The tubes were incubated in the Peltier thermal cycler PTC-200 at the following conditions: 95°C for 10 minutes, 30 cycles: 93°C for 45 seconds, 55°C for 60 seconds, 72°C for 45 seconds and 72°C for 10 minutes.

The primers used for the control PCR correspond to the HPRT housekeeping gene (Sigma) and their sequence is: Down: 5'-CGAGAGGTCCTTTTCACCAGC, Up: 5'-GATTATGGACAGGACTGAAAG.

Agarose gel electrophoresis

Electrophoresis was performed on a horizontal gel at 1.8% agarose (peqGold Universal Agarose, PeqLab biotechnologies Gmbh, Erlangen, Switzerland) in TBE 0.5X buffer. 10μl of PCR products were loaded in the wells after addition of 2μl of loading buffer (0.25% bromophenol blue, 40% sucrose in water, PeqLab biotechnologies Gmbh). Electrophoresis was run for 5 minutes at 22V and for 60 minutes under a constant voltage of 100V. DNA fragments were visualized by staining the gel in an ethidium bromide solution for 10 minutes and by placing the gel on an ultraviolet transilluminator (Image Master VDS, Pharmacia Biotech). The size of the fragments obtained was evaluated by comparison to the 50bp DNA-Leiter marker (PeqLab biotechnologies Gmbh). The size of the fragment obtained using the HPRT housekeeping gene primers was of 389bp.

Semi-quantitative Real Time RT-PCR

The PCR reaction was carried out using the SensiMix DNA kit (Quantance, London, UK) in a final volume of 25μl by adding 5μl of cDNA to the following reaction mixture: 2X SensiMix (dU), 4mM $MgCl_2$, 0.5 Units UNG, 20X TaqMan probe (Applied Biosystems). Amplification of 18s rRNA was done for each sample as endogenous control of the amount and quality of total RNA added to each reaction. All samples were amplified in duplicate. The relative quantification of gene expression was done in the Rotor gene 2000 (Corbett Research, Sydney, Australia) by setting the thermal cycling conditions according to the manufacturer instructions and using the Rotor Gene Software to determine the threshold cycle (Ct value). The formula to determine the arbitrary units is $2^{-\Delta\Delta Ct}$, where ΔCt is the target gene Ct value minus the 18s Ct value and the $\Delta\Delta Ct$ is the ΔCt of the treated sample minus the ΔCt of the control sample. Results are presented as the gene expression fold increase between treated samples and control. The arbitrary thresholds of 2-fold increase and 0.5-fold increase were chosen as biologically significant.

ELISA (enzyme linked immunosorbent assay)

To quantitate protein production of IL-6, IL-10, IL-12, IL-17, IL-23 and IFN-γ in culture supernatant, the Ready-Set-Go! kit (eBioscience) was used. Briefly, a NUNC Maxisorp 96 (NUNC, Roskilde, Danemark) wells ELISA plate was coated with capture antibody diluted 1/250 in Coating buffer overnight at 4°C. The following day, the plate was washed extensively with Wash Buffer (PBS 1X, 0.05% Tween-20) and blocked with 1X Assay Diluent (eBioscience) for 1 hour at RT. Afterwards, appropriately diluted standards and samples were added to the plate and incubated for 2 hours at RT. Next, the Detection Antibody diluted 1/250 in 1x Assay Diluent was added to the wells for 1 hour at RT. The plate was then incubated with the Avidin-HRP enzyme diluted 1/250 in 1x Assay Diluent for 30 minutes. Finally, the substrate solution was added to each well and after 15 minutes the reaction was stopped by adding 1M H_3PO_4. After each step, the plate was washed extensively with the Wash Solution. Optical density was determined at 450nm in MRX Microplate Reader (Dynex Technologies Inc., Chantilly, UK).

Role of the lymphatic conduit system

To study whether reagents injected intradermally could reach the draining lymph node by entering directly the lymphatic vessels, mice were injected i.d. with 9μg/mouse of PGN in presence or absence of 2.5μg/mouse of PRO in the pinna of the ear using an Hamilton syringe fitted with a 30.5 gauge needle. Control mice were injected with the same volume of PBS1X. Three hours later, 5μg/mouse of ovalbumin conjugated to Alexa Fluor 488 (OVA-Alexa 488, Invitrogen) was also injected in the same site. In some experiments the site of injection was surgically removed 5 hours after the pretreatment. To reduce the pain associated with the procedure, mice were given an intraperitoneal injection of a cocktail of xylazine (0.2mg/mouse, Rompun 2%, Bayer AG, Leverkusen, Germany) and ketamin (1.5mg/mouse, Ketalar, Pfizer, New York, USA) at the time of treatment and ear removal and the drinking water was supplemented with paracetamol (1.3mg/ml, Dafalgan, Bristol Myers SquibbCo., New York, USA). Twenty-four hours later the auricular draining lymph node was collected for single cell suspension preparation and FACS analysis.

DCs adoptive transfer and Th cell polarization

DCs, sorted as described above, were incubated at 10^6 cells/ml in a petri dish with a combination of the TLR-2 and the NOD2 agonists in presence of the soluble protein keyhole limpet hemocyanin (KLH, Calbiochem) and in presence or absence of the β2-AR agonist salbutamol (Sigma). Notably, 10μg/ml PAM, 5μg/ml MDP, 100μg/ml KLH and 10^{-6} M salbutamol were used. After three hours cells were collected and mice were injected in the right hint footpad with 10^6 cells. To asses the adoptive response to KLH in terms of cytokine production, mice were sacrificed 7 days later and cells from the draining popliteal lymph nodes were obtained and incubated in complete medium at 5. 10^5cells/250μl/well in presence of 100μg/ml of KLH at 37°C for 48h. The culture supernatants were then collected and the concentration of IFN-γ and IL-17 were quantified by ELISA.

Single cell suspension from lymph nodes

Draining lymph nodes were collected in 1ml RPMI 1640 supplemented with 5% FCS and digested with 0.5mg/ml of Collagenase A (Roche Diagnostic) and 40μg/ml of DNase I (Roche Diagnostic) for 10 minutes at 37°C. Lymph nodes were then gently teased and cells were filtered through a 40μm cell strainer (BD Falcon, Bedford, USA). Cells were washed by spinning 10 minutes at 4°C with PBS1x supplemented with 5% FCS, 5mM EDTA and 5μg/ml DNase I. The pellet was resuspended in PBS1x+ 1% FCS and cell number was counted using the Neubauer chamber (Paul Marienfeld Gmbh, Lauda-Königshofen, Germany)

Flow cytometry (FACS)

For all FACS analysis, 0.5. 10^6 cells were first incubated 5 minutes at 4°C with the purified antibody anti-mouse CD16/CD32 (BD Bioscience Pharmingen, San Jose, USA) to ensure blocking of Fc receptor. Afterwards, cells were labelled with phycoerythrine PE-conjugated anti CD11c antibody (BD Bioscience Pharmingen) or PE-conjugated m-PDCA-1 antibody mixture (Miltenyi Biotech) allowing a single color identification of CD11c+, CD45R+, B220+ and Ly-6C+, which identifie plasmacytoid dendritic cells (pDCs). The corresponding isotypes, PE conjugated Hamster IgG1, λ and PE conjugated rat IgG2b, κ respectively, were used as negative control.

Statistics

Differences were analyzed by the Statview software and the repeated measures ANOVA test.

III.

Manni, M., Maestroni, G.J., **Sympathetic nervous modulation of the skin innate and adaptive immune response to peptidoglycan but not lipopolysaccharide. Involvement of β-adrenoceptors and relevance in inflammatory diseases.** Brain Behav Immun. 2008 Jan; 22 (1):80-88.

3.1 Introduction

The skin is the largest organ of the body and plays a central role in host defense. The epidermis is composed of keratinocytes, which function both as physical barrier and early warning system. Immune cells of the epidermis include Langerhans cells and intraepithelial lymphocytes. The derma is composed of connective tissue produced by fibroblasts. Immune cells resident in the derma include dermal DCs, mast cells and cutaneous lymphocyte antigen-positive memory T cells. Recently, we and others reported that catecholamines may affect skin Langerhans cells and bone marrow derived dendritic cells (DCs) migration and antigen presenting ability via adreneoceptors (ARs), (Maestroni, 2000; Maestroni, 2002a; Maestroni and Mazzola, 2003). However, these studies primarily investigated contact hypersensitivity responses using contact allergens. No in vivo evidence was available about a possible adrenergic modulation of innate or adaptive responses elicited by pattern recognition receptors (PRRs) that recognize specific microbial components (Takeda et al., 2003). Besides DCs and immune cells, epithelial cells, endothelial cells and fibroblasts express PRRs and are important players in cutaneous infections and inflammatory diseases (Faure et al., 2000; Kang et al., 2006; Sandor and Buc, 2005). Most cells that may react to PRR ligands, including Langerhans cells, DCs and epidermis keratinocytes express also β-ARs (Chen et al., 2002; Elenkov et al., 2000; Maestroni, 2006). The importance of PRRs (in particular of TLRs) and β-ARs have been both suggested to play a role in skin inflammation. However, a possible interplay between TLRs and β-ARs has not been investigated. As far as it concerns TLRs the evidence of their important role in skin disorders such as psoriasis and atopic dermatitis is steadily growing (Kang et al., 2006). The TLR-induced responses may be dependent or independent on the adaptor protein MyD88. Current opinion views MyD88 as essential for inflammatory cytokine production via all TLRs, whereas TRIF is involved in TLR3- and TLR4-mediated MyD88-independent induction of IFN-β (Takeda et al., 2003). In particular, the TLR2 pathway activated by PGN, (Dziarski and Gupta, 2005b) is MyD88-dependent and should lead to NFkB activation and production of inflammatory cytokines only, while that of TLR4 that sense LPS, should lead to the production of both inflammatory cytokines and IFN-β (Takeda, 2005).

For the β-adrenergic system, a dysregulation of catecholamine biosynthesis with epidermis norepinephrine levels associated with high numbers of β2-ARs in differentiating keratinocytes has been reported in vitiligo (Schallreuter, 1997). In atopic eczema, a point mutation in the β2-AR gene could alter the structure and function of the receptor, thereby leading to a low density of receptors on both keratinocytes and peripheral blood lymphocytes (Schallreuter, 1997). In psoriasis, β-ARs are downregulated and, interestingly, β-AR blockers may cause psoriasiform, lichen planus-like, and eczematous "chronic" rashes (Halevy and Livni, 1993; Hodl, 1985; Steinkraus et al., 1993). Last but not least, the onset and course of dermatological disorders may be significantly influenced by stress, emotional disturbances, or psychiatric disorders (Chuh et al., 2006).

Here we present evidence that β-ARs may affect the skin response to peptidoglycan (PGN) from *S. Aureus* but not to LPS from *E. Coli*. β−ARs inhibition and PGN injection before primary immunization with ovalbumin (OVA) dissolved in saline resulted in a Th1 shift of the recall memory response. This effect evidently depended on enhanced local expression of inflammatory cytokines as well as of IFN-β and CXCR3 ligands, which were associated with an increased number of pDCs in the draining lymph node. Both β1- and β2-ARs inhibition was apparently involved in the observed effects,

3.2 Methods

Mice

BALB/c (H-2^d) inbred mice were purchased from Harlan, Italy and transgenic DO11.10 (H-2^d) mice, expressing on 90% of CD4+ T cells a T cell receptor specific for the OVA peptide 323-339, were a generous gift of Dr. Martin-Fontecha, Institute of Research in Biomedicine, Bellinzona, Switzerland. All the mice used in the experiments were female, 2-4 months old and were maintained in our animal room under a standard 12 h photoperiod, at 21 ± 1°C, with food and water ad libitum. All experiments were authorized by the local veterinary authority.

Real time RT-PCR

We measured the mRNA coding for TNF-α, IL-1α, IL-7, IL-12, IL-18, IL-23, IFN-γ, IL-10 and the chemokines CCL-1, CCL-2, CCL5, CCL27. Groups of mice were injected intradermally (i.d.) with PGN or LPS ± the non-selective β-ARs antagonist propranolol (PRO). Total RNA isolated from skin samples was reverse transcribed using random hexamers and the TaqMan Reverse Transcription kit (Perkin-Elmer Applied Biosytems, Foster City, CA). A relative quantification of cytokine mRNA was done in a Rotor Gene 2000

(Corbett Research) using pre-developed reagents (Applied Biosystems, Foster City, CA, US). Amplification of 18S rRNA was done for each sample as endogenous control of the amount and quality of total RNA added to each reaction. Thermal cycling conditions were according to the manufacturer instructions. All samples were amplified in duplicate. Threshold cycle Ct, which correlates inversely with the target mRNA levels, was measured as the cycle number at which the reported fluorescence emission increased above a threshold level. The amount of inflammatory cytokine or IFN-β and CXCR3 ligands mRNA upon PGN or LPS injection was expressed as a n-fold difference relative to the amount of mRNA in controls injected with saline only. The formula used to determine this value is $2^{-\Delta\Delta Ct}$, where ΔCt is determined by subtracting the average 18S rRNA value from the average target Ct value. The calculation of ΔΔCt involves the subtraction of the ΔCt of control from the target ΔCt value.

Adoptive transfer and immunization

Brachial, axillary, inguinal and popliteal lymph nodes were collected from DO11.10 mice. The lymph nodes were teased and the cells were filtered through a 40 µm cell strainer (Falcon) to prepare a single cell suspensions in RPMI 1640 with 5% FCS. Lymph node cell suspensions from DO11.10 mice contained 40 ± 1.5 % of cells expressing the OVA specific T cell receptor as assessed by flow cytometry using PE-conjugated anti- KJ1-26 mAb (Caltag Laboratories Invitrogen, Basel, Switzerland) The cells were then centrifuged, resuspended in PBS and 7.5 x 10^6 lymph node cells, containing 3 x10^{6} transgenic KJ1-26+ cells, were injected i.v. into syngeneic BALB/c mice. 48 h later the mice were then injected intradermally (i.d.) in the shaved back with 100 µl of PBS containing 50 µg of S. Aureus peptidoglycan (PGN, Sigma, Co., St. Louis, USA) or 2 µg of E. Coli lipopolysaccharide (LPS, 0127:B8, Sigma) in presence or absence of propranolol (5 µg, Sigma) or ICI 118,551 (5 µg, Tocris Cookson Ltd, UK). After 3 h the mice were injected i.d. in the same site of the back with 100 µl PBS containing 50 µg of ovalbumin (OVA, Sigma). Ten days later the mice were injected sc with 50 µg of OVA dissolved in 25 µl of PBS and emulsified in 25 µl of FCA. After 7 days the mice were challenged again sc with OVA to assess the DTH response and cytokine production in lymph node cell suspensions.

Cytokines in lymph node cells and delayed hypersensitivity (DTH) response

Groups of mice were immunized as reported above and 17 days later (recall response) challenged with 50µg OVA in 50 µl PBS in the right hind footpad. The DTH response was assessed by measuring the degree of foot swelling of the OVA-injected foot compared with that of the vehicle–treated contralateral foot at 48 hours after challenge using a digital micrometer (Mitutoyo, Japan). To assess cytokine production, the mice were sacrificed 17 days after immunization and the draining inguinal lymph node cells were obtained, seeded in microplates at 2 x 10^5 cells / 200 µl / well, and incubated in RPMI 1640, 10 % FCS at 37° C for 48 h in presence of 0, 10 and 100 µg/ml of OVA. Next, culture supernatants were collected and the concentration of IFN-γ and IL-4 were quantitated by ELISA (Becton Dickinson: Allschwill, Switzerland).

In vivo migration

Mice were injected id with PGN or LPS ± propranolol, ICI 118,551 or betaxolol (5µg, Tocris Cookson Ltd, UK) in the hind left footpad and 3 hours later with ovalbumin conjugated with Alexa Fluor 488 (5 µg /mouse, Invitrogen, Molecular Probes, Carlsbad, CA, USA) in the same site. In certain experiments PGN ± the combination of ICI 118,551 (5µg) and betaxolol (5µg) was also injected. 24 hours later the mice were killed and single-cell suspensions were prepared from the popliteal draining lymph nodes. Lymph nodes were incubated in Collagenase A, 0.5 mg/ml (Boehringer Mannheim, Rot Kreuz, Switzerland) and DNAse I (40 µg/ml, Perkin Elmer, Rot Kreuz, Switzerland) for 10 min at 37° C. Afterwards the tissue was gently teased and cells were filtered through a 40 µm cell strainer (Falcon). Cells were washed and labelled with PE-conjugated anti-CD11c monoclonal antibody or with a PE-conjugated mixture of monoclonal antibodies recognizing paces (Miltenyi Biotech) and analysed by flow cytometry to assess the percentage and number of CD11c+, Alexa Fluor 488+ and pDCs, Alexa Fluor 488+ cells. Anti-mPDCA-1 antibodies from Miltenyi allow a single color identification of CD11c+, CD45R+ (B220+), Ly-6C+ pDCs.

Statistics

Differences were analyzed by one way analysis of variance with Dunnett's test using the JMP software.

3.3 Results

It has been known for many years that the decision of the T cell to mount a productive response (immunity) or to remain silent (tolerance) is controlled by the form in which the antigen is administered. Antigen with adjuvant leads to immunity, whereas soluble antigen without adjuvant leads to tolerance. Therefore we used PGN or LPS as adjuvant and studied whether the innate inflammatory response and the following adaptive responses to a soluble protein was under a sympathetic β-adrenergic control.

Effect of β-ARs inhibition on the skin innate response upon PGN or LPS injection

We investigated whether the pharmacological inhibition of the skin β-ARs coupled with activation of TLRs could influence inflammatory cytokines and chemokines gene expression in the skin. Three hours and six hours later the mice were sacrificed and the skin was excised at the site of injection for cytokine and chemokine gene expression analysis. We found that among the genes tested, both PGN and LPS enhanced the expression of TNF-α, IL-1α, IL-12, IL-23, IFN-γ, CCL2 and CCL5 especially at 3 hours after injection (data not shown). Figure 9A shows that addition of PRO to PGN, but not to LPS, increased the skin expression of the IL-12, IL-23, and IFN-γ genes 3 hours after injection; the expression of IL-1α and CCL5 was around twofold and that of TNF-α and CCL2 was increased (data not shown) but did not reach the twofold increase chosen as arbitrary threshold to regard an effect as biologically relevant. The expression of the remaining genes was almost undetectable when compared to saline. Relative to LPS, PRO was able to induce only a weak increase of IFN-γ expression, an effect that was however much lower than that observed with PGN. Next we investigated whether the β2-AR specific antagonist ICI 118,551 could reproduce the effect of PRO on the PGN action. The effect was a slightly increased expression of IL-1α only (Fig. 9B). It should be noted that the cytokine gene expression induced by LPS was generally higher than that induced by PGN alone (data not shown). Only with the addition of PRO to PGN, the figures approached those of LPS.

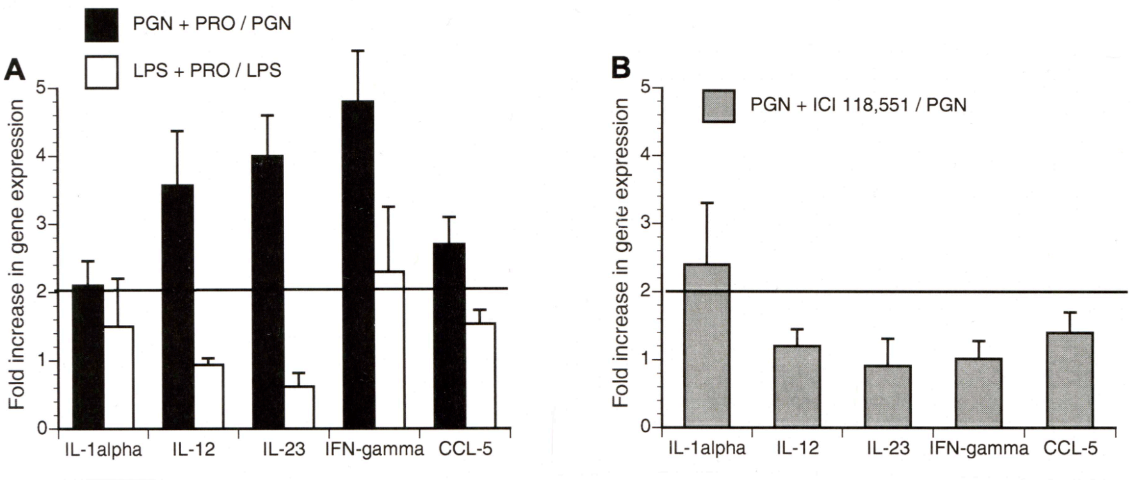


Figure 9: Effect of β-ARs inhibition on cytokine and chemokine mRNA expression in the skin response to PGN or LPS. Mice were injected i.d. with PGN or LPS ± the non specific β-ARs antagonist propranolol (PRO) or with PGN ± the specific β2-ARs antagonist ICI 118,551. Panel A shows the fold increase in gene expression as evaluated 3 hours after treatment by real time RT-PCR. The bars are the mean ± SE of the ratios of the arbitrary units induced by injection of PGN or LPS in presence of the adrenergic antagonists divided by the arbitrary units induced by PGN or LPS alone obtained in 4 experiments (3 mice per group, 4 ratios). Panel B show the folds increase in the expression of the same genes 3 hours after injection of PGN in presence or absence of ICI 118,551. The mean of three experiments ± SE is shown. In all experiments, the arbitrary units were calculated taking as reference the gene expression after i.d. injection of saline. Twofold increase was taken as arbitrary threshold.

We also verified if the lack of response in the LPS groups could depend on the dose by injecting a ten fold lower dose (0.2 µg LPS / mouse). We got a much lower cytokine response but even in this suboptimal situation, PRO failed to affect cytokine expression (data not shown). These results indicate that β1-ARs with a likely contribution of β2-ARs may influence the expression of IL-12, IL-23, and IFN-γ genes upon PGN but not LPS injection in the skin.

Impact of β-ARs inhibition and PGN on the recall memory response to a soluble protein

It is widely accepted that IL-12, IL-23 and IFN-γ are crucial for the development of Th1 immune responses. Thus, we studied whether the boosting effect of β-ARs blockade in presence of PGN before primary immunization to OVA dissolved in PBS could influence the recall memory response. As a model we used BALB/c mice adoptively transferred with lymph node cells from transgenic DO11.10 mice containing 40% of CD4+ T cells expressing a T cell receptor specific for the OVA peptide 323-339. PGN in presence or absence of PRO or ICI 118,551 was injected intradermally and 3 hours later, the mice were injected in the same skin site with OVA dissolved in saline. Ten days later the mice were boosted with OVA embedded in CFA. The delayed type hypersensitivity (DTH) response and cytokine production in the draining lymph nodes were assessed 7 days later.

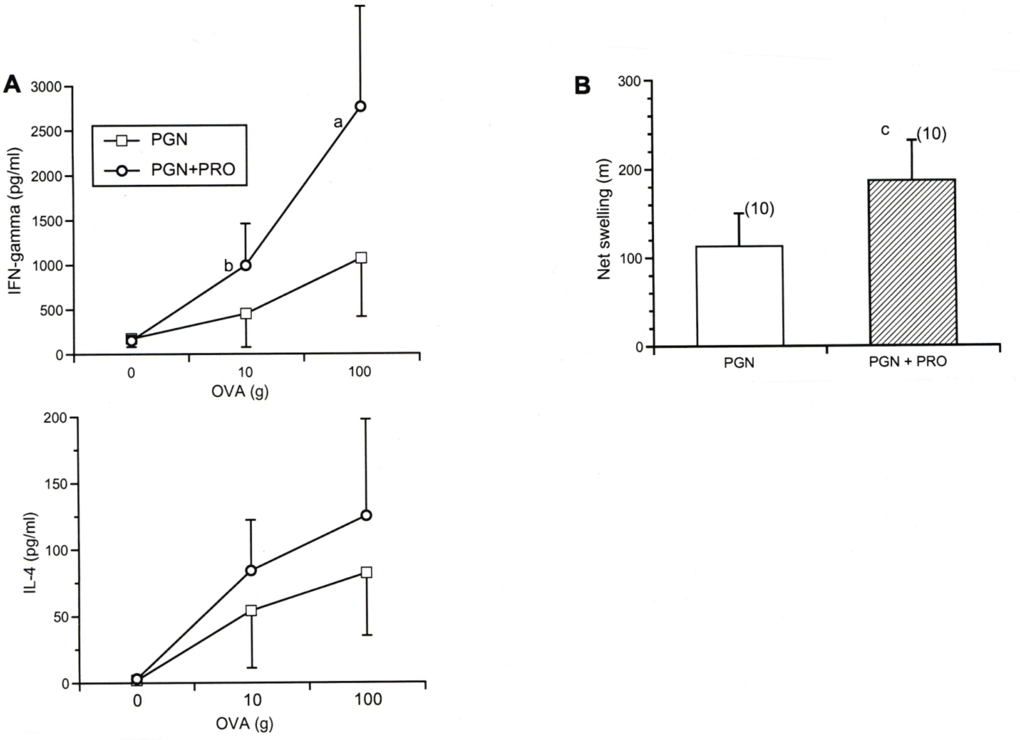


Figure 10: Effect of β-ARs inhibition and PGN injection before primary sensitization to OVA on the recall memory response. KJ1-26+ cells transferred mice were injected i.d. with PGN ± the non specific β-ARs antagonist PRO, 3 hours before immunization with OVA dissolved in saline. 17 days later the in vitro IFN-γ and IL-4 production in lymph node cells upon incubation with various concentrations of OVA (A) and the DTH response (B) were assessed. The curves in A represent the mean values ± the standard deviation per group (3 experiments). The figures in brackets in B represent the number of mice tested per group. a: F(1,28) = 6.40, $p < 0.02$; b F (1, 40) = 4.47: $p < 0.05$; c: F (1, 18) = 6.64, $p < 0.02$ (ANOVA).

Figure 10 shows that preconditioning the skin with PGN in presence of PRO resulted in a significantly stronger and Th1-shifted recall memory response as assessed by the pattern of cytokine production (Fig. 10A) and higher DTH response (Fig. 10B). When LPS was injected instead of PGN, the resulting adaptive response was of the Th1 type but PRO did not exert any influence on both cytokine production (Fig. 11A) and DTH response (Fig. 11B). Then, we investigated whether the specific β2-ARs antagonist ICI 118,551 could exert any effect when combined with PGN. The results reported in figure 12 show that ICI 118,551 did not affect the cytokine production (A) or the DTH response (B). These results were consistent with the influence of PRO on the inflammatory response and suggest that the β1-ARs expressed in the skin participate in modulating the inflammatory response to PGN and influence the subsequent adaptive response to a soluble antigen.

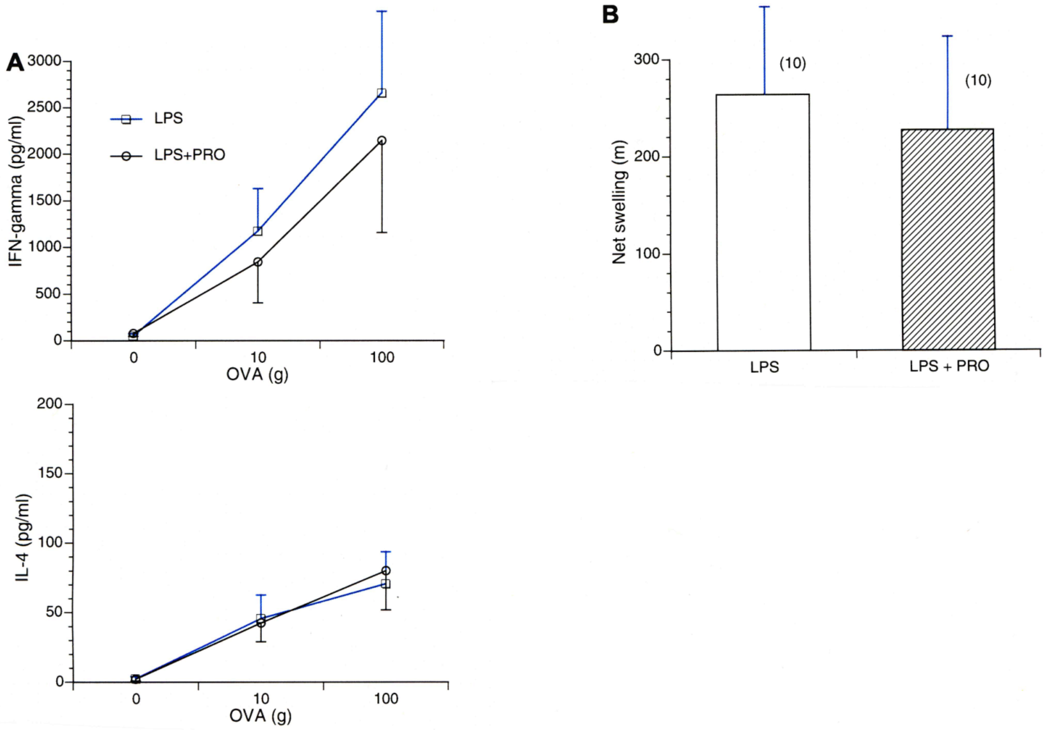


Figure 11: Effect of β-ARs inhibition and LPS injection before primary sensitization to OVA on the recall memory response. KJ1-26+ cells transferred mice were injected i.d. with LPS ± the non specific β-ARs antagonist PRO, 3 hours before immunization with OVA dissolved in saline. 17 days later the in vitro IFN-γ and IL-4 production in lymph node cells upon incubation with various concentrations of OVA (A) and the DTH response (B) were assessed. The curves in A represent the mean values ± the standard deviation of 12 mice per group (3 experiments). The figures in brackets in B represent the number of mice tested per group.

Effect on skin DCs migration

We wondered whether the amplified Th1 priming induced by the β-ARs blockade was due to an increased DCs migration and consequent antigen presentation. As cutaneous DCs include epidermal Langerhans cells, interstitial/dermal DCs as well as pDCs that, however, seems to appear in the skin mainly under pathological conditions (Valladeau and Saeland, 2005), we

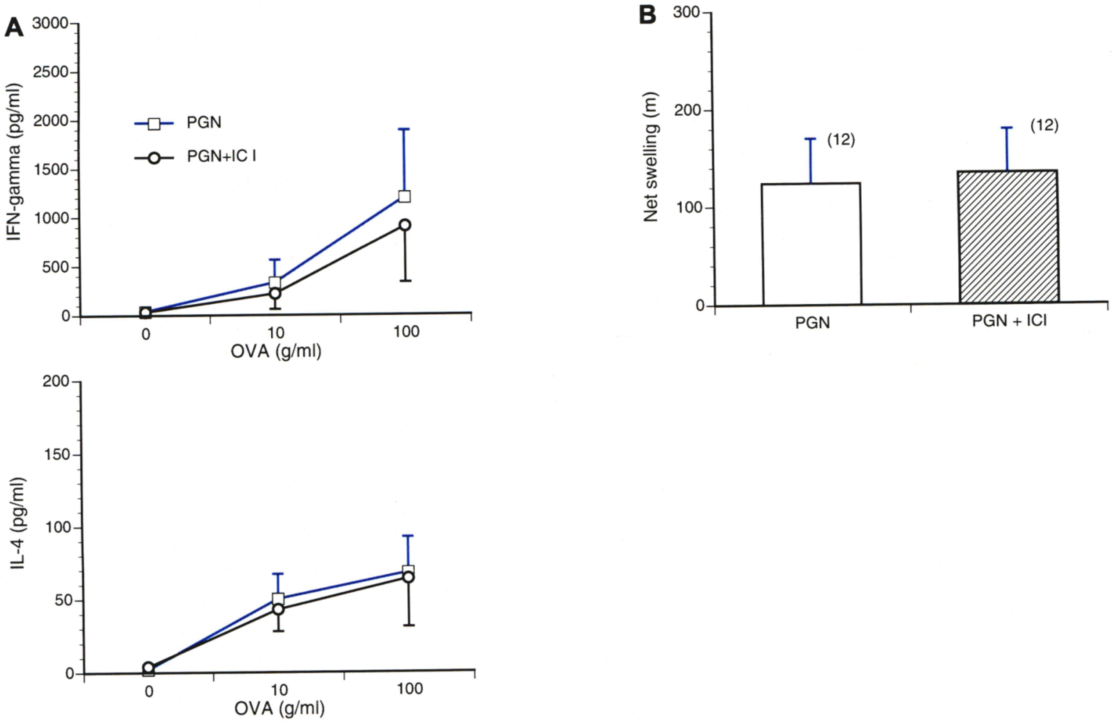
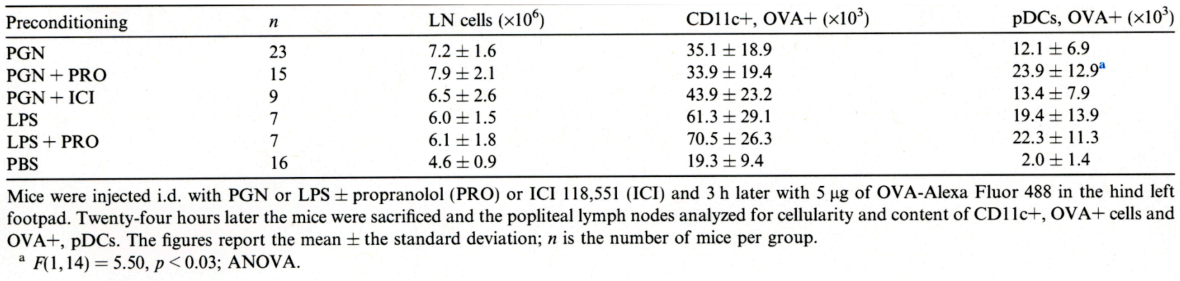


Figure 12: Effect of β2-ARs inhibition and PGN injection before primary sensitization to OVA on the recall memory response. KJ1-26+ cells transferred mice were injected i.d. with PGN ± the specific β2-ARs antagonist ICI 118,551 (ICI), 3 hours before immunization with OVA dissolved in saline. 17 days later the in vitro IFN-γ and IL-4 production in lymph node cells upon incubation with various concentrations of OVA (A) and the DTH response (B) were assessed. The curves in A and the bars in B represent the mean values ± the standard deviation of 12 mice per group (3 experiments).

considered both myeloid and pDCs. Table 4 shows the lymph node cellularity and number of CD11c+, OVA+ (Alexa Fluor 488+) and pDCs, OVA+ in the popliteal draining lymph nodes 24 hours after i.d. injection of PGN or LPS ± PRO or ICI 118,551 followed 3 hours later by OVA-Alexa Fluor 488 in the hind left footpad. The results indicate OVA+, pDCs were significantly increased in the mice whose skin was pre-conditioned by LPS before OVA-Alexa Fluor 488 injection. No effect of PRO was however evident. In the PGN treated groups, the number of OVA+, pDCs was lower but the injection of PRO but not of ICI 118,551 increased significantly their number. Hence, the increased Th1 priming induced by the PGN and β-ARs blockade might depend on a selective recruitment of pDCs, that in presence of a soluble antigen act as antigen presenting cells in the draining lymph node.

Table 4: In vivo migration of antigen-positive DC subset upon skin preconditioning



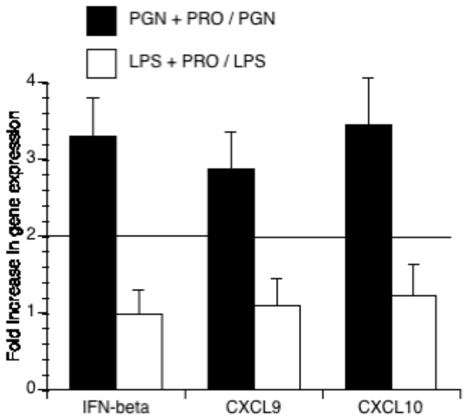
Preconditioning	*n*	LN cells ($\times 10^6$)	CD11c+, OVA+ ($\times 10^3$)	pDCs, OVA+ ($\times 10^3$)
PGN	23	7.2 ± 1.6	35.1 ± 18.9	12.1 ± 6.9
PGN + PRO	15	7.9 ± 2.1	33.9 ± 19.4	23.9 ± 12.9[a]
PGN + ICI	9	6.5 ± 2.6	43.9 ± 23.2	13.4 ± 7.9
LPS	7	6.0 ± 1.5	61.3 ± 29.1	19.4 ± 13.9
LPS + PRO	7	6.1 ± 1.8	70.5 ± 26.3	22.3 ± 11.3
PBS	16	4.6 ± 0.9	19.3 ± 9.4	2.0 ± 1.4

Mice were injected i.d. with PGN or LPS ± propranolol (PRO) or ICI 118,551 (ICI) and 3 h later with 5 µg of OVA-Alexa Fluor 488 in the hind left footpad. Twenty-four hours later the mice were sacrificed and the popliteal lymph nodes analyzed for cellularity and content of CD11c+, OVA+ cells and OVA+, pDCs. The figures report the mean ± the standard deviation; *n* is the number of mice per group.

[a] $F(1, 14) = 5.50$, $p < 0.03$; ANOVA.

Effect on IFN-β and CXCR3 ligands expression in the skin

pDCs precursors may be recruited from the blood by CXCR3 ligands in the skin (Asselin-Paturel et al., 2005; Kohrgruber et al., 2004). The question we asked was therefore whether IFN-β and the inducible chemokines CXCL9, CXCL10 were induced in the skin upon PGN + PRO treatment. We thus repeated the skin preconditioning and measured the IFN-β, CXCL9 and CXCL10 gene expression in the skin 3 hours later. Fig. 13 shows that indeed the combination of PGN + PRO but not of LPS + PRO could induce an increase in the expression of these mediators in the skin 3 hours after preconditioning. It should be noted, however, that LPS per se could induce a higher expression of these genes. As the LPS induced a Th1 memory response to soluble OVA (Fig. 11) was associated with high numbers of OVA+, pDCs in the draining lymph node (Table 4), the induction of IFN-β in the skin during the innate phase of the response is probably the critical event for recruitment of pDCs and for instruction of the consequent innate response to OVA. Therefore, we investigated the relative contribution of β1-ARs and β2-ARs in the IFN-β boosting following PGN injection in the skin by using the specific antagonists betaxolol (β1-AR) or ICI 118,551 (β2-ARs). Figure 14 shows that neither betaxolol nor ICI 118,551 alone could augment the expression of IFN-β and of the CXCR3 ligands CXCL9, CXCL10 when injected together with PGN.



	PGN	PGN+PRO	LPS	LPS+PRO
IFN-beta	5.4± 3.3	16.2± 6.2	21.6± 7	21.5± 6.5
CXCL-9	2.5± 1.8	6.3±3.1	54.8± 6.2	60.2± 6.3
CXCL-10	8.8± 5.6	19.3±9.1	132.1± 8.2	163.6± 32.3

Figure 13: Effect of β-ARs inhibition on IFN-β and CXCR3 ligands mRNA expression in the skin response to PGN or LPS. Mice were injected i.d. with PGN or LPS ± PRO. The bars show the folds increase in gene expression as evaluated 3 hours after treatment by real time RT-PCR. The figures reported are the mean ± SE of the ratios of the arbitrary units induced by injection of PGN or LPS in presence of the β-ARs antagonists divided by the arbitrary units induced by PGN or LPS alone in 3 experiments (3 mice per group, 3 ratios). The table shows the mean of the arbitrary units ± SE in the various groups. In all experiments, the arbitrary units were calculated taking as reference the gene expression after i.d. injection of saline. Twofold increase was taken as arbitrary threshold.

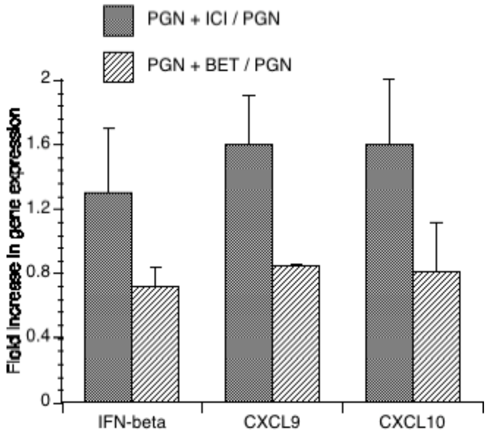
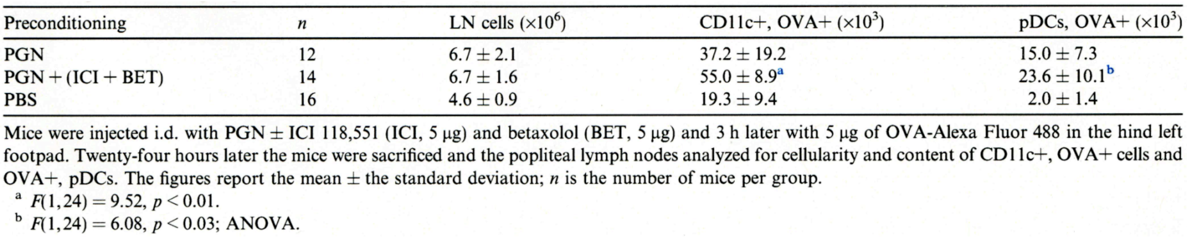


Figure 14: Effect of selective β1-ARs or β2-ARs inhibition on IFN-β and CXCR3 ligands mRNA expression in the skin response to PGN. Mice were injected i.d. with PGN ± the specific β1-ARs antagonist betaxolol (BET) or the specific β2-ARs antagonist ICI 118, 551 (ICI). The bars show the folds increase in gene expression as evaluated 3 hours after treatment by real time RT-PCR. The figures reported are the mean ± SE of the ratios of the arbitrary units induced by injection of PGN in presence of the β-ARs antagonists divided by the arbitrary units induced by PGN alone in 3 experiments (3 mice per group, 3 ratios).

Effect of the combination of ICI 188,551 and betaxolol on pDCs recruitment

The present results suggests that blockade of both β1-and β2-ARs enhanced recruitment and/or migration of pDCs that appears to be the crucial event linking the innate and adaptive immune response and producing the Th1 shift. In fact, the number of antigen positive pDCs in the draining lymph node was high in all groups in which the response to OVA was of the Th1 type, that is, in the LPS ± PRO and PGN + PRO treated mice (Table 4). To further confirm that inhibition of both β-AR subtypes is needed to affect the response to PGN, we investigated whether the combination of ICI 118,551 and betaxolol could reproduce the effect of PRO. Table 5 shows the lymph node cellularity and number of CD11c+, OVA+ (Alexa Fluor 488+) and pDCs, OVA+ in the popliteal draining lymph nodes 24 hours after i.d. injection of PGN ± (ICI 118,551+ betaxolol) followed 3 hours later by OVA-Alexa Fluor 488 in the hind left footpad. The data show that blockade of both β1-and β2-ARs by betaxolol and ICI 118,551 respectively resulted in a significant increase of pDCs in the draining lymph node just as it was the case with PRO (Table 4). However, at variance with PRO, the combination of the two specific antagonists produced also a significant increase of the general CD11c+, OVA+ DCs population.

Table 5: In vivo migration of antigen-positive DC subset upon skin preconditioning with PGN± (ICI 118,551+betaxolol



Preconditioning	*n*	LN cells ($\times 10^6$)	CD11c+, OVA+ ($\times 10^3$)	pDCs, OVA+ ($\times 10^3$)
PGN	12	6.7 ± 2.1	37.2 ± 19.2	15.0 ± 7.3
PGN + (ICI + BET)	14	6.7 ± 1.6	55.0 ± 8.9^{a}	23.6 ± 10.1^{b}
PBS	16	4.6 ± 0.9	19.3 ± 9.4	2.0 ± 1.4

Mice were injected i.d. with PGN ± ICI 118,551 (ICI, 5 μg) and betaxolol (BET, 5 μg) and 3 h later with 5 μg of OVA-Alexa Fluor 488 in the hind left footpad. Twenty-four hours later the mice were sacrificed and the popliteal lymph nodes analyzed for cellularity and content of CD11c+, OVA+ cells and OVA+, pDCs. The figures report the mean ± the standard deviation; *n* is the number of mice per group.

[a] $F(1,24) = 9.52$, $p < 0.01$.

[b] $F(1,24) = 6.08$, $p < 0.03$; ANOVA.

3.4 Discussion

On the basis of the findings reported we suggest that inhibition of both β1- and β2–ARs is needed to heighten the inflammatory response to PGN but not to LPS in the skin. The adrenergic blockade affects the inflammatory cytokines IFN-γ, IL-12, IL-23 and IFN-β inducible chemokines. These chemokines are CXCR3 ligands that may attract CXCR3+ cells including pDCs (Kohrgruber et al., 2004). The increase of pDCs in the draining lymph node might depend on recruitment of specific precursors either in the skin followed by migration to the lymph node or directly in the lymph node. In fact, interstitial fluid is constantly drained into lymph nodes via afferent lymph vessels. This conduit enables monocyte-derived macrophages and DCs to access lymph nodes from peripheral tissues. However, during inflammation in the skin, a second recruitment pathway is evoked that recruits large numbers of blood-borne cells to lymph nodes via high endothelial venules. In fact, inflamed peripheral tissues may project their local cytokine/ chemokine profile to high endothelial venules in draining lymph nodes and thereby exert "remote control" over the composition of leukocyte populations that home to these organs from the blood (Palframan et al., 2001). In any case, the combination of Th1 type inflammatory cytokines and pDCs recruitment lead to a Th1 – type adaptive response to the foreign protein. This conclusion was also supported by the inability of ICI 118,551 to affect the adaptive response to OVA (Fig. 12) as well as IFN-β expression and pDCs number (Table 4). On the other hand, also the β1-ARs antagonist betaxolol failed to increase IFN-β expression (Fig.14) and the number of OVA+, pDCs in the draining lymph node when injected together with PGN (data not shown). On the contrary, the combination of ICI 118,551 with betaxolol reproduced the effect of PRO on the pDCs number (Table 5) confirming that both β1- and β2-ARs need to be inhibited to affect the response to PGN. In addition, the combination of the two specific antagonists led also to an increase of the general antigen positive CD11c+ cell population, an effect that was not observed with PRO (Table 4). This effect might depend on the dose (5 μg ICI 118,551 + 5 μg betaxolol in comparison with 5 μg of PRO) or on possible different pharmacological properties of the combination of the specific antagonists.

The involvement of both β1- and β2-ARs might depend on a different cell source of the cytokines and chemokines implicated in the phenomenon or on the expression of both receptors on the target cell(s). Many cell types in the skin may express both β1- and β2-ARs: DCs (Maestroni, 2002b; Seiffert et al., 2002), macrophages (Sigola and Zinyama, 2000), fibroblasts (Yoshida et al., 1996), CD4+, CD25+ T cells (Maestroni, unpublished results) and, of course, endothelial cells. As all these cell types contribute in the inflammatory response with production of cytokines that may in turn exert a paracrine role on other cell types, it seems difficult to identify the primary target of both PGN and PRO without additional studies. In addition, β1- and β2-AR can form heterodimers and have altered pharmacological properties from the receptor homodimers (Lavoie and Hebert, 2003). In regard to the difference in the skin response to PGN and LPS, mast cells might play a role. In fact, it has been reported that activation of TLR2 and TLR4 by respective microligands in mast cells produces a different cytokine response. Furthermore, TLR2- but not TLR4-dependent stimulation resulted in mast cell degranulation (Supajatura et al., 2002) This phenomenon might account for the different innate and adaptive immune response elicited by PGN and LPS. In fact, the response to PGN alone was shifted toward the Th2 type while the response to LPS was clearly of the Th1 type as evidenced by the high IFN-γ/IL-4 ratio and by the strong DTH response (Fig.11). However, the involvement of mast cells cannot fully explain the different sensitivity to PRO and the need of inhibiting both β1- and β2-ARs. In fact, mast cell production of inflammatory mediators is regulated by β2-ARs only (Kay and Peachell, 2005). Mast cells have been also described to exert an important role in the mobilization of Langerhans cells in response to PGN (Jawdat et al., 2006). In our case, the effect of β-ARs inhibition was mainly exerted on pDCs, a DCs subset that is not normally present in the skin. However, the different mast cell response to PGN and LPS might involve a different paracrine effect on other cell types bearing both β-AR subtypes.

Perhaps, the reason for the different β2-ARs influence on the PGN and LPS response depends on signaling pathways involved. The scarce influence of the β2-ARs inhibition on the LPS response would suggest that β-ARs affect neither the MyD88-dependent nor –independent pathway. As a consequence, also the TLR2, Myd88-dependent, pathway should not be affected. Yet, in our hands the β-ARs blocking could equally enhance the inflammatory cytokine and IFN-β response upon PGN injection. PGN is a unique and essential component of the cell wall of virtually all bacteria, is not present in eukaryotes, and is an excellent target for the innate immune system. Indeed, unlike TLR4 that is the sole receptor for LPS (Palsson-McDermott and O'Neill, 2004), there are numerous PGN recognition molecules that are distinct from TLR2. These include CD14, nucleotide oligomerization domain (Nod)-containing proteins, a family of peptidoglycan recognition proteins, and PGN-lytic enzymes (Dziarski and Gupta, 2005a). Thus, it is possible that β-ARs have an effect on one or more of these latter PGN recognition molecules. This interpretation is also supported by the observation that activation of TLR2 alone does not lead to IFN-β production (Toshchakov et al., 2002). Nonetheless, a most recent report describes a so far unknown cross talk between the MyD88 and TRIF pathways originating by a TLR synergy (Ouyang et al., 2007). Accordingly, it is conceivable that the various PRRs stimulated by PGN, including TLR2, in presence of PRO realize a similar synergy resulting in a stimulation of IFN-β and related genes.

In previous studies we showed that β2-ARs in bone marrow derived DCs could affect the inflammatory cytokine response when the cells were activated in vitro with both PGN or LPS (Maestroni, 2002a). The apparent discrepancy of the present findings suggest that in the skin, the cell target of both PGN and LPS are dissimilar or react differently from bone marrow derived DCs. In any case, the present findings call attention to the role of the skin adrenergic system in our understanding of acquired inflammatory diseases. It is recognized that the sympathetic and parasympathetic nervous systems generally inhibit innate immune responses at systemic and regional levels, whereas the peripheral sensory nervous system tends to amplify local innate immune responses. These systems work together to first activate and amplify local inflammatory responses that contain or eliminate invading pathogens, and subsequently to terminate inflammation and restore host homeostasis (Sternberg, 2006). Thus, the physiological role of the skin adrenergic system might be that of limiting the Th1 response to pathogens that are recognized by certain PRRs. This mechanism might have evolved to shape the appropriate immune response to bacteria or viruses that may hit the skin more frequently. Disorders of the adrenergic (β-ARs) regulation of the skin immune response may thus results in excessive or reduced Th1 priming and have pathological consequences. Such disorders may be secondary to emotional stress that has been reported to affect skin diseases such as atopic dermatitis and plaque psoriasis (Chuh et al., 2006). Pertinent to the present findings, excessive Th1-priming has been associated with an augmented risk for organ-specific autoimmune diseases (Kramer et al., 1996) and current pathogenesis concepts consider skin disorders such as psoriasis vulgaris, vitiligo and alopecia aerata as T cell mediated autoimmune diseases (Chow et al., 2005). What's more, pDCs in the skin were shown to play a critical pathogenetic role in psoriasis and atopic dermatitis (Nestle et al., 2005; Novak and Bieber, 2005). IL-23 was reported to stimulate epidermis hyperplasia with implication for psoriasis pathogenesis (Chan et al., 2006). Likewise, skin colonization with S. Aureus has important implications in the pathogenesis of atopic dermatitis and psoriasis (Tomi et al., 2005). Thus, we might have disclosed part of the pathogenetic mechanisms of these skin disorders by linking the skin β-adrenergic system to selected PRR(s). Future studies should be aimed at identifying such PRR(s) and their possibly relevance in other immune-based skin disorders and in skin cancers. It is also conceivable that similar mechanisms might be at work in modulating mucosal immunity. Finally, our findings might find application in vaccines design.

Acknowledgements

This study has been supported by the Swiss National Science Foundation, grant no. 310000-107524/1. We thank Mrs. Elisabeth Hertens and Mrs. Paola Galli for the excellent technical assistance.

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IV. Additional results to β-adrenoceptor and relevance in inflammatory diseases

4.1 TLR-2 and TLR-4 gene expression in murine skin

In order to evaluate the expression of TLRs in murine skin cells, mice skin was incubated in dispase 5U/ml supplemented with 0.375% Trypsin for 1 hour at 37°C. Then, the dorsal part, corresponding to the epidermal sheets, was separated from the derma using sterile forceps. The gene expression of TLR-2 and TLR-4 in the whole skin, the epidermis and the derma was evaluated by Real time RT-PCR compared to the XS52 cell line. The results are expressed as the ratio of the arbitrary units of the derma or the whole skin compared to epidermis.

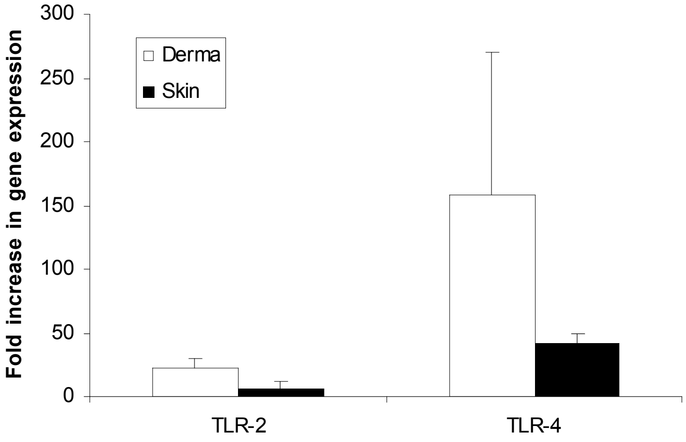

Figure 15: TLRs distribution in mice skin. Mice skin was separated in epidermal sheets and derma and the mRNA expression of TLR-2 and TLR-4 was evaluated by Real time RT-PCR. The bars represent the ratio±SD of the arbitrary units of the derma or the whole skin compared to epidermis. In all experiments, the arbitrary units were calculated using the gene expression of the XS52 cell line as reference.

The results presented in Fig. 15 show that TLR-2 and TLR-4 gene expression were respectively 23 and 158 times higher in the derma compared to the epidermis. In regard to the whole skin, the TLR-2 and TLR-4 mRNA expression was also increased compared to the epidermis 6 and 42 times respectively. This indicates that TLR-2 and TLR-4 are expressed ubiquitously in mice skin but predominantly in the dermal compartment.

4.2 Effect of PRO and ICI 118,551 on cytokine gene expression in murine skin

We investigated whether the pharmacological inhibition of β-ARs per se may affect the gene expression of the proinflammatory cytokines IL-12, IFN-γ and IL-23 by real time RT-PCR in mice skin treated with 5μg of the specific β2-ARs antagonist ICI 118,551 or of the non-specific β-ARs antagonist propranolol.

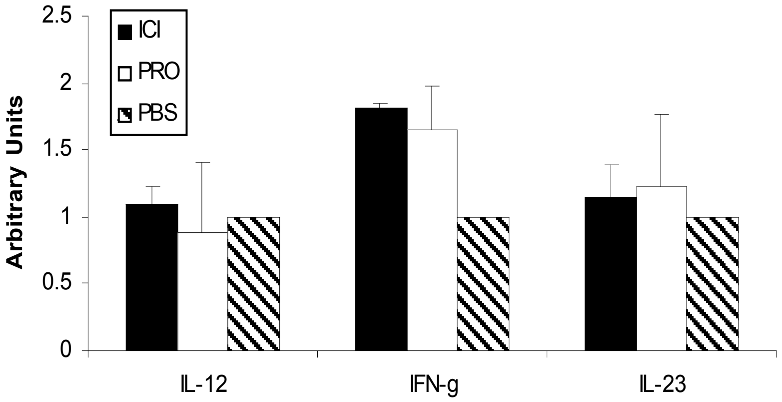

Figure 16: mRNA expression of the proinflammatory cytokines IL-12, IFN-γ and IL-23. Mice were intradermally injected with 5μg of the specific β2-ARs antagonist ICI 118,551 or the non-specific β-ARs antagonist propranolol. 3 hours later, mice were sacrificed and the skin at the site of injection was collected for evaluation of the proinflammatory gene expression by Real time RT-PCR. The bars represent the arbitrary units± SD calculated using the gene expression of mice treated with the vehicle alone as reference.

Fig. 16 shows that injection of ICI 118,551 and PRO in mice skin had no significant influence on the gene expression of IL-12, IFN-γ and IL-23. There was no increase in arbitrary units in β-ARs antagonists treated mice compared to vehicle treated mice. We confirmed that pharmacological inhibition of β-ARs per se with the specific β2-ARs antagonist ICI 118,551 or the non-specific β-ARs antagonist propranolol, had no effect on the mRNA expression of the proinflammatory cytokines IL-12, IFN-γ and IL-23.

4.3 Effects of β-ARs blockade on pDCs migration from ear

Plasmacytoid DCs may be recruited into the draining lymph node directly from the blood via high endothelial venules[56] or may enter the skin by CXCR3-chemotaxis and then migrate from the skin into the draining lymph node[57,58]. To test if the increased number of plasmacytoid DCs in the draining lymph node upon TLRs activation and OVA injection depended on a selective recruitment of these cells from the skin or directly from the blood, mice were injected i.d. with PGN in presence or absence of PRO in the ear pinna. Three hours later, OVA-Alexa 488, was injected in the same site and two hours later the ear was completely removed. Finally, after 24 hours the auricular draining lymph node was collected for single cell suspension preparation and FACS analysis. We reasoned that if pDCs in the draining lymph node had only a skin origin, we should not have found any increase in their number after the surgical removal of the ear.

Table 6: In vivo migration of antigen positive DC subsets after ear skin preconditioning and surgical removal.

Preconditioning	n	LN cells ($x10^6$)	CD11c+, OVA+ ($x10^3$)	pDCs, OVA+ ($x10^3$)
PGN	10	11.5± 4.1	65.5± 22.7	39.7± 24.4
PGN+PRO	10	11.8± 3.6	80.7± 29.7	68.6± 42.1
PBS	5	4.9± 1.7	41.8± 15.4	5.4± 4.2

Mice were injected i.d. with PGN± PRO in the ear pinna and three hours later with OVA-Alexa 488. Five hours after the preconditioning, the ear was completely removed. Twenty-four hours later, the mice were sacrificed and the auricular draining lymph node analyzed for lymph node cellularity and content of CD11c+,OVA+ cells and OVA+, pDCs. The table reports the mean ± SD; n is the number of mice per group.

The lymph node cellularity and the recruitment of CD11c+, OVA+ cells and OVA+, pDCs were significantly increased in mice preconditioned with PGN or PGN+PRO compared to control mice (Table 6). This suggests that PGN and OVA-Alexa 488 migrated from the injection site to the draining lymph node before the ear removal and influenced the recruitment of these cells from the blood. However, in contrast to the results of DCs migration reported in Table 4, the number of OVA+, pDCs was not significantly increased by addition of PRO in the preconditioning. This indicated that removal of the ear prevented the effect of PRO suggesting that pDCs may be recruited either from the blood or from the skin and that PRO affected especially the latter mechanism.

V. *In vitro* modulation of cytokines production by β-adrenoceptors in pattern recognition receptor activated dendritic cells

5.1 *In vitro* modulation of cytokines production by NE in PRRs activated DCs

In order to better characterize the observed modulation of the PGN signaling by β-ARs *in vivo*, we decided to investigate the effect of β-ARs on cytokine production in DCs activated *in vitro* with different PRRs. Unlike LPS, which is a specific TLR-4 ligand, PGN is recognized by different type of molecules, such as CD14, NOD receptors, PGN-lytic enzymes and PGN recognition proteins[59]. In particular, it has been reported that PGN from Gram positive and Gram negative bacteria may be internalized in cells and cleaved in subunits of MDP. Next, MDP may bind to the NOD2 receptor and activate the NFκB pathway and the transcription of inflammatory genes. Therefore, we investigated the influence of the β-ARs activation on the specific signaling pathway of the TLR-2 and NOD2 receptors. DCs were incubated either with PGN, the specific TLR-2 agonist PAM, the specific NOD2 agonist MDP and a combination of PAM and MDP in presence or absence of NE. Although it is well known that the physiological sympathetic neurotransmitter NE is not a specific agonist for β-ARs, previous studies showed that in DCs NE modulates cytokine production mainly by β-ARs[54]. Six hours later, the supernatants were collected for the determination of the pro- and anti-inflammatory cytokines concentration by ELISA.

5.1.1 NE effect on IL-12 production

The production of the proinflammatory cytokine IL-12 was increased in DCs incubated with the TLR-2/NOD2 agonist PGN compared to control cells incubated with culture medium alone. Addition of the specific TLR-2 agonist PAM or the specific NOD2 agonist MDP to DCs had practically no effect on the production of IL-12, which remained under the detection threshold of the ELISA kit (15pg/ml) (Fig. 17). In contrast, when a combination of PAM and MDP was added, there was an appreciable increase in IL-12 production compared to control cells. However, this increase was considerably lower than that observed in DCs incubated with PGN. This difference may be explained by a possible difference in the receptor binding affinity of these different compounds to TLR-2 and NOD2 receptors as well as by the fact that PGN may be recognized by additional PRRs, as reported above.

Addition of the NE to DCs incubated with PGN or with PAM+MDP dramatically reduced the production of IL-12, which in the case of PAM+MDP treatment decreased under the minimum detection threshold. In contrast, addition of NE to DCs treated with either PAM, MDP or medium alone, had no effect on IL-12 production that still remained under the detection threshold.

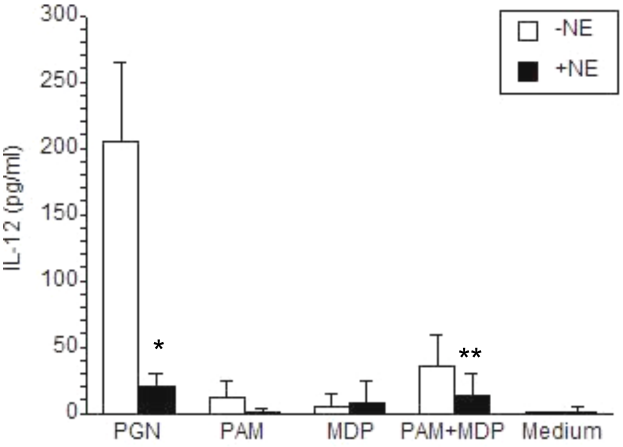

Figure 17: Effect of NE on IL-12 production in DCs incubated with PRRs agonists. 1.10^6/ml DCs were incubated either with 10µg/ml of PGN, 10µg/ml of PAM, 2 µg/ml MDP or a combination of 10µg/ml PAM+ 2 µg/ml MDP in presence or absence of 10^{-6}M NE. Six hours later, the supernatants were collected and IL-12 concentration was determined by ELISA. The bars represent the mean of the cytokine production of 5 independent experiments±SD. (ANOVA, *p<0.0001; **p<0.005).

5.1.2 NE effect on IL-10 production

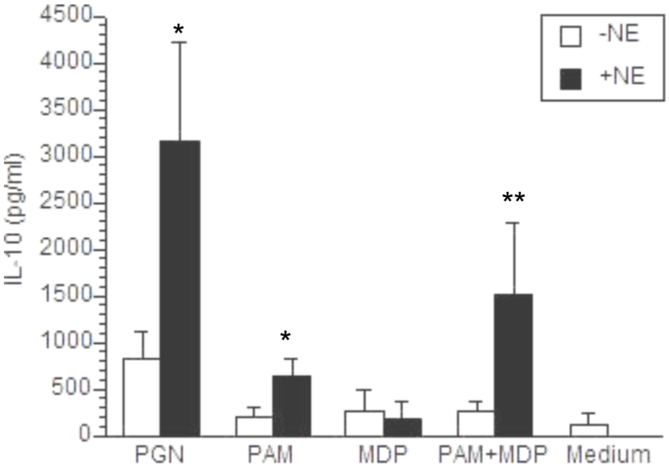

Figure 18: Effect of NE on IL-10 production in DCs incubated with PRRs agonists. DCs were incubated either with 10µg/ml of PGN, 10µg/ml of PAM, 2 µg/ml MDP or a combination of 10µg/ml PAM+ 2 µg/ml MDP in presence or absence of 10^{-6}M NE. Six hours later, the supernatants were collected and the IL-10 concentration was determined by ELISA. The bars represent the mean of the cytokine production of 3 independent experiments±SD. (*p<0.0001; **p<0.001).

In general, addition of either PGN, PAM, MDP or PAM+MDP to DCs cultures *in vitro* augmented the production of the anti-inflammatory cytokine IL-10 compared to control cells incubated with medium alone. PGN induced a greater production of IL-10 than PAM, MDP and PAM+MDP (Fig 18). As for the proinflammatory cytokine IL-12, this difference may be due to a lower binding affinity of PAM and MDP for the TLR-2 and NOD2 receptors compared to PGN as well as to the ability of PGN to bind to other PRRs.

Addition of NE further increased the amount of IL-10 produced by DCs incubated with either PGN, the specific TLR-2 agonist PAM or the combination of PAM+MDP. Interestingly, all these compounds signal through TLR-2. In contrast, the IL-10 production resulting from NOD2 activation by MDP was not influenced by addition of NE in the culture medium. This suggested that the increased IL-10 production in DCs incubated with PGN in presence of NE, was due to a modulation of TLR-2 but not of NOD2 signaling. Addition of NE to control cells incubated with medium alone had no effect on IL-10 production.

5.1.3 NE effect on IL-6 production

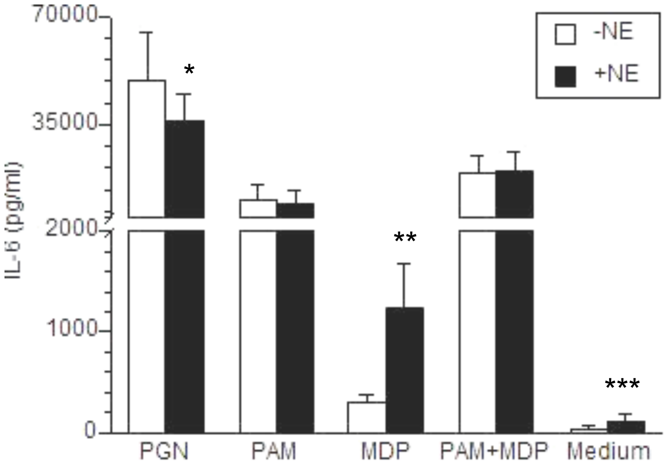

Figure 19: Effect of NE on IL-6 production in DCs incubated with PRRs agonists. DCs were incubated either with 10μg/ml of PGN, 10μg/ml of PAM, 2 μg/ml MDP or a combination of 10μg/ml PAM+ 2 μg/ml MDP in presence or absence of 10^{-6}M NE. Six hours later, the supernatants were collected and IL-6 concentration was determined by ELISA. The bars represent the mean of the cytokine production of 5 independent experiments±SD. (*p<0.001; **p<0.0001;*** p<0.005).

DCs incubated with either PGN, PAM or the combination of PAM+MDP, increased the IL-6 cytokine production compared to control cells incubated in medium alone. When DCs were incubated with the specific NOD2 receptor agonist MDP, there was also an increase in IL-6 but to a much lower extent (Fig. 19).

Addition of NE significantly increased the production of IL-6 in MDP treated DCs, while it had no effect on PAM and PAM+MDP treated cells. Moreover, as previously reported by other authors[46], NE also significantly increased the production of IL-6 in control cells incubated in absence of proinflammatory stimuli. Surprisingly, in contrast to the MDP and PAM+MDP groups, addition of NE to DCs incubated with PGN, significantly decreased the amount of this cytokine in the culture supernatant compared to DCs incubated with PGN alone. This discrepancy might be explained by the ability of PGN to bind receptors different from TLR-2 and NOD2 and that might also induce transcription of proinflammatory cytokine genes. Moreover, it is known that IL-10 may inhibit the production of IL-6. Here, the increased IL-10 concentration in culture supernatants of DCs incubated with PGN, PAM and PAM+MDP in presence of NE may possibly affect the IL-6 production.

5.1.4 NE effect on IL-23 production

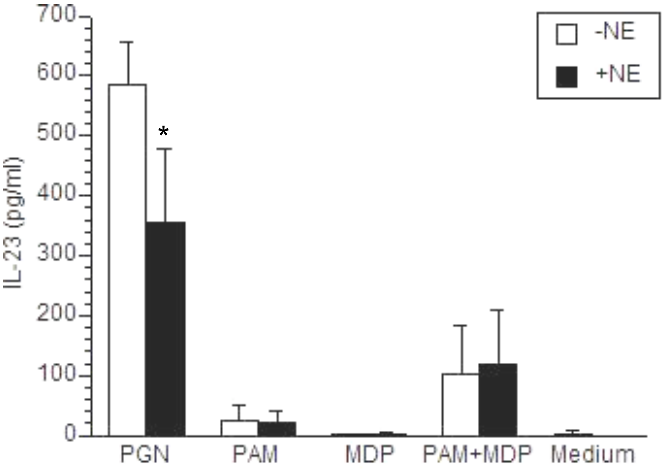

Figure 20: Effect of the addition of NE on IL-23 production in DCs incubated with PRRs agonists. DCs were incubated either with 10µg/ml of PGN, 10µg/ml of PAM, 2 µg/ml MDP or a combination of 10µg/ml PAM+ 2 µg/ml MDP in presence or absence of 10^{-6}M NE. Six hours later, the supernatants were collected and IL-23 concentration was determined by ELISA. The bars represent the mean of the cytokine production of 3 independent experiments±SD. (*p<0.0005).

IL-23 production was increased in DCs incubated both with PGN or the combination of PAM+MDP compared to control cells. In contrast, PAM and MDP treated cells barely produced IL-23, which remained under the detection limit of the ELISA kit (30pg/ml).

Addition of NE to DCs treated with PGN, significantly reduced the production of this cytokines, while it had no effect in all the other groups (Fig. 20). Again also for this cytokine a difference in the NE effect on PGN and PAM+MDP activated DCs was evident. The explanation might attain to the consideration quoted above for the other cytokines.

5.1.5 NE effect on cytokines production in LPS stimulated DCs

Although no effect of the β-ARs inhibition on the skin innate response to LPS was found *in vivo* (Fig. 9), we went back *in vitro* to test the effect of the addition of NE on cytokines production in DCs to investigate whether we could trace any difference between the PGN and LPS signaling.

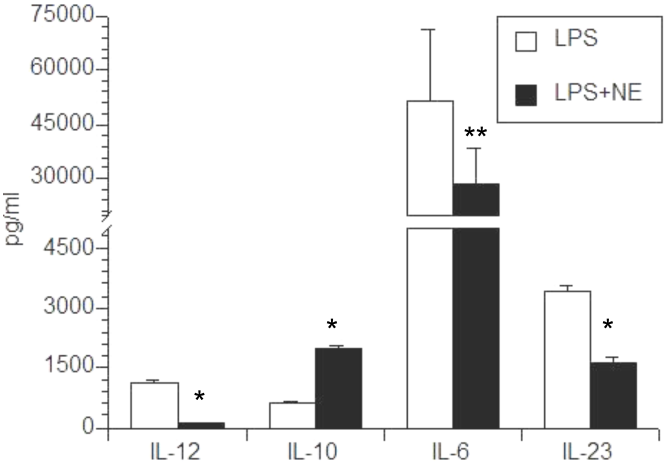

Figure 21: Effect of the addition of NE on cytokines production in DCs incubated with LPS. DCs were incubated with 1μg/ml of LPS, in presence or absence of 10^{-6}M NE. Six hours later, the supernatants were collected and the cytokines concentration was determined by ELISA. The bars represent the mean of the cytokine production of 2 independent experiments±SD. (*p<0.0001, **p<0.01).

As already reported in part in previous studies[52,54] and in contrast with the *in vivo* experiments, addition of NE to DCs incubated with LPS significantly increased the production of the anti-inflammatory cytokine IL-10 while decreasing the production of IL-12, IL-6 and IL-23 (Fig. 21). The difference between the *in vivo* and *in vitro* experiments may probably be due to the presence in mice skin of many different cell types, such as mast cells and fibroblast, which may react to LPS± βARs inhibition in a different way than DCs. Comparing DC cytokines modulation by NE upon PGN and LPS stimulation, we observed that LPS induced a significant ($p<0.0001$) greater production of IL-12 and IL-23 than PGN and that addition of NE to LPS treated DCs had a stronger effect on IL-6 and IL-23 (45% and

53% reduction respectively) than in PGN (27% and 39% reduction respectively) stimulated cells.

5.2 Effect of IL-10 neutralization on IL-6 and IL-23 production

Previous studies have demonstrated that IL-10 may inhibit in a dose dependent manner the expression and the production of proinflammatory cytokines, notably of IL-6 in human monocytic cell line[60], rat mast cells[61] and murine microglial cell line[62]. Here, we wondered if the decrease in IL-6 production in PGN and NE treated cells could be explained by an IL-10 inhibition of the NOD2-dependent IL-6 production. We demonstrated that addition of NE to PGN treated cells increased the production of IL-10 by affecting the TLR-2 signaling pathway (Fig. 18). Possibly, this augmented production of IL-10 inhibited the production of IL-6 by the NOD2 pathway resulting in a decreased IL-6 concentration in the supernatant of PGN+NE treated DCs compared to PGN alone. Furthermore, we wondered if this effect might also influence the IL-23 production. Therefore, DCs were incubated *in vitro* either with PGN, the specific TLR-2 agonist PAM and the combination of PAM+MDP in presence or absence of NE and of the purified antibody anti-mouse IL-10. Six hours later, the supernatants were collected for determination of the IL-6 and IL-23 concentration by ELISA.

5.2.1 Effect of IL-10 neutralization on IL-6 and IL-23 production upon PGN± NE treatment of DCs

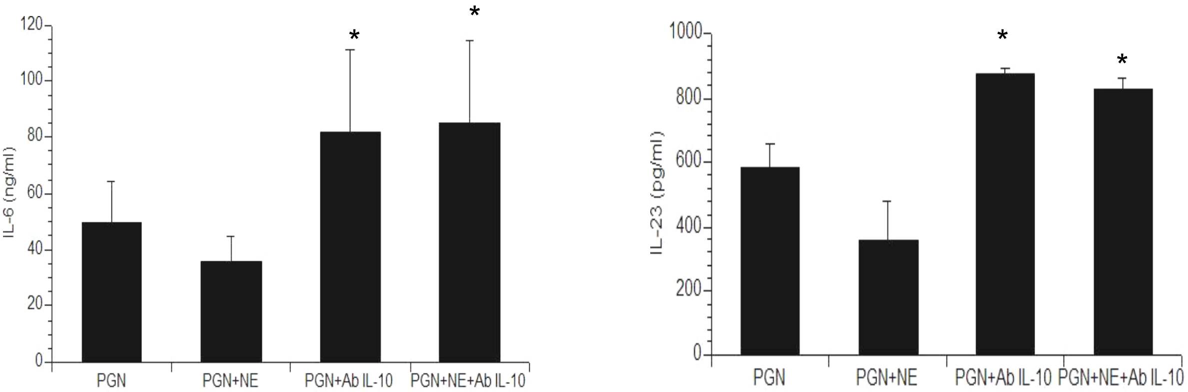

Figure 22: Effect of IL-10 neutralization on IL-6 and IL-23 production in DCs incubated with PGN±NE. DCs were incubated with 10µg/ml of PGN in presence or absence of 10^{-6}M NE. In some experiments 20µg/ml of the purified antibody anti-mouse IL-10 was also added. Six hours later, the supernatants were collected and IL-6 and IL-23 concentration determined by ELISA. The bars represent the mean of the cytokine production of 3 independent experiments±SD. (*p<0.0001).

Addition of the anti-mouse IL-10 antibody to DCs treated with PGN or PGN+NE apparently abolished the production of IL-10 confirming the neutralization ability of the antibody (data not shown). Furthermore, production of IL-6 and IL-23 in these cells was significantly increased compared to DCs treated with PGN or PGN+NE. However, no difference was detected between DCs incubated with PGN+Ab IL-10 and PGN+NE+Ab IL-10 (Fig. 22). This indicated that the increased production of IL-10 in DCs treated with PGN and NE was responsible for the decreased production of IL-6 and IL-23.

5.2.2 Effect of IL-10 neutralization on IL-6 and IL-23 production upon PAM± NE treatment of DCs

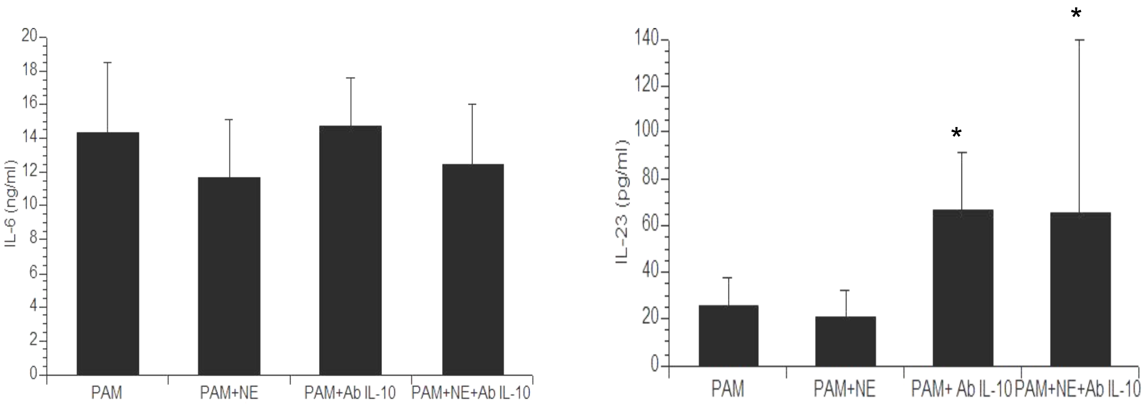

Figure 23: Effect of IL-10 neutralization on IL-6 and IL-23 production in DCs incubated with PAM±NE. DCs were incubated with 10μg/ml of PAM in presence or absence of 10^{-6}M NE. In some experiments 20μg/ml of the purified antibody anti-mouse IL-10 was also added. Six hours later, the supernatants were collected and the IL-6 and IL-23 concentration determined by ELISA. The bars represent the mean of the cytokine production of 2 independent experiments±SD (* $p<0.01$).

In contrast with the results obtained in DCs incubated with PGN±NE, addition of the antibody to DCs stimulated by PAM in presence or absence of NE had no effect on IL-6 production (Fig. 23). This suggested that IL-10 may inhibit IL-6 production triggered by NOD2 but not by TLR2. On the contrary, neutralization of IL-10 significantly increased the production of IL-23 in DCs stimulated with PAM in presence or absence of NE. However, there were no significant difference between DCs stimulated with PAM+Ab IL-10 and PAM+NE+Ab IL-10. In conclusion, TLR-2 dependent IL-23 production might be modulated by IL-10.

5.2.3 Effect of IL-10 neutralization on IL-6 and IL-23 production upon PAM+MDP± NE treatment of DCs

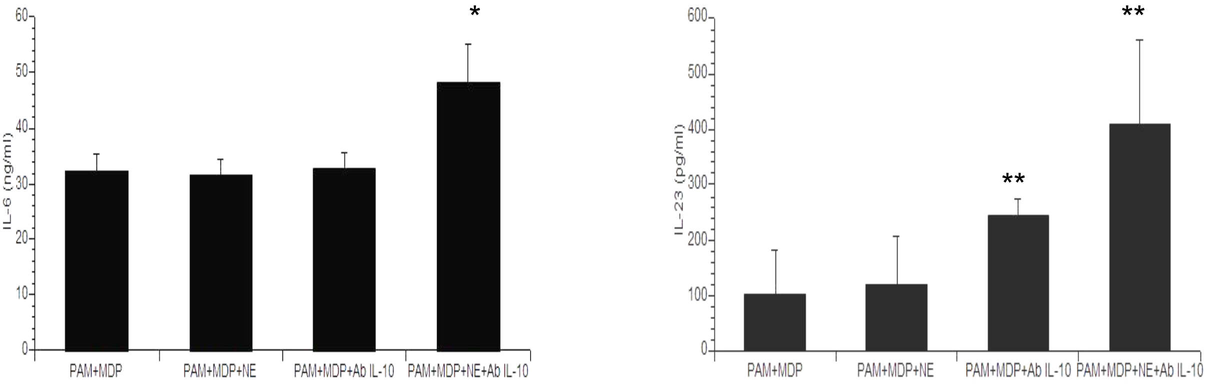

Figure 24: Effect of IL-10 neutralization on IL-6 and IL-23 production in DCs incubated with PAM+MDP±NE. DCs were incubated with 10µg/ml of PAM+2µg/ml of MDP in presence or absence of 10^{-6}M NE. In some experiments 20µg/ml of the purified antibody anti-mouse IL-10 was also added. Six hours later, the supernatants were collected and the IL-6 and IL-23 concentration was determined by ELISA. The bars represent the mean of the cytokine production of 2 independent experiments±SD. (*$p<0.05$; **$p<0.0001$).

The anti-mouse IL-10 antibody increased IL-6 production in PAM+MDP stimulated DCs in presence but not in absence of NE (Fig. 24). This may be due to the existence of a minimum concentration that IL-10 has to reach to be able to inhibit IL-6. In fact, the concentration of IL-10 was considerably higher in PAM+MDP+NE stimulated DCs than in those activated by PAM+MDP alone (Fig. 18). Consequently, addition of the anti-mouse IL-10 antibody had an effect only on DCs in presence of NE. This consideration is supported also by the observation that the anti-mouse IL-10 antibody was effective in increasing IL-6 in PGN and PGN+NE treated DCs. In fact, in both these groups the concentration of IL-10 was higher than that found in supernatant from DCs stimulated with PAM+MDP alone. Moreover, the TLR-2/NOD2 agonists ratio used in the experiment might be different from the ratio in PGN probably in favor of NOD2.

Concerning IL-23 production, neutralization of IL-10 significantly increased the concentration in PAM+MDP stimulated DCs in presence or absence of NE.

5.3 *In vitro* modulation of cytokine mRNA expression by NE in PRRs activated DCs

In order to test if the *in vitro* modulation of the DC cytokines production by NE upon PRRs activation was exerted at the level of mRNA expression, DCs were incubated either with PGN, PAM, MDP, PAM+MDP or LPS in presence or absence of NE. Three hours later, cells were collected, the total RNA extracted and the cytokines gene expression analyzed by real time RT-PCR.

5.3.1 NE effect on cytokines mRNA expression in PGN stimulated DCs

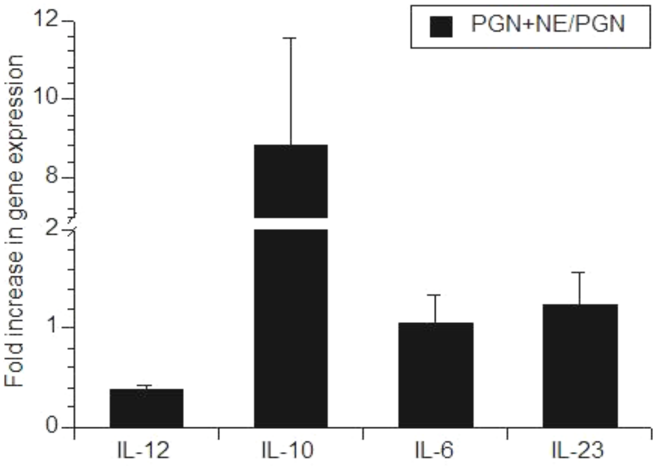

Figure 25: Effect of NE on cytokines mRNA expression in DCs incubated with PGN. DCs were incubated with 10µg/ml of PGN in presence or absence of 10^{-6}M NE. Three hours later, the cells were collected and the cytokines mRNA expression evaluated by Real Time RT-PCR. The bars represent the ratio±SD of the arbitrary units of PGN+NE treated cells over that of PGN treated cells as obtained in 3 independent experiments. In all experiments, the arbitrary units were calculated using the gene expression of DCs incubated with culture medium alone as reference. Twofold increase and 0.5 fold increase were taken as arbitrary threshold for biological significance.

In agreement with the results obtained for protein production, IL-12 and IL-10 mRNA expression was respectively decreased and increased in DCs stimulated with PGN+NE compared to DCs stimulated with PGN alone. On the contrary, there was no difference between IL-6 and IL-23 mRNA expression in the two groups (Fig. 25), while, at the protein level, these two cytokines were both significantly decreased (Fig. 19 and 20).

5.3.2 NE effect on cytokines mRNA expression in PAM stimulated DCs

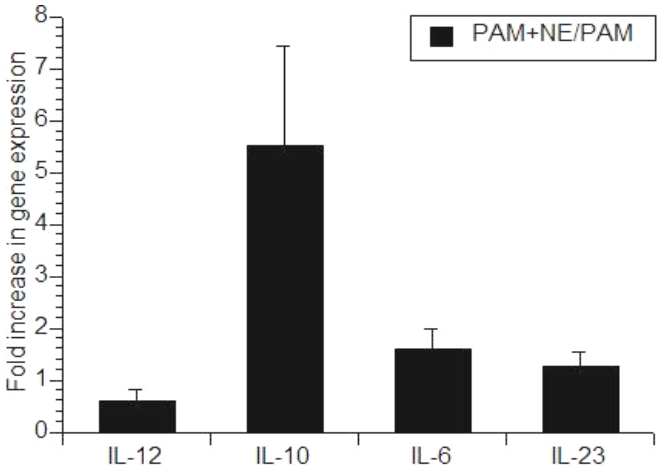

Figure 26: Effect of NE on cytokines mRNA expression in DCs incubated with PAM. DCs were incubated with 10µg/ml of PAM in presence or absence of 10^{-6}M NE. Three hours later, the cells were collected and the cytokines mRNA expression evaluated by Real Time RT-PCR. The bars represent the ratio±SD of the arbitrary units of PAM+NE treated cells over that of PAM treated cells of 5 independent experiments. In all experiments, the arbitrary units were calculated using the gene expression of DCs incubated with culture medium alone as reference. Twofold increase and 0.5 fold increase were taken as arbitrary threshold for biological significance.

The IL-10 mRNA expression was increased in DCs treated with PAM+NE compared to DCs treated with PAM alone (Fig. 26). This is in agreement with the effect on the IL-10 protein concentration in culture supernatants. Furthermore, there was no difference in gene expression of IL-12, IL-6 and IL-23 in the two groups. In accordance, IL-6 was not modulated by NE at the protein level. As far as it concerns IL-12 and IL-23, the protein concentration in the DC supernatants were under the lower detection threshold of the ELISA kit (Fig. 17, 19 and 20), indicating that these cytokines were not activated.

5.3.3 NE effect on cytokines mRNA expression in MDP stimulated DCs

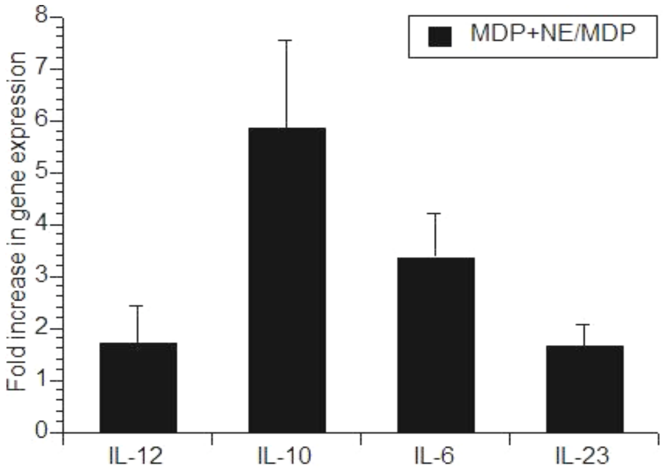

Figure 27: Effect of NE on cytokines mRNA expression in DCs incubated with MDP. DCs were incubated with 2µg/ml of MDP in presence or absence of 10^{-6}M NE. Three hours later, the cells were collected and the cytokines mRNA expression evaluated by Real Time RT-PCR. The bars represent the ratio±SD of the arbitrary units of MDP+NE treated cells over that of MDP treated cells obtained in 3 independent experiments. In all experiments, the arbitrary units were calculated using the gene expression of DCs incubated with the culture medium alone as reference. Twofold increase and 0.5 fold increase were taken as arbitrary thresholds for biological significance.

Consistently with the results obtained for IL-6 concentration in DC supernatants, IL-6 gene expression in DCs stimulated with MDP+NE was also increased compared to DCs stimulated with MDP alone. No difference in IL-12 and IL-23 gene expression was apparent between the two groups (Fig. 27). However, the production of these two cytokines in the culture supernatant was under the detection limit of the ELISA kit (Fig. 17 and 20). In contrast with the protein result, the IL-10 mRNA expression was increased in DCs incubated with MDP+NE compared to DCs incubated with MDP alone. This may be explained by the very low level of IL-10 gene expression induced by MDP in DCs (Table 7). In contrast with PAM stimulated DCs, where there was an effective increase in IL-10 mRNA and protein, the arbitrary units for the IL-10 gene expression in MDP treated DCs were not increased compared to control cells incubated with medium alone. Although the addition of NE may increment the arbitrary units of MDP treated cells, this increment was probably not sufficient for an effective increase of the IL-10 production at the protein level.

Table 7: NE effect on IL-10 mRNA expression in MDP and PAM stimulated DCs

MDP (AU)	MDP+NE (AU)	PAM (AU)	PAM+NE (AU)	Medium (AU)
1.60	6.63	46.52	233.94	1
1.59	9.09	43.26	148.57	1
2.64	20.11	21.70	183.54	1

DCs were incubated with 2μg/ml of MDP or 10μg/ml of PAM in presence or absence of 10^{-6}M NE. Three hours later, the cells were collected and the cytokines mRNA expression evaluated by Real Time RT-PCR. The table report the arbitrary units of three independent experiments. In all experiments, the arbitrary units were calculated using the gene expression of DCs incubated with the culture medium alone as reference (AU=1). AU: Arbitrary Units.

It has been reported that NE affect DCs mainly through β-ARs[54]. However, no evidence of a β-ARs modulation of the NOD2 signaling pathway has been reported. To investigate if NE increased IL-6 gene expression through a β2-ARs-dependent modulation of NOD2 signaling, DCs obtained from β2-AR gene deficient mice were incubated *in vitro* with MDP in presence or absence of NE. Three hours later, cells were collected for determination of the IL-6 gene expression by Real Time RT-PCR.

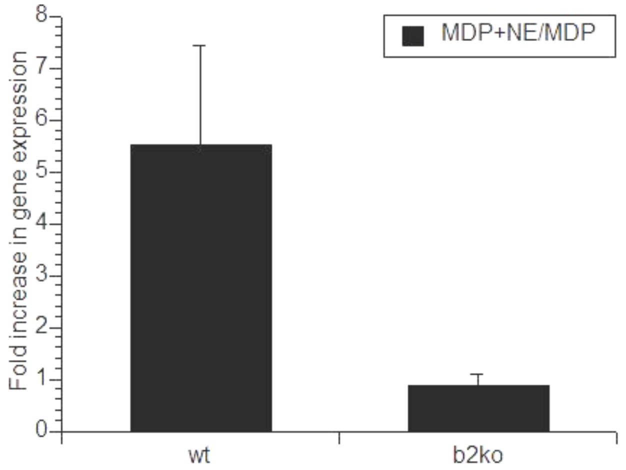

Figure 28: Effect of NE on IL-6 gene expression in wt and β2ko derived DCs stimulated with MDP. Wt or β2ko derived DCs were incubated with 2μg/ml of MDP in presence or absence of 10^{-6}M NE. Three hours later, cells were collected and the cytokines mRNA expression evaluated by Real Time RT-PCR. The bars represent the ratio±SD of the arbitrary units of MDP+NE treated cells over that of MDP treated cells obtained in 3 independent experiments. In all experiments, the arbitrary units were calculated using the gene expression of DCs incubated with the culture medium alone as reference. Twofold increase and 0.5 fold increase were taken as arbitrary thresholds for biological significance.

As expected, in contrast with wild type mice, where the addition of NE augmented the IL-6 gene expression, in β2ko derived DCs addition of NE to MDP treatment had no effect on the IL-6 gene expression (Fig. 28). This demonstrates that, as for others PRRs, NE modulated the NOD2 signaling in DCs by β2-ARs.

5.3.4 NE effect on cytokines mRNA expression in PAM+MDP stimulated DCs

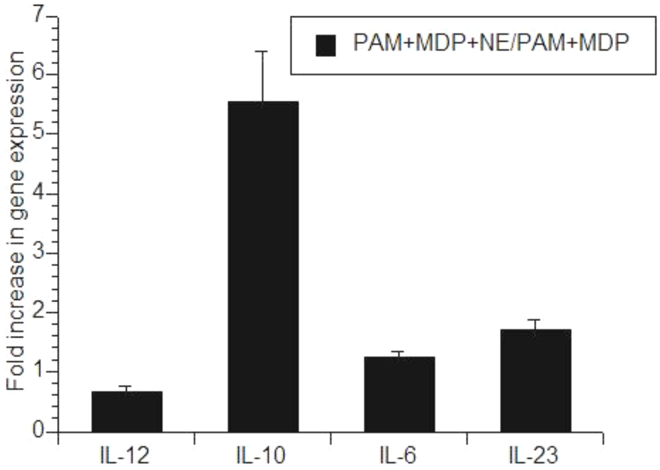

Figure 29: Effect of NE on cytokines mRNA expression in DCs incubated with PAM+MDP. DCs were incubated with 10μg/ml of PAM+ 2μg/ml of MDP in presence or absence of 10^{-6}M NE. Three hours later, the cells were collected and the cytokines mRNA expression evaluated by Real Time RT-PCR. The bars represent the ratio±SD of the arbitrary units of PAM+MDP+NE treated cells over that of PAM+MDP treated cells obtained in 3 independent experiments. In all experiments the arbitrary units were calculated using the gene expression of DCs incubated with the culture medium alone as reference. Twofold increase and 0.5 fold increase were taken as arbitrary thresholds for biological significance.

As in protein studies, IL-10 gene expression was increased in DCs incubated with PAM+MDP in presence of NE compared to DCs incubated with PAM+MDP alone (Fig. 29). No difference in gene expression was detected for IL-12, IL-6 and IL-23. These results are consonant with the protein determinations for IL-6 and IL-23 but not for IL-12. The absence of a IL-12 mRNA modulation by NE may be explained by the low level of IL-12 protein production in PAM+MDP±NE stimulated DCs (Fig. 17). The protein concentration is next to the detection threshold of the ELISA kit, so that the decreasing trend observed at the gene expression level for PAM+MDP treated DCs in presence of NE might be sufficient for a significant protein decrease.

5.3.5 NE effect on cytokines mRNA expression in LPS stimulated DCs

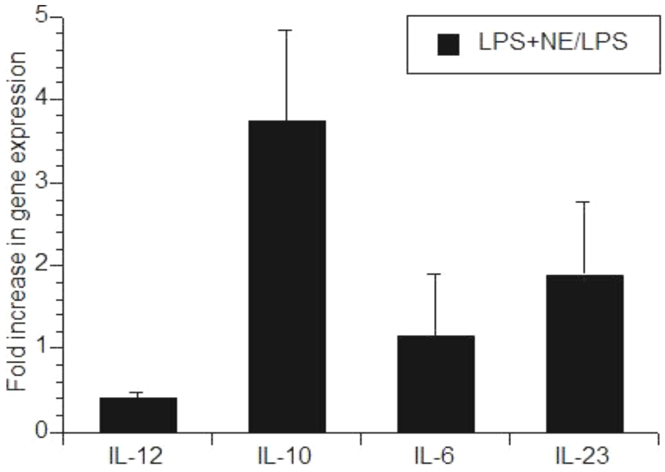

Figure 30: Effect of NE on cytokines mRNA expression in DCs incubated with LPS. DCs were incubated with 1μg/ml of LPS in presence or absence of 10^{-6}M NE. Three hours later, the cells were collected and the cytokines mRNA expression evaluated by Real Time RT-PCR. The bars represent the ratio±SD of the arbitrary units of LPS+NE treated cells over that of LPS treated cells obtained in 3 independent experiments. In all experiments the arbitrary units were calculated using the gene expression of DCs incubated with the culture medium alone as reference. Twofold increase and 0.5 fold increase were taken as arbitrary threshold for biological significance.

In agreement with our previous results, IL-12 and IL-10 gene expression were respectively decreased and increased in LPS+NE stimulated DCs compared to LPS treated DCs. However, in contrast with the results obtained for the protein concentration in DCs supernatant, no difference in gene expression was detected for IL-6 and IL-23 cytokines (Fig. 30).

5.4 Impact of β2-ARs activation in TLR-2 and NOD2 stimulated DCs on the adaptive response to a soluble protein upon adoptive transfer *in vivo*

It is widely accepted that IL-12 is crucial for the development of Th1 immune response while IL-6 and IL-23 are involved in the Th17 development[24]. To test if β2-ARs activation in TLR-2 and NOD2 stimulated DCs may influence the adaptive response to a soluble protein, DCs were incubated with PAM+MDP in presence of the soluble protein KLH and in presence or absence of the β2-AR agonist salbutamol. Three hours later, DCs were injected i.d. in the mice hint footpad and seven days later the IFN-γ and IL-17 production was assessed by ELISA in draining lymph node cells upon *in vitro* restimulation with KLH.

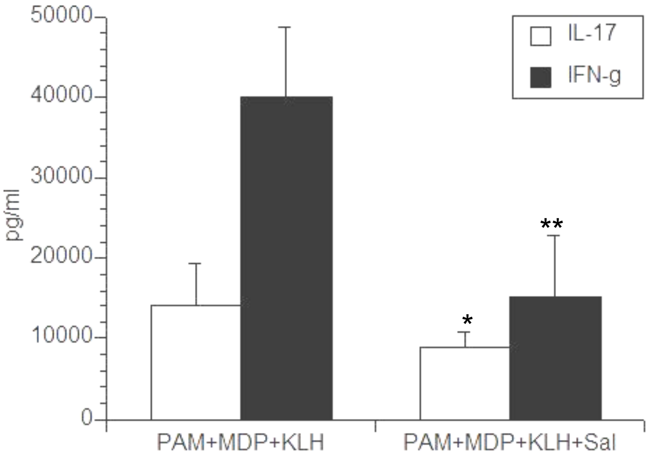

Figure 31: Effect of β2-ARs activation in TLR-2 and NOD2 stimulated DCs on the adaptive response to a soluble protein. DCs were incubated with 10μg/ml PAM+ 5μg/ml MDP+ 100μg/ml of the soluble protein KLH in presence or absence of 10^{-6}M of the β2-ARs agonist salbutamol. Three hours later, DCs were washed and injected in mice hint footpad and 7 days later the *in vitro* IL-17 and IFN-γ production in lymph node cells reincubated with 100μg/ml KLH was assessed by ELISA. The bars represent the mean of the cytokine production of 3 mice ±SD. (*p<0.05, **p<0.0001).

Consistently with our *in vitro* results, showing that addition of NE inhibited the production of the proinflammatory cytokine IL-12 but not that of IL-6 and IL-23 in DCs stimulated with PAM+MDP, here we found that PAM+MDP stimulation of DCs induced a Th17 response to the protein. Activation of β2-ARs in these DCs did reduce the Th1 priming but not the Th17 priming as assessed by the strong reduction of IFN-γ and the weak albeit significant effect on IL-17 production (Fig. 31). The ratio of IFN-γ over IL-17 was consequently strongly decreased in mice injected with DCs preconditioned with sabutamol (1.69) compared to mice injected with DCs preincubated with PAM+MDP+KLH (2.8).

VI. Effects of the salivary gland extract (EGS) from *Ixodes ricinus* on pDCs migration

Mice were injected i.d. in the pinna of the ear using an Hamilton syringe fitted with a 30.5 gauge needle with 5µg/mouse of PGN in presence or absence of 2µl/mouse EGS (generous gift of Prof. M. Brossard, University of Neuchâtel, Switzerland). Control mice were injected with EGS alone or with PBS 1X. Twenty-four hours later the auricular draining lymph node was collected for single cell suspension preparation and FACS analysis. Cells were labelled either with the PE-conjugated-m-PDCA-1 antibody, identifying pDCs, or with the PE-conjugated-CD8a antibody and the FITC-conjugated-CD11c antibody, identifying lymphoid DCs.

Table 8: In vivo migration of antigen-positive pDC subset upon skin preconditioning

Preconditioning	n	LN cells (x10^6)	CD8a+,CD11c+ (x10^3)	pDCs (x10^3)
PGN	8	9.4± 2.2	46.6± 14.2	67.4± 18.0
PGN+EGS	9	11.7± 2.6	59.7± 13.1	96.9± 27.5 a
EGS	6	5.8± 1.2	35.9± 10.7	46.9± 13.8
PBS	6	5.3± 0.9	34.6± 9.8	49.9± 10.6

The figure report the mean± the standard deviation; n is the number of mice per group. a: $p<0.05$.

Treatment of mice skin with PGN increased the migration of pDCs and CD8a+,CD11c+ cells into the auricular draining lymph node compared to control mice injected with EGS or PBS alone. However, addition of the salivary gland extract to the treatment significantly increased the number of pDCs, but not that of CD8+,CD11c+ cells. These results suggested, as our previous results showed in Table 4 and 6, that PGN and EGS exerts their effects either at local level, by influencing pDCs migration from the site of inflammation to the node, or at the draining lymph node level. In fact, the local induced cytokine/chemokines and PGN and EGS themselves might migrate from the site of injection through the lymphatic vessels into the node. There, they might influence the recruitment of pDCs from the blood into the node by inducing the expression of CXCR3 ligands and adhesion molecules on the high endothelial venules. This would also indicate that EGS might contain either some PAMPs or chemokines that would mediate the effect observed.

VII. Discussion

In our previous studies we demonstrated the existence of a β-ARs modulation of the skin innate and adaptive immune response; β-ARs blockade in mice skin enhanced the inflammatory cytokine and the IFN-β inducible chemokine gene expression in response to PGN but not to LPS. This had also an influence on the DTH response and on the cytokine production in the draining lymph node that switched towards the Th1 type. Moreover, the recruitment of pDCs into the draining lymph node was also increased. The use of a mixture of PGN and EGS also increased the pDCs migration compared to PGN alone (Table 8). This result suggested that EGS might contain either some PAMPs or chemokines that would act in synergy with PGN to influence the pDCs migration. These cells may be recruited to the draining lymph node either directly from the blood via the high endothelial venules or from the blood in the skin followed by migration in the lymph node.

We found that removal of the ear after skin preconditioning with PGN±PRO followed by OVA-Alexa 488 injection had no influence on the lymph node cellularity and on the recruitment of CD11c+, OVA+ cells and OVA+, pDCs, which were significantly increased compared to control mice injected with saline (Table 6). This suggests that preconditioning of the ear exerts a fast and direct influence on the draining lymph node possibly by direct PGN and OVA-Alexa 488 migration from the site of injection before ear removal or by projection of the local induced inflammatory cytokines/chemokines profile to the draining lymph node. Both mechanisms have been indeed described, exogenous substances injected in the skin and/or skin-generated cytokines or chemokines may directly reach the lymph node through the lymphatic conduit system[63]. Once in the node, PGN, cytokines and chemokines may affect the recruitment of blood cells and consequently change the lymph node cell composition.

Addition of PRO in the preconditioning before ear removal had no significant effect on recruitment of pDCs in contrast with the results obtained in intact mice (Table 6). This indicates that removal of the ear prevented the effect of PRO suggesting that pDCs may be recruited either from the blood or from the ear skin and that PRO affect especially the latter mechanism.

Taken together our *in vivo* findings show that NE exerts anti-inflammatory effects on the PGN induced cytokines production in DCs and confirm our previous results obtained *in vivo* and, in part, *in vitro*[54]. Here, the adrenergic activation along with PGN stimulation decreased the production of the inflammatory cytokines IL-12, IL-6 and IL-23 and increased that of IL-10. However, PGN is sensed not only by TLR-2 but also by other different PRRs, including the NOD2 receptor. Hence, using specific agonists for the TLR-2 and NOD2 receptors, we analyzed more in detail the NE modulation of the TLR-2 and NOD2 signaling pathway.

First of all, we confirmed that NE reduced IL-12 production in PGN treated DCs. In contrast, PAM or MDP stimulated DCs in presence or absence of NE practically did not produce IL-12. However, when a combination of PAM+MDP was used, there was an increased production of IL-12, which was just over the minimum detection threshold of the ELISA kit and was abolished by the addition of NE (Fig. 17). This suggested that both TLR-2 and NOD2 receptors were implicated in the PGN induced IL-12 production and that their agonists may operate synergistically. However, the IL-12 production observed in PAM+MDP±NE groups was considerably lower than in PGN±NE groups. This difference might be explained by a different receptor binding affinity of these compounds for TLR-2 and NOD2 receptors as well as by the ability of PGN to bind additional PRRs other than TLR-2 and NOD2. These results were further confirmed by the IL-12 gene expression in PGN±NE, PAM±NE and MDP±NE treated DCs. In PAM+MDP+NE, there was a decrease in IL-12 mRNA expression that, however, did not reach the arbitrary threshold for biological significance (0.5). The contrasting NE effect observed at the protein level might depend on the low concentration, next to the detection threshold, of IL-12 found in the culture supernatant. Possibly, the slight decrease observed at the gene expression level for PAM+MDP treated DCs in presence of NE was sufficient to induce a significant decrease of the protein concentration. Nevertheless, one should consider that values obtained under the detection threshold were not reliable.

Next, we demonstrated that the TLR-2, but not the NOD2, component of the PGN signaling is responsible for the increased IL-10 production induced by NE. In fact, NE was unable to enhance IL-10 production in MDP treated cells, while it strongly increased IL-10 production in PGN, PAM and PAM+MDP stimulated DCs (Fig. 18). These results were also confirmed by the cytokine gene expression analysis. NE amplified the IL-10 mRNA expression in DCs treated with PGN, PAM and PAM+MDP, indicating that NE may act on IL-10 production by modulating its gene expression. Contrary to what we expected on the basis of the protein results, NE also increased the IL-10 mRNA expression in MDP stimulated DCs. This apparent discrepancy might depend on the very low induction of IL-10 gene expression in MDP treated DCs. In fact, although NE augmented the IL-10 gene expression in MDP treated cells, the increment was minimal and probably not sufficient for an effective increase of the IL-10 production at the protein level. As a matter of facts, the arbitrary units relative to the

IL-10 gene expression in MDP+NE treated DCs were much lower than those induced by all other treatments in absence of NE (Table 7).

Afterwards, we demonstrated that NE might modulate the NOD2 signaling pathway affecting the production of IL-6. Indeed, MDP stimulated DCs significantly increased the IL-6 production in presence of NE. On the contrary, no influence of NE on PAM induced IL-6 production was found (Fig 19). This provided evidence of a NE dependent modulation of IL-6 production through NOD2 but not TLR-2 signaling pathway. These results were also confirmed by the increased IL-6 gene expression induced in MDP, but not in PAM, treated cells in presence of NE. This suggests that, as found for IL-10, NE may influence the NOD2-dependent IL-6 production by modulating its gene expression.

Although it has been reported that NE affect DCs mainly through β-ARs[52], until now, no evidence of a β-ARs modulation of the NOD2 signaling pathway was demonstrated. Therefore, we investigated if the increased IL-6 gene expression in MDP treated DCs in presence of NE was mediated by β2-ARs using DCs derived from β2ko mice. In effect, no difference in IL-6 gene expression was detected in DCs treated with MDP in presence or absence of NE. This result provided evidence of the involvement of β2-ARs in the NE modulation of the NOD2 mediated IL-6 production.

Surprisingly, in PGN stimulated DCs, NE exerted an inhibitory effect also on the production of IL-6. We hypothesized that this unexpected result could depend on the NE and TLR-2 induced increase of IL-10. In fact, it has been demonstrated that IL-10 may inhibit in a dose dependent manner the expression and production of IL-6[60,61,62]. Therefore, we wondered if the decreased IL-6 production in PGN+NE treated cells could be explained by an IL-10 inhibition of the NOD2-dependent IL-6 production. As previously reported, the IL-10 concentration in the supernatant of PGN+NE, but not of MDP+NE, treated cells was increased compared to DCs treated with the PAMPs alone. Possibly, this increment affected the IL-6 production by the NOD2 pathway. In fact, neutralization of IL-10 by an anti-IL-10 antibody significantly increased the IL-6 production in PGN stimulated DCs both in presence or absence of NE (Fig. 22). This demonstrated that the augmented IL-10 production in PGN+NE stimulated DCs was indeed responsible for the decreased IL-6 concentration in DCs culture supernatant. Moreover, neutralization of IL-10 in DCs incubated with PAM in presence or absence of NE had no significant effect on IL-6 (Fig. 23), further demonstrating that IL-10 may inhibit IL-6 production triggered by NOD2 but not by TLR-2 signaling. In contrast with PGN, NE had no significant effect on the PAM+MDP induced IL-6 production. Nevertheless, addition of the

IL-10 neutralizing antibody significantly increased IL-6 in PAM+MDP stimulated DCs in presence but not in absence of NE (Fig. 24). This seemingly contrasting results might be due to the concentration of IL-10 needed for an effective inhibition of IL-6. In fact, the IL-10 concentration in PGN stimulated DCs was three times higher than in PAM+MDP treated DCs. In addition, the PAM/MDP ratio used in our experiments was possibly different from the ratio between TLR-2 and NOD2 agonists in PGN and such a difference was probably in favor of NOD2. In fact, NE did not influence IL-6 production in PAM+MDP treated DCs even if the concentration of IL-10 was slightly increased, probably because of the high proportion of MDP compared to PAM.

In regard to the IL-6 gene expression in PGN, PAM and PAM+MDP treated DCs, we found that addition of NE to the culture medium had no significant effect on the IL-6 mRNA expression. This suggested that IL-10 may exerts its inhibitory effect on PGN treated DCs at a post-transcriptional level. Nevertheless, it is known that IL-10 exerts its anti-inflammatory effects indirectly primarily by inhibition of proinflammatory gene transcription. IL-10 activates the transcription factor STAT3, which may then activate the expression of other anti-inflammatory genes which subsequently may inhibit the proinflammatory genes[64]. Perhaps, in our case this anti-inflammatory genes might exerts their immunosuppressive action by inhibiting the IL-6 production at a post-transcriptional level.

Finally, we demonstrated that NE inhibits IL-23 in DCs incubated with PGN. PAM or MDP stimulated DCs did not produce substantial amounts of IL-23 either in presence or absence of NE. However, when DCs were incubated with PAM+MDP, an appreciably production of this cytokine was found, suggesting that, as for IL-12, PAM and MDP may act synergistically to induce IL-23 production (Fig. 20). Interestingly, addition of NE in DCs incubated with PAM+MDP had no significant effect on IL-23 production. Again, the difference between PGN and PAM+MDP treated cells might be due to the ability of PGN to bind to receptors other than TLR-2 and NOD2 and to the different proportion of TLR-2 and NOD2 agonists in PGN.

As for IL-6, we wondered if the decreased production of IL-23 might be due to the anti-inflammatory properties of IL-10. Neutralization of IL-10 in PGN±NE, PAM±NE and PAM+MDP±NE treated cells, significantly augmented IL-23 production suggesting that, in contrast with IL-6, IL-23 induced from TLR-2 activation might be inhibited by IL-10. Again, as demonstrated by the analysis of the mRNA expression and as hypothesized for IL-6, the inhibition of IL-23 by IL-10 might take place at a post-transcriptional level.

In conclusion, these results provided evidence of a discrete noradrenergic modulation via β-ARs of the cytokines production in DCs stimulated with different PRRs. Hence, we wondered if such a modulation could also influence Th development and differentiation during an immune response to a soluble protein. Our preliminary results clearly indicated that addition of the β2-ARs agonist salbutamol to DCs stimulated *in vitro* with PAM+MDP and loaded with KLH before injection, inhibited IFN-γ and, to a much lesser extent, IL-17 production in draining lymph node cells restimulated *in vitro* with the soluble protein (Fig. 31). Interestingly, although the IL-17 production was not increased, the ratio of IFN-γ over IL-17 switched in favor of IL-17. These results suggested that β2-ARs activation in DCs may selectively inhibit Th1 cell differentiation while sparing the TLR-2 and NOD2 induced Th17 cell polarization.

Thus, it is possible that in case of stimulating DCs with PGN instead of PAM+MDP, addition of β2-AR agonists might induce Th17 cell differentiation. Accordingly, our *in vitro* results showed that addition of NE to DCs stimulated with PGN reduced by 90% the IL-12 production, known to be essential for Th1 primig, while IL-6 and IL-23, which are involved in Th17 differentiation, were just reduced by 27% and 39% respectively. This modification of the cytokine pattern suggested that, as for PAM+MDP treated cells, in PGN stimulated DCs, β2-ARs may influence the balance between Th1 and Th17 cell differentiation in favor of Th17.

These results are in agreement also with our *in vivo* studies. In fact, preconditioning of mice skin with PGN+PRO significantly increased the gene expression of IFN-γ and of others proinflammatory cytokines. Moreover, the IFN-γ production in the draining lymph nodes assessed after mice skin preconditioning with PGN+PRO followed by injection of a soluble protein was also increased, while no effect on the IL-4 production was observed. IFN-γ, which is a Th1 cytokine, is known to exert an inhibitory effect on Th17 cell development. Thus, these results suggested that *in vivo* the Th1 polarization induced by β-ARs blockade was not at the expense of Th2 priming but rather at that of Th17 polarization.

When we tested *in vitro* the effect of the addition of NE on cytokines production in DCs incubated with LPS, we observed that the proinflammatory cytokines IL-12, IL-6 and IL-23 were decreased while the anti-inflammatory cytokine IL-10 was increased. These results were in contrast with our *in vivo* results, where the pharmacological blocking of the β-ARs along with TLR-4 activation by LPS had no effect on the cytokine gene expression in mice skin.

The different cytokines modulation in the *in vivo* and *in vitro* experiments may be due to the presence in the skin of many different cells types, which may react to β-ARs inhibition in a different way than DCs. For instance, TLR-4 and β-ARs may be expressed in mice skin on DCs but also on macrophages, mast cells, fibroblasts and endothelial cells. The different contribution of each cell subpopulation to the inflammatory response may thus account for the diverse results obtained *in vitro* and *in vivo*.

We observed also that LPS induced a greater production of IL-12 and IL-23 than PGN and that addition of NE to LPS treated DCs had a stronger effect on IL-6 and IL-23 production, reduced respectively by 45% and 53%, than in PGN treated DCs. This difference is probably due to the ability of PGN to bind to PRRs different from TLR-2, in particular to the NOD2 receptor. We demonstrated that activation of the NOD2 receptor in presence of NE increased IL-6 production. This might explain the minor reduction of IL-6 in PGN treated cells compared to LPS treated cells in presence of NE.

However, these results do not explain the different adrenergic modulation of PGN and LPS *in vivo* but suggests the existence of a possible difference in the β-ARs modulation. Maybe, in LPS treated cells β-ARs activation may decrease not only Th1 cell differentiation by inhibiting IL-12 but, in contrast to PGN, also the Th17 cell development by inhibiting IL-6 and IL-23. Further experiments are needed to verify this working hypothesis of Th differentiation ensuing upon PGN versus LPS injection.

VII. Conclusion

In conclusion, we supported our previous findings obtained *in vivo* (Results part I) by demonstrating that in DCs NE may differently modulate pro- and anti-inflammatory cytokines production according to the type of PRRs activation. In particular, we confirmed the inhibitory ability of NE on IL-12 production and we demonstrated that activation of the NOD2 receptor in presence of NE leads to an augmented production of IL-6. In contrast, activation of TLR-2 in presence of NE resulted in an increased production of the anti-inflammatory cytokine IL-10. Moreover, we demonstrated that the TLR-2 induced IL-10 might inhibit IL-6 and IL-23 production by exerting indirectly its action possibly at the post-transcriptional level.

IL-6 is a pleiotropic cytokine involved in many different mechanisms, notably in the inflammatory response, hematopoiesis and T cells differentiation. However, IL-6 has also been reported to be involved in many different diseases such as chronic inflammatory bowel diseases and rheumatoid arthritis. In Crohn’s disease and in ulcerative colitis, which are inflammatory bowel diseases, IL-6 may activate the transcription factor STAT-3 in T cells by binding the soluble IL-6 receptor. Then, STAT-3 may induce some anti-apoptotic factors resulting in T cell resistance to apoptosis. T cells may then accumulate in tissues and cause chronic inflammation[14]. In murine models of rheumatoid arthritis, IL-6 knock out mice failed to develop joint swelling and lesions characteristic of this disease[15]. Moreover high levels of IL-6 in serum were correlated with high arthritis indexes.

Thus, a deregulation of the mechanisms controlling IL-6 production may leads to an increased IL-6 concentration and signaling and to the development of related pathologies. Our findings may be relevant in understanding the mechanisms regulating IL-6 production in PRRs activated DCs and, in particular, they demonstrate the involvement of β-ARs. Consequently, these results may be helpful in our understanding of the pathogenesis of many diseases. In addition, IL-6, together with IL-23, has been implicated in the development of the newly discovered Th17 cell lineage. Most interesting, it has been reported that stimulation of the NOD2 receptor induced the production of IL-17 in human memory T cells[65]. Consistently, we found that activation of NOD2 receptor leads to IL-6 production, which may be increased by NE. Moreover, our preliminary results demonstrated that adoptive transfer of DCs stimulated *in vitro* in presence of the β2-AR agonist salbutamol influenced the Th differentiation by inhibiting the Th1 development in favor of Th17 cells. In addition, mice skin preconditioning with PGN+PRO followed by injection of a soluble protein increased the IFN-γ production in the draining lymph nodes probably affecting the balance between Th1 and Th17.

The Th17 cell lineage is known to be necessary in the immune response against extracellular bacteria and parasitic worms. Nevertheless, high levels of IL-17 production have been associated with the development of cancer and autoimmune and inflammatory diseases. For instance IL-17 is produced in rheumatoid arthritis synovium[66], psoriasis[67] and in mouse experimental autoimmune encephalomyelitis (EAE)[29] and collagen induced arthritis (CIA). Absence of IL-6, which is needed for Th17 priming, protected mice against both EAE and CIA[68,69]. It has also been reported that IL-23 and IL-17 are increased in human tumors and that IL-23 deficient mice develop a tumor resistance associated with a decreased inflammation to cutaneous chemical carcinogenesis[70].

Our *in vivo* and *in vitro* findings provide evidence of a β-ARs implication in modulating the Th lineage development by modulating the cytokines production upon different PRRs activation. Consequently, β-ARs might also be implicated in the development of cancer, autoimmune and inflammatory disease by promoting Th17 polarization. A deregulation of the adrenergic system might result in a deregulated response to bacteria involving an inappropriate Th development causing an increased risk for autoimmune or inflammatory diseases. In addition, our findings might provide new perspectives in the therapeutic utilization of β2-AR agonists and in the interpretation of their clinical efficacy. In conclusion, a complete understanding of the β-ARs influence on the Th differentiation might have a far reaching pathophysiological relevance.

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