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DISSERTATION

Investigation of neuro-immune interactions under the influence of dydrogesterone and interferon alpha

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°C: Degrees Centigrade

µg: Microgram

µl: Microliter

APC: Allophycocyanin

BSA: Bovine Serum Albumine

CD: Cluster of Differentiation

CGRP: Calcitonin Gene Related Peptide

CNS: Central Nervous System

EAE: Experimental Autoimmune Encephalomyelitis

ENS: Enteric nervous system

EPM: Elevated Plus Maze

FACS: Fluorescence Activated Cell Sorting

Fc: Fragmant crystallizable

FCS: Fetal Calves Serum

FITC: Fluoresceinisothiocyanat

FST: Forced Swimming Test

HBSS: Hank's Buffered Salt Solution

HPA axis: Hypothalamic-pituitary-adrenal axis.

ICAM-1: Intercellular adhesion molecule-1

IDO: indoleamine-2,3-dioxygenase

IFN: Interferon

IL: Interleukin

ip: Intraperitoneal

l: Liter

LFA-1: Leukocyte function-associated antigen-1

mg: Milligram

MHC: Major Histocompability Complex

ml: Milliliter

MS: Multiple Sclerosis

NE: Norepinephrine

PBS: Phosphate Buffered Saline

PE: Phyoerythrin

PerCP: Peridinin chlorophyll protein

RPMI: Roswell Park Memorial Institute

TCR: T cell receptor

Th: T helper

TST: Tail Suspension Test

U: Unit

VIP: vasoactive intestinal peptide

1. Summary

Nervous, immune and endocrine systems work together to maintain the homeostasis by using a large variety of mediators. Understanding the communication pathways of these systems may lead to more effective regulation of these systems in disease conditions. In this work, we investigated the mechanisms of communication between nervous, immune, and endocrine systems in two different models. In our first study, we investigated the endocrine regulation of nervous and immune systems. The neuropeptide substance P (SP) is involved in inflammation and pain perception via its high affinity neurokinin 1 receptor (NK-1R). Decreased pain sensitivity is found to be associated with high plasma progesterone levels. We hypothesized that progesterone may attenuate nociception and associated inflammatory response via NK-1R-dependent pathways. To address this, we incubated murine lymphocytes with progesterone derivative dydrogesterone. Subsequently, the expressions of NK-1R and T helper (Th1)-type cytokines were analyzed by flow cytometry. We found that dydrogesterone induced a decrease in the percentage of NK-1R+ lymphocytes *in vitro* and *in vivo*. Increase in Th2-type and decrease in Th1-type cytokines could be detected *in vitro*. Dydrogesterone injection was associated with an increased pain threshold. These results reveal that progesterone modulates the cross talk of the nervous, endocrine and immune systems in inflammation and pain. In our second study, we investigated neuro-immune interactions under the influence of interferon alpha (IFN-α). IFN-α is used in the treatment of many viral and malignant diseases. Although IFN-α administration is highly efficacious, treatment is often complicated by depression which may require discontinuation of the therapy. The mechanisms underlying IFN-α-induced depression are still not understood. We explored behavioural and immune effects of IFN-α administration in mice. BALB/c mice received daily injections of IFN-α. We monitored depression and anxiety-like behavior in mice using forced swimming test (FST), tail suspension test (TST), and elevated plus maze (EPM). We studied the expression of adhesion molecules on peripheral blood leukocytes, and analyzed the recruitment of lymphocyte subsets into the brain. IFN-α resulted in increased immobility in FST, without significant effects in TST and EPM. Increased percentages of NK cells and lymphocytes expressing the LFA-1 or Mac-1 were observed on blood lymphocytes. The percentages of CD4+ and CD8+ lymphocytes as well as LFA-1-expressing CD4+ and CD8+ lymphocytes were increased in the brains of IFN-α group. Our data suggest that

IFN-α leads to an increase in blood cells with migratory potential, accompanied by an increased number of lymphocytes in the brain, whilst the detectable modulation of the behaviour was rather modest. Taken together, our data from the two studies support the notion of reciprocal functional interactions between the nervous and immune systems.

2. Introduction

Survival of organisms in the presence of various internal and external challenges is maintained by a complex and dynamic equilibrium of the internal milieu, a process called homeostasis. Homeostasis is regulated by a large variety of mediators, including neurotransmitters, neuropeptides, integrins, cytokines, chemokines, hormones, and other molecules all of which are the products of mainly nervous, immune and endocrine systems. When homeostasis is disturbed, a range of molecular, cellular and behavioral responses arise. Various internal and external challenges such as infection, inflammation, autoimmunity, and mood disorders trigger those responses which results in the activation of immune, nervous, and endocrine systems. These systems attempt to counteract the disturbing factors in order to re-establish homeostasis through enhancement of memory, mobilization and replenishment of energy, and trafficking of immune cells to places in the body where they are needed to counter-balance different kinds of challenges. The two major systems of homeostasis, nervous and immune systems, appear to have different ways of organization (Haddad et al., 2002; Chapman et al., 2008).

2.1. Nervous system

The nervous system consists of two main divisions: the central nervous system (CNS) and the peripheral nervous system (PNS). The nervous system receives, and processes information from external and internal environment and regulates the responses according to the received information. The nervous system sends signals from brain to the peripheral tissues and receives signals from periphery to the brain along fixed pathways which are distributed throughout the body. The brain is among the key organs of homeostasis which works together with endocrine and immune systems to reestablish the balance; however brain has long been considered as an immunoprivileged organ. This consideration was largely based on the following observations: 1) passage of cells and molecules from the blood vessels into the brain is under strict control of the blood-brain barrier (BBB), a layer consisting of highly specialized cells; 2) T lymphocyte trafficking, a physiological surveillance function of the immune system is lower in brain tissue compared with other tissues; 3) in addition to diminished access of immune cells to the brain tissue, immune reactions such as antigen presentation or response to antigens occurs at very low levels in the brain; 4)

grafts implanted into the brain have longer survival times, indicating the lower immunoreactivity of the brain tissue; 5) microglial cells, the resident macrophage population of the brain, express low levels of major histocompability complex (MHC)-II, demonstrating lower antigen presentation functions in the brain tissue, and 6) dendritic cells, the professional antigen presenting cells are absent or at very low levels in the brain (Billingham and Boswell, 1953; Karman et al., 2004; Becher et al., 2006). These characteristics point out the restrictive nature of brain for the access and functions of the immune system.

2.2. Immune system

The immune system is the primary system of detection and defense for the threats arising from the environment. The cells involved in the immune response are organized into tissues and organs to perform their functions most effectively. The major lymphoid organs and tissues are classified into primary and secondary lymphoid organs. Thymus and bone marrow constitute the primary lymphoid organs which function mainly in the production and maturation of lymphocytes. Secondary lymphoid organs and tissues comprise spleen, lymph nodes, and mucosa associated lymphoid tissue (MALT), where antigens are gathered and presented to the naïve lymphocytes (Abbas and Lichtman, 2003). Consequently, naïve lymphocytes are activated following stimulation and differentiate into effector cells. Differentiated lymphocytes can be characterized by specific sets of cytokines that they secrete upon re-stimulation. For instance, naïve $CD4^+$ helper T cells can develop into at least 4 types of cells including Th1, Th2, Th17, and regulatory T cells. Th1-type cells secrete mainly inflammation promoting cytokines such as Interferon (IFN)-γ, interleukin (IL)-12, tumor necrosis factor (TNF)-α; whereas Th2-type cells secrete mainly anti-inflammatory cytokines such as IL-4, IL-10, IL-5, and IL-13. Th17 cells secrete mainly the proinflammatory cytokine IL-17, while regulatory T cells have anti-inflammatory effects and maintain tolerance to self components. The dominance of the Th1-type or Th2-type cytokines in an immune reaction determines the type, and character of the immune response. Moreover alterations in the levels of certain types of cytokines are found related to many diseases (Maier and Watkins, 1998; Chatila, 2005; Kulmatycki and Jamali, 2005; Afzali et al., 2007).

The immune system has also been regarded as a system working mostly autonomously. This view was based on the fact that the cellular components of

immunity require very little communication with cells of the body that are distant from the site of pathogen invasion. As opposed to the nervous system, immune system is a decentralized system, and the communication is carried out by mediators instead of neural wires. Trafficking of immune cells between the tissues also lacks a central organization, and immune system usually sustains a localized immune response. The activation of immune cells occurs at the site where effector actions are started, which is usually the place of tissue damage, inflammation, or antigen challenge (Abbas and Lichtman, 2003).

2.3. Interaction of nervous and immune systems

Due to the differences in their organization, nervous system and immune system have long been thought to function separately from each other. However, the nervous and immune systems are more similar than different in aspects of receiving, recognizing, and integrating signals and in their structural design for accomplishing these tasks. CNS and immune system have sensory elements, which receive information from the environment and other parts of the body, and motor elements, which carry out appropriate responses. Additionally, both can be characterized by their specificity, diversity and memory capacity. Accumulating evidence started to change the view that these systems function separately from each other (Fig. 1, modified from (Chapman et al., 2008))

2.4. Immune system interacts with nervous system

The immunoprivileged state of the brain has long been attributed to BBB and the lack of lymphatics in the brain tissue. However, the vigorous immune reaction observed following invasion of CNS by infectious agents, and the presence of peripheral immune responses directed towards CNS self-antigens in autoimmune conditions such as multiple sclerosis (MS) and experimental autoimmune encephalomyelitis (EAE) suggest the presence of an effective immune surveillance function in the brain tissue (Engelhardt, 2006). It is now known that leukocytes readily cross the BBB, and antigens in the brain drain into the cervical lymph nodes (Cserr and Knopf, 1992). Moreover, the neuro-immune communication is not limited only to immune surveillance functions, but also includes many other components of immunity, such as the effects of cytokines.

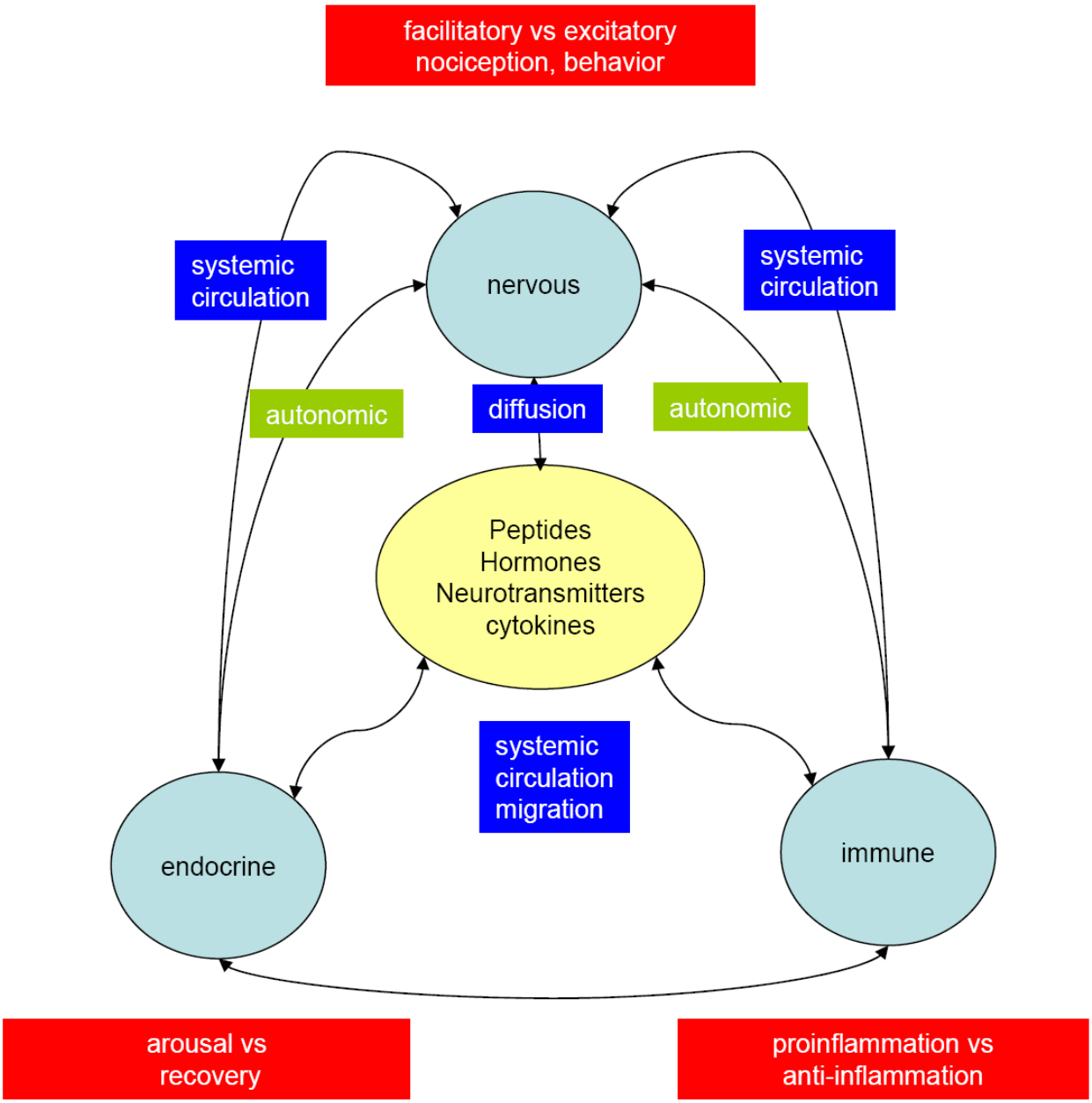

Fig. 1: Nervous, endocrine, and immune systems communicate dynamically using the language of common chemical substances. The major elements are peptides, hormones, neurotransmitters, and cytokines. These substances exert different effects depending on the context. Circulation, diffusion and migration are some of the processes of information transmission. Systemic circulation and autonomic nervous system activity are other vehicles of information transmission. Because the nervous, endocrine, and immune systems have constant reciprocal communication, they tend to react to a stressor in a highly orchestrated manner, as a single unit (modified from (Chapman et al., 2008)).

Locally or systemically released cytokines can affect nervous system functions in different ways: 1) under normal conditions, BBB protects the CNS from passage of potentially dangerous molecules but also from the cytokines that come from blood. However, certain cytokines can pass through leaky areas of the brain and induce CNS responses. Particularly, TNF- α, IL-1 and IL-6 have the ability to induce both sympathetic nervous system and hypothalamic-pituitary-adrenal (HPA) axis activity (Cassidy and O'Keane, 2000). These cytokines induce secretion of corticotrophin releasing factors which in turn stimulate adrenal corticosteroid production. 2) Additionally, BBB may become more permeable during inflammatory conditions, which causes cytokines to gain access to the brain (Persidsky et al., 2006). 3) On the other hand, cytokines bind to their receptors in the linings of the brain blood vessels and stimulate the release of secondary chemical signals into the brain tissue (Hashimoto et al., 1991; Wong et al., 1995). 4) Cytokines can also signal the brain directly via neural routes. For instance, injection of IL-1 into the abdominal cavity activates the nucleus tractus solitarius, the principal region of the brain stem for receiving visceral sensor signals (Goehler et al., 1998), and cutting the vagal nerve results in blocking the IL-1 mediated activation of this nucleus (Maier et al., 1998). Therefore, immune cells and their products have access to the nervous system, and interact with many aspects of nervous system functions. Behavior and pain response are important examples of the nervous system functions that can be modulated by immune system.

2.4.1. Immune system affects behavior

During infection and inflammatory conditions a wide range of responses including nervous, immune and endocrine systems arise which results in endocrine, immune, autonomic and behavioral changes. The patient feels tired, loses appetite and interest, feels depressed, irritable, and experiences attention difficulties, and memory loss. These symptoms resemble the symptoms of depression (Dantzer, 2006). This response is mainly mediated by proinflammatory cytokines. Certain proinflammatory cytokines such as IL-1, IL-6, TNF-α, and soluble IL-2 and IL-6 receptors are found elevated in depressed patients (Maes et al., 1991; Maes et al., 1993; Maes et al., 1995; Muller and Ackenheil, 1998; Anisman et al., 1999), and the magnitude of the cytokine secretion is associated with the severity of depression (Maes, 1999). Furthermore, stimulation with several cytokines and other immune challenges provokes depressive-like

symptomatology. For instance, healthy volunteers who receive a low dose of LPS or are vaccinated with live attenuated rubella virus display depressed mood, anxiety, and memory deficits up to 10 weeks following the challenge (Yirmiya et al., 2000). Immune challenges with cytokines are used in medical practice for therapeutic purposes. For example, IFN-α, IL-2, and TNF-α are used in the treatment of many types of cancers and viral infections. However, treatment with those cytokines triggers severe depression and many other psychiatric side-effects, complicating the treatment (Schleifer et al., 1999; Yirmiya et al., 2000). Therefore, it is essential to perform therapeutic drug monitoring and to investigate behavioral changes in these patients. Nevertheless, understanding the underlying mechanisms of cytokine-induced behavioral changes may result in the possibility of effectively protecting or reducing the psychiatric side-effects of these cytokines.

2.4.2. Immune system affects pain response

Pain can be defined as an unpleasant perception of a nociceptive sensation. Nociception results from the activation of primary sensory neuron populations that transmit the nociceptive information to the spinal cord from where it is relayed to the supraspinal levels. Stimulation of sensory nerve endings results in the pain perception. Stimulants that have the potential to cause tissue damage such as heat, oxygen deprivation, exposure to chemicals sensitize nociceptors in such a way that previously slight or ineffective stimulations become painful. The sensitization of nociceptors following injury or inflammation results from the release of a variety of chemicals from the damaged cells and tissues in the vicinity of the injury. These substances include bradykinin, histamine, prostaglandins, leukotriens, acetylcholine, serotonin, and Substance P (SP) (Verri et al., 2006). Those substances originate from different population of cells, but all act to decrease the threshold for activation of nociceptors. SP is a peptide synthesized by the nociceptors themselves, and transported to the peripheral terminal, where they are stored and released upon depolarization of the terminal. Injury/inflammation leads to the release of SP and calcitonin gene-related peptide (CGRP) from nociceptive nerve endings. These two neuro-peptides contribute to the spread of edema by causing vasodilatation. Activation of one branch of a nociceptor axon can lead to the secretion of SP by the other branches of that axon in the neighboring skin. SP causes vasodilatation and the release of histamine from mast cells (Brain et al., 1995). Local administration of SP can reproduce all the symptoms of

inflammation, heat, redness, and swelling. Since this inflammation is mediated by neural activity, it is referred to as neurogenic inflammation. Antagonists of SP can completely block neurogenic inflammation in humans (Agro and Stanisz, 1993; Kataeva et al., 1994; Jacoby et al., 2000).

During pain processing, the nervous system interacts effectively with the immune system. It is known that cytokines constitute a link between cellular injuries, immunological recognition and the local or systemic signs of inflammation (Verri et al., 2006). Indeed, it has been shown that a cascade of cytokines/chemokines precedes the release of nociceptive mediators (Cunha et al., 1999a; Lorenzetti et al., 2002). It is also reported that cytokines affect nociception pathways. For instance, inflammatory cytokines such as TNF-α and IL-1 increase nociception (Clatworthy, 1998; Clatworthy and Grose, 1999), while anti-inflammatory cytokines IL-4 and IL-10 reduce inflammation and nociception (Mertz et al., 1994; Poole et al., 1995; Cunha et al., 1999b; Cunha et al., 1999a). Additionally, immune cells also contribute to the pain processes, for example macrophages are reported to contribute to the nerve injury-induced pain responses (Watkins and Maier, 2005), and this response can be altered by delaying the recruitment of macrophages to the injury region (Myers et al., 1996). Conversely, pain is enhanced by attracting activated macrophages to the injured nerve (Clatworthy et al., 1995). Evidence implicates proinflammatory cytokines as key immune molecules in these pathological changes (Kleinschnitz et al., 2004; Sommer and Kress, 2004). Therefore, investigating the interactions between nervous system and immune system in pain processes may give us the opportunity to modulate the nociceptive responses in pathological conditions.

2.5. Nervous system interacts with immune system

Similarly, the immune system is also under the influence of the nervous system. Indeed, most of the primary and secondary lymphoid tissues receive extensive innervations predominantly from the sympathetic nervous system. Upon stimulation of these sympathetic nerve terminals located in lymphoid tissues, norepinephrine (NE) is released. Target immune cells in lymphoid tissues express NE receptors. Leukocytes also express a wide range of receptors for neurotransmitters, neuropeptides and hormones (Tanriverdi et al., 2003; Nance and Sanders, 2007). Therefore, the type of response to these molecules varies between different lymphocyte and monocyte

populations, such that the effect of different transmitters may vary in different circumstances. Stimulation of these receptors, via locally released or systemically circulating catecholamines, affects the function of these cells resulting in alteration of lymphocyte trafficking, circulation, proliferation, and cytokine production (Elenkov et al., 2000).

In addition to the catecholaminergic innervation, lymphoid organs receive sensory peptidergic innervations, specifically SP, neurokinin A, CGRP, and vasoactive intestinal peptide (VIP). There is a close spatial relationship between peptidergic nerve fibers and immune cells, especially mast cells, T cells, and macrophages. These close contacts form a general structural pattern in all lymphoid tissues. Furthermore, neuro-mast cell, neuro-macrophage, and neuro-T cell contacts, are not restricted to the lymphoid tissues, but are also regularly encountered in all somatic and visceral tissues. In general, innervating nerves of an organ have important functions in the establishment of inflammatory reactions (Baluk, 1997). Supportingly, stimulation of sensitive nerve fibers with a stimulant results in an inflammatory response in a large variety of tissues such as skin, eye, dental pulp, joints, and lung (Foreman, 1987; Meggs, 1993; Arnalich et al., 1994; McKay and Bienenstock, 1994). Moreover, the intensity of the inflammatory response is regulated by the innervating nerves. For example, arthritis can be induced more intensely in the joints which have denser innervations, and when the innervating nerve of the inflamed joint is cut, arthritis can be no longer induced in that joint (Levine et al., 1984; Levine et al., 1987).

Neuropeptides especially SP released from the nerve terminals play important roles in the modulation of inflammation (Otsuka and Yoshioka, 1993). Application of SP to the skin results in inflammation and trafficking of lymphocytes to the region of inflammation (Brain et al., 1995). Additionally SP contributes to the inflammation by causing the formation of small gaps between vascular endothelial cells (Green et al., 1993). SP executes its inflammatory functions through its receptors, predominantly NK-1 receptor (NK-1R). Lymphocytes, macrophages, and mast cells express receptors for SP, and they can be stimulated by SP to produce cytokines (Ho et al., 1997; De Giorgio et al., 1998; Tripp et al., 2002). SP increases vascular permeability and increases the migration of leukocytes into the inflamed tissue (Pernow, 1983). The trafficking of leukocytes to inflamed areas can be enhanced by SP-induced expression of adhesion

molecules on leukocytes. Furthermore, it has been demonstrated that SP can induce the expression of adhesion molecules Mac-1 (CD11b) on neutrophils, and ICAM (intercellular adhesion molecule)-1and leukocyte function-associated antigen (LFA)-1 on endothelial cells and lymphocytes, respectively (Matis et al., 1990; DeRose et al., 1994; Vishwanath and Mukherjee, 1996). SP is also present in the nervous system and is widely distributed in CNS, PNS and enteric nervous system (ENS) (Black and Garbutt, 2002). Expression of SP receptor in the brain are suggested to be related to behavior and stress responses (Santarelli et al., 2002). SP is also involved in nociceptive systems in the dorsal horn of the spinal cord (Black and Garbutt, 2002). Taken together, SP and its receptor NK-1R which are distributed throughout the tissues and cells of the immune system, CNS, PNS and ENS, play important roles in behavior and perception of pain.

2.6. Neuro-immune-endocrine interaction

Nervous-immune system interactions are modulated and fine-tuned by the endocrine system. Many hormones are implicated in the process of neuro-endocrine-immune communication. Most of the hormones have some influences on immune system functions (McEwen et al., 1997). Indeed, catecholamines and glucocorticoids influence the trafficking of immune cells and modulate the type of immune response by influencing cytokine release from leukocytes (Engelhardt, 2000). These actions are carried out by either direct modulation of immune cells, or by their direct interaction with neurons (McEwen et al., 1997).

The sex steroids progesterone and estrogen are key to the sexual dimorphism present in immune responses (e.g., increased incidence of autoimmune disease in females) and to changes in immune status which occur, for example, during pregnancy. The sex steroid progesterone is also referred to as a neurosteroid and an immunosteroid due to its potent effects on nervous and immune systems (Miyaura and Iwata, 2002). Progesterone supports the production of anti-inflammatory Th2-type cytokines (IL-4, IL-10). The balance between Th1 and Th2-type cytokines is crucial for successful pregnancy. An inflammatory response mediated by Th1 cytokines (IL-2, TNF-α, IL-12, IFN-γ) is suggested to have deleterious effects on mammalian pregnancy, while anti-inflammatory Th2 cytokines (IL-4, IL-10) appear to be pregnancy protective by supporting the immune tolerance to the fetus and inhibiting the fetal rejection (Blois et

al., 2005). In addition to its immune effects, progesterone has neuroprotective effects, induces neurogenesis, and alters memory and cognitive functions (Brinton et al., 2008). On the other hand, progesterone can also be affected by the nervous system, for instance, psycho-emotional stress is known to inhibit the female reproductive system primarily through inhibition of progesterone production (Wiebold et al., 1986; Chrousos et al., 1998). Further, in a murine stress-triggered abortion model, it is demonstrated that stressed animals show lower systemic levels of progesterone and a reduced expression of progesterone receptor at the feto-maternal interface, and injection of the progesterone derivative dydrogesterone, a progesterone with a binding profile highly selective for the progesterone receptor, can inhibit the stress induced effects on those mice (Joachim et al., 2003). The dorsal horn of the spinal cord, which is important in the transmission of pain stimuli, is also known as a neurosteroidogenic center. Enzymes converting progesterone to its neuroactive metabolites are found in this region (Patte-Mensah et al., 2004). However, the action mechanism of this steroid hormone regarding pain perception remains unknown.

2.7. Aims: Study of the interaction mechanisms of neuro-immune-endocrine systems

As described above, it is widely accepted that neuronal, immune, and endocrine systems can modulate one another. Understanding the communication pathways may lead to more effective regulation of these systems in disease/ disorder conditions. In this study, we therefore aimed to investigate the mechanisms of communication between nervous, immune, and endocrine systems in two different models.

Study 1: Investigation of neuro-immune interactions under the influence of dydrogesterone: We hypothesized that progesterone may attenuate nociception and associated inflammatory response via NK-1R dependent pathways. In order to investigate this hypothesis, we employed a progesterone derivative, dydrogesterone, to investigate the endocrine regulation of nervous and immune systems. We investigated the effect of dydrogesterone on nociception, on NK-1 receptor expression and on the immune system in terms of cytokine expression.

Study 2: Investigation of neuro-immune interactions under the influence of IFN-α: IFN-α is used in the treatment of many viral (e.g. Hepatitis C) and malignant diseases due to

its antiviral and antiproliferative effects. Although IFN-α administration is highly efficacious, treatment is often complicated by psychiatric side effects. Depression is observed in 20-40 % of the patients receiving IFN-α, and may require discontinuation of the therapy (Bonaccorso et al., 2002a; Schaefer et al., 2003b). However, the underlying mechanisms of IFN-α-induced behavioral changes are unknown. Therefore, we aimed to model cytokine-induced behavioral changes in mice. We administered IFN-α to mice and monitored anxiety-like and depression-like behavior. Then, we investigated the changes in the immune system, regarding SP-NK1R expression, cytokine secretion profiles and leukocyte trafficking into the brain as potential underlying mechanisms of IFN-α-induced behavioral changes.

3. STUDY 1: INVESTIGATION OF NEURO-IMMUNE INTERACTIONS UNDER THE INFLUENCE OF DYDROGESTERONE

3.1. Introduction

The immune, nervous and endocrine system can each be characterized by its specificity, diversity and memory capacity (Vitetta et al., 1991; De Kloet, 2004). So it seems not surprising that an intense cross-talk between these 'super-systems' has evolved, reflected by an exponentially rising number of publications (Licinio and Frost, 2000; Sakane and Suzuki, 2000; Espinosa and Bermudez-Rattoni, 2001; Corcos et al., 2002; Haddad et al., 2002; Beishuizen and Thijs, 2003; Brazzini et al., 2003; Dhabhar, 2003; O'Connor et al., 2004). The cross talk between these 'super-systems' is now known to be possible due to a common 'chemical language', e.g. by the expression of receptors for endocrine (Baulieu and Schumacher, 2000) or neural mediators on immune cells (Haddad et al., 2002), or by the receptor presence for segregates from immune cells on nerve fibers (Steinhoff et al., 2003).

Nervous, immune, and endocrine system functions are modulated by an intense cross-talk between these systems. Particularly, Th1 dominated neurogenic inflammation is an intriguing example of such a cross talk between systems. Here, the activation of sensory nerve fibers innervating the tissue causes secretion of active neuropeptides, e.g. SP, into the microenvironment (Steinhoff et al., 2003; Liddle and Nathan, 2004). Upon release, SP triggers inflammatory processes via interaction with endothelial, arterial and immune cells (Maggi, 1995). SP triggers the secretion of IL-1, IL-6, IL-10, IL-12, TNF-α from monocytes, and IL-2, IL-4, IL-10, IFN-γ from T cells (Lotz et al., 1988; Laurenzi et al., 1990; Ho et al., 1996; Kincy-Cain and Bost, 1997; Levite, 1998). In turn, the SP-triggered release of pro-inflammatory cytokines from a variety of immune cells activates pain-responsive sensory nerve terminals, hence facilitating pain perception (Watkins and Maier, 2002). SP has the highest affinity for NK-1R (Laird et al., 2000), which was found to be upregulated during inflammation (Adcock et al., 1993; Goode et al., 2003), and NK-1R knockout mice have suppressed immunity (Svensson et al., 2005). Blockade of NK-1R could result in an overall immunosuppressant effect, which may also lead to a decreased pain perception (Quartara and Maggi, 1998). Further, SP could be involved in the sensitization of primary afferent nerves during inflammation (Heppelmann and Pawlak, 1997). To date, our understanding of cross-talk between

immune system and pain pathways *in vivo* is still limited; and only little information is available on the expression and-likely more important- the modulation of NK-1R expression.

Highly conserved hormones like progesterone (Baker, 1997) ensure that the existence of mankind is protected, so it seems likely that progesterone -in addition to its role in gestation- has effects on primary non-gestation related tissues and systems. Indeed, due to its modulatory function in the nervous system, progesterone is now also being referred to a neurosteroid (Mensah-Nyagan et al., 1999; Baulieu and Schumacher, 2000; Schumacher et al., 2000; Melcangi et al., 2005). Moreover, progesterone may even be referred to as an 'immunosteroid' since it alters the Th (T Helper) 1/Th2 ratio in favor of Th2, e.g. in stress triggered SP-mediated abortions (Moro et al., 2001; Miyaura and Iwata, 2002).

Besides natural progesterone which is produced and secreted in females by corpus luteum, placenta and in small quantities by adrenal cortex, there is a broad spectrum of steroids with progesterone-like actions which are derived from different parent compounds (Schindler et al., 2003). Close to the natural progesterone are retro-progesterones, e.g. dydrogesterone, which is a stereoisomer of progesterone with an additional double bond between carbon 6 and 7. Strikingly, dydrogesterone has the property to bind almost exclusively to the progesterone receptor (Schindler et al., 2003). Although the receptor binding affinity appears to be lower compared to progesterone, the equivalence dose of dydrogesterone is 10–20 times lower than progesterone, due to the ameliorated bioavailability and the progestogenic nature of the dydrogesterone metabolites (Schindler et al., 2003). Additionally, dydrogesterone is used as a medication. The main indication for treatment with dydrogesterone is menstrual cycle irregularity due to irregular corpus luteum functioning, as well as (together with estrogen) hormonal replacement therapy in the postmenopausal period.

Interestingly, progesterone metabolism actively takes place in the dorsal horn of the spinal cord. Since the dorsal horn of the spinal cord also has great importance in the transmission of the pain stimuli, this region is referred as a neurosteroidogenic center (Patte-Mensah et al., 2004).This neurosteroidogenic center synthesizes progesterone, and converts it to its active metabolites (Dawson-Basoa and Gintzler, 1997, 1998).

Moreover, the inflammatory mediator SP is found to inhibit the conversion of progesterone to its active metabolites in the dorsal horn of the spinal cord which was mediated through NK-1R (Patte-Mensah et al., 2005).

Considering the anatomical and functional interaction between SP-NK-1R system, progesterone, and the immune system, we hypothesized that the pain threshold might be affected by progesterone, and also the anti-inflammatory effects of progesterone may contribute to the pain process. Therefore, we decided to test this hypothesis by investigating the effects of the progesterone derivative dydrogesterone on pain perception, NK-1R expression and immune system. Because of its higher activity and therapeutic usage, we used dydrogesterone in our experiments. To address our hypothesis, we investigated the effects dydrogesterone on:

1) the NK-1R expression of cultured lymphocytes *in vitro*
2) the cytokine expression in lymphocytes *in vitro*
3) the NK-1R expression on blood and uterine lymphocytes *in vivo*
4) the NK-1R expression on peritoneal mast cells *ex vivo*
5) the perception of pain *in vivo*

3.2. Materials and Methods

3.2.1. Animals

Six to 8 weeks-old female mice of the CBA/J strain were purchased from Charles River (Sulzfeld, Germany). The mice were housed in community cages at the animal facility of Charité, Virchow Hospital (Berlin, Germany) with a 12:12 hour light/dark cycles. Animal care and experimental procedures were followed according to institutional guidelines and conformed to requirements of the state authority for animal research conduct (LaGeSo, Berlin, Germany). The detailed information on the chemicals, reagents, antibodies, equipment, and software used in this study can be found in the appendix section.

3.2.2. Cell culture

Female CBA/J mice were sacrificed and spleens were removed. Mononuclear cells were isolated from the spleens as previously described (Arck et al., 1995). Briefly, spleens were cut and passed through a 100 µm mesh (BD Biosciences, Erembodegem, Belgium) by using a plunger. The cells were collected in a petri dish containing Roswell Park Memorial Institute (RPMI) medium, washed, and mononuclear cells were separated under sterile conditions by density gradient centrifugation with Lympholite /M (Cedarlane Labs, Hornby, Ontario, Canada). Two hundred µl of cell suspension (10^4 cells /ml) were incubated per well under conditions of 5% CO_2 at 37 °C. Cells were cultured on a 96 well plate in RPMI media supplemented with 10 % fetal calves serum (FCS), streptomycin penicillin and 10^{-6}, 10^{-7}, 10^{-8} or 10^{-9} M respectively of dydrogesterone (9β,10α-pregna-4,6-diene-3,20 dione), also known as 6-dehydro-retroprogesterone, 10α-isopregnenone (Schindler et al., 2003), provided by Solvay Pharmaceutical, Hannover, Germany). Cell concentration and incubation times were modified according to published protocols (Franke et al., 2003). After 24 hours of incubation, cells were harvested from the wells and used for the flow cytometric analysis.

3.2.3. Preparation of uterine cell suspension

To obtain uterine cell suspension, a previously described method was used (Blois et al., 2004a). Briefly, uteri were removed, minced, collected in tubes containing Hank's buffered salt solution (HBSS), and digested for 20 min at 37°C under slight agitation in HBSS with 200 U/ml hyalurinodase (Sigma-Aldrich, St Louis, USA), 1mg/ml collagenase (Sigma-Aldrich, Steinheim, Germany), 0.2 mg/ml DNase I (Boehringer Mannheim, Germany), and 1 mg/ml Bovine Serum Albumin(BSA) /fraction V (Sigma-Aldrich, Steinheim, Germany). Thereafter, the isolated cells were collected in a fresh tube through a 100 um net (BD Biosciences) and washed with RPMI 1640/10% FCS. The procedure was repeated twice, with HBSS containing no mixture of enzymes. Cells were resuspended in Lympholite M solution and centrifuged at 800xg for 20 min at room temperature. The low-density fraction at the interface was collected and washed several times. The cell pellets were used for subsequent flow cytometry analysis.

3.2.4. Peritoneal Lavage and isolation of mast cells

In order to evaluate the expression of NK-1R on mast cells, peritoneal lavage was performed according to a published protocol using a specific mast cell medium (Silverman et al., 2000). Then, mast cells were separated by CD117-conjugated magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany). At the end of this process the purity of the enrichment was 70-80 %. Mast cells were cultured on a 96 well plate in RPMI media supplemented with 10 % FCS, streptomycin penicillin and 10^{-8} M dydrogesterone. 200 µl of cell suspension (10^6 cells /ml) were incubated per well under conditions of 5% CO_2, and 37 °C. After 24 hours of incubation, cells were harvested from the wells and used for the flow cytometric analysis by labeling with the mast cell marker CD117 (BD Biosciences) and NK-1R primary and secondary antibodies (van der Kleij et al., 2003).

3.2.5. Administration of dydrogesterone

CBA/J female mice were randomized into the following groups: 1) control mice injected with 200 µl sterile sesame oil, 2) dydrogesterone treated mice injected with 1.25 µg dydrogesterone, dissolved in 200 µl sterile sesame oil. Injections were performed subcutaneously in the dorsal neck region. The dydrogesterone dosage was selected according to the previous reports from our laboratory (Joachim et al., 2003; Blois et al.,

2004b). After 48 hours mice were sacrificed, blood samples were obtained by retro-orbital puncture and collected into tubes containing heparin. Following treatment with sterile ammonium chloride lysis buffer for 10 min to deplete erythrocytes, blood cells were washed twice with phosphate buffered saline (PBS), and the cell pellets were used for flow cytometric analysis.

3.2.6. Flow Cytometry

Flow cytometry was performed using our laboratory's standard protocol (Blois et al., 2004b). All monoclonal antibodies were purchased from BD Biosciences (see appendix). Briefly, isolated mononuclear cells were incubated for 3 hours with Brefeldin A (10^6 cells/ml medium with 1 µl of golgi plug ^{TM}, BD Pharmingen, Heidelberg, Germany) in RPMI and 10% FCS in a humidified incubator at 37 °C with 5 % CO_2. After that, the cells were washed and blocked with 2 % normal mouse serum to avoid nonspecific binding of Fragment crystallizable (Fc) receptors. Cells were incubated for 30 min at 4°C with antibodies against surface antigens (1:50, goat anti NK-1R antibody, Santa Cruz Biotechnology, Inc, Heidelberg, Germany, 1:100 Allophycocyanin (APC) conjugated donkey anti goat antibody Santa Cruz Biotechnology, Inc, Heidelberg Germany). Then, the cells were washed and fixed using a commercially available fixing solution (Becton Dickinson, Erembodegem, Belgium). Upon fixation, cells were permeabilized by using the Fluorescence Associated Cell Sorting (FACS) permeabilizing solution (Becton Dickinson, Erembodegem, Belgium) and then stained with intracellular antibodies (IL-4, IL-10, TNF-α, all purchased at BD Biosciences) using FACS permeabilizing solution. Controls cells were stained with the corresponding isotype matched monoclonal antibodies. The acquisition was performed using a FACSCalibur system (BD Biosciences). Instrument compensation was set in each experiment using single color stained samples. Data were analyzed by using Cell Quest software and expressed as percentage of cells positive for the surface marker evaluated.

3.2.7. Tail- Flick Test

Nociceptive thresholds to radiant heat stimuli were evaluated using an analgesiometer (IITC Life Sciences, Woodland Hills, CA, USA). The mice were placed on a glass

surface and a light beam was focused on the distal 2 cm of the tail (Fig. 2). The time mice take before flicking their tail away from the thermal challenge is referred to as the tail flick latency time. The latencies were measured before the drug application and in 30 minutes intervals until 180 minutes post-injection. A 10-second cut-off maximum was programmed into the timer to prevent tissue damage. Mice were habituated to the testing apparatus for 4 days prior to testing. An increase in the latency time compared with that of control was considered as hypoalgesia and a decrease in latency time was defined as hyperalgesia (Le Bars et al., 2001).

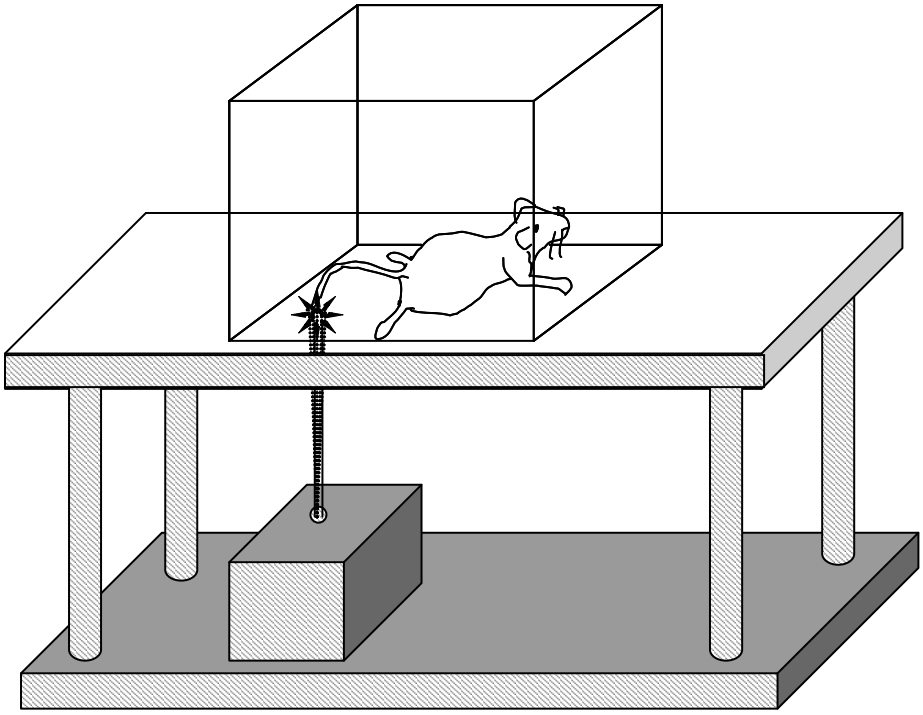

Fig. 2: Illustration of tail Flick Test. Nociceptive thresholds to radiant heat stimuli were evaluated using an analgesiometer. The mice were placed on a glass surface and a light beam was focused on the distal 2 cm of the tail.

3.2.8. Immunohistochemistry

Mice were anesthetized with a lethal dose of ketanest and perfused with a mixture of 4% paraformaldehyde and 14% saturated picric acid. Uteri were removed in order to perform fluorescent staining. Rabbit anti NK-1R primary antibody (Chemicon International Inc, CA, USA) was used in a 1/50 dilution for the detection of NK-1R expression on the cells. Rhodamine conjugated goat anti rabbit secondary antibody

(Jackson Immunoresearch, USA) was used in a 1/100 dilution for detection by fluorescent microscopy.

3.2.9. Statistics

Significance of differences among groups was determined using the non-parametric Mann Whitney U test. Significance was set at $p \leq 0.05$ (*) and $p \leq 0.01$(**). The results are illustrated in bars; box plots depicted similar trends.

3.3. Results

3.3.1. Dydrogesterone decreases the percentage of NK-1R$^+$ lymphocytes in vitro

To investigate the effects of dydrogesterone on the expression of NK-1R on lymphocytes, we cultured spleen cells with the progesterone derivative dydrogesterone for 24 h. A decrease in the percentages of NK-1R$^+$ splenic lymphocytes of dydrogesterone groups could be detected by flow cytometry, which reached levels of significance when the cells were incubated with dydrogesterone concentrations at 10^{-8} M and 10^{-9} M (p<0.05) (Fig.3)

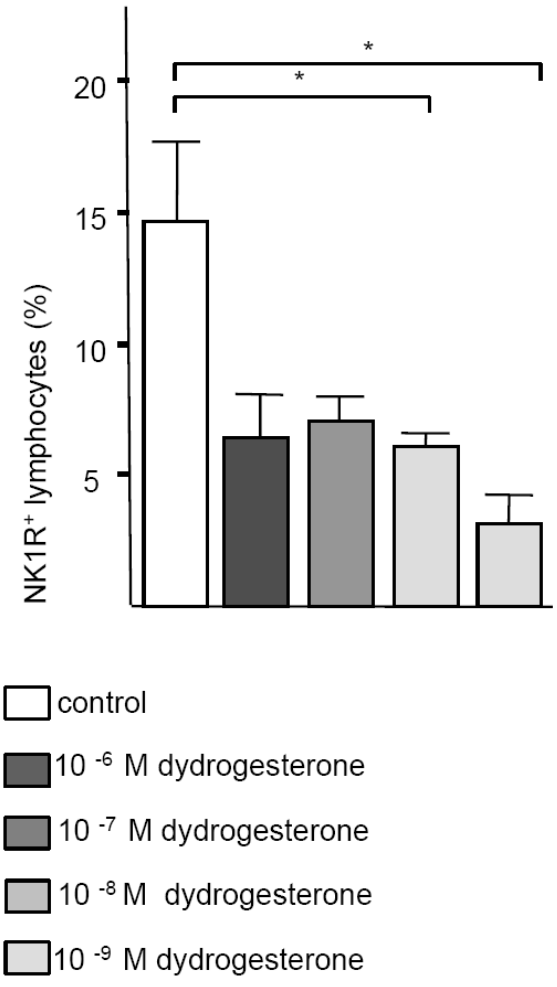

Fig. 3: *In vitro* effects of dydrogesterone on NK-1R expression on splenic lymphocytes. Dydrogesterone induced a decrease in the percentage of NK-1R$^+$ lymphocytes. Data are means ± SEM. y axis represents the percentage of NK-1R$^+$ lymphocytes in relation to the total number of lymphocytes. *p<0.05, as calculated by the Mann–Whitney U-test

3.3.2. Dydrogesterone skews the cytokine expression on lymphocytes towards a Th2 dominance in vitro

Further, the presence of cytokines in splenic lymphocytes cultured in the presence of dydrogesterone was detected by intracellular flow cytometry. As shown in Fig. 4A, coculture of lymphocytes with dydrogesterone significantly increased the percentage of IL-4^+ lymphocytes in all dydrogesterone concentrations tested ($p< 0.01$). Similarly, as presented in Fig. 4B, the percentage of spleen lymphocytes positive for additional Th2 type cytokine, IL-10, was also increased in spleen cells which were cultured in the presence of dydrogesterone. Levels of significance were reached for the dydrogesterone concentrations of 10^{-6}, 10^{-7} and 10^{-9} M ($p< 0.01$). Further, the percentages of Th1 type, TNF-α^+ splenic lymphocytes tended to be decreased following incubation with dydrogesterone without reaching statistical significance (Fig. 4C).

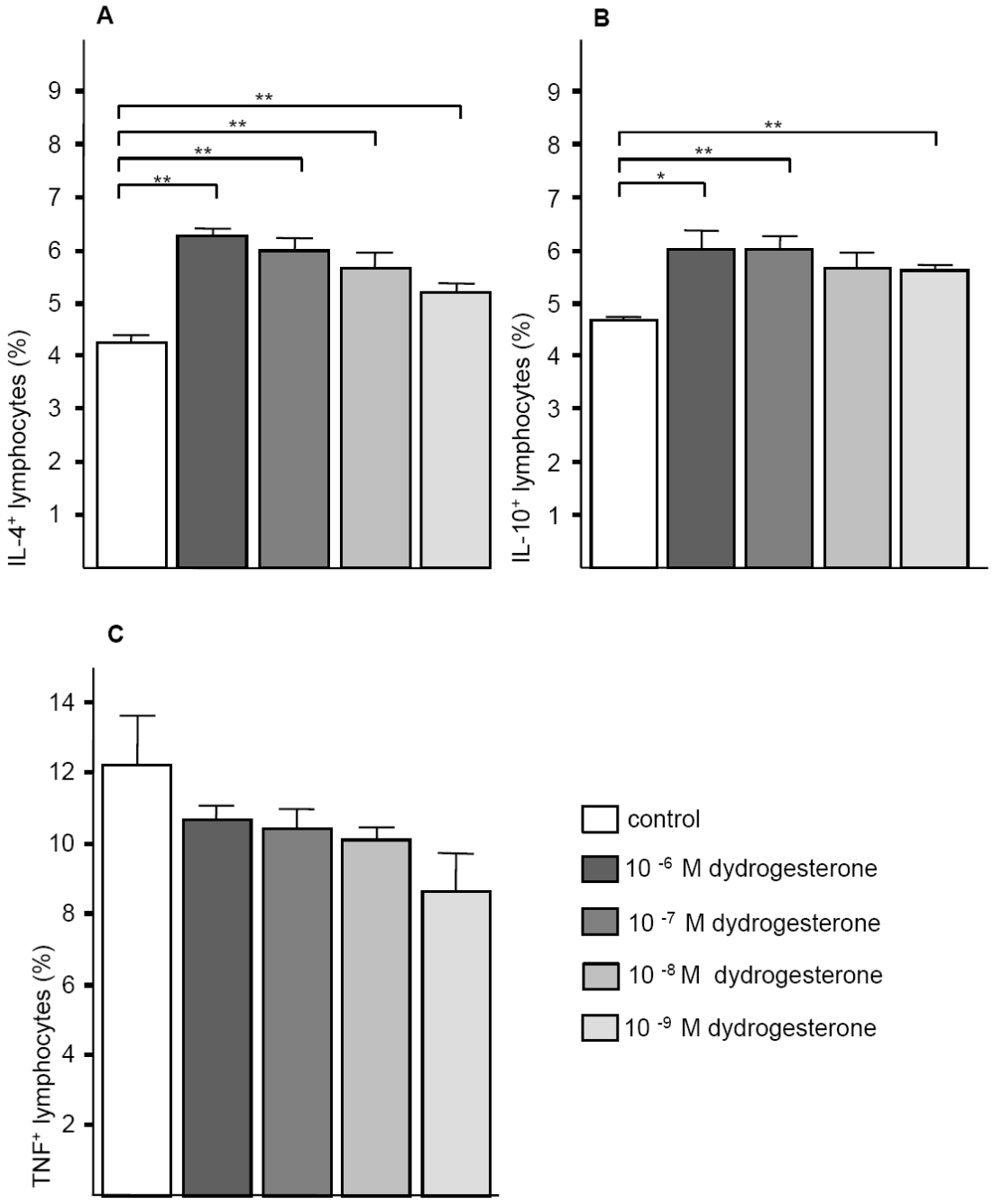

Fig. 4: *In vitro* effects of dydrogesterone on cytokine production in splenic lymphocytes. Dydrogesterone caused an increase in the percentage of the Th2-type cytokine IL-4 (A), and the percentage of IL-10$^+$ splenic lymphocytes (B), compared to lymphocytes cultured in the absence of dydrogesterone. Additionally, a trend towards decreased production of the Th1-type cytokine TNF-α was detected which did not reach levels of significance (C). Data are means ± SEM. y axis represents the percentage of lymphocytes expressing cytokines. *p<0.05 and **p<0.01, as calculated by the Mann–Whitney U-test.

3.3.3. Expression of NK-1R on lymphocytes was decreased upon dydrogesterone treatment in vivo

The effect of dydrogesterone on the expression of NK-1R on lymphocytes derived from blood and uterus was also analyzed by flow cytometry. As depicted in Fig. 5, dydrogesterone decreased the NK-1R expression on uterine lymphocytes *in vivo* ($p < 0.05$). Dydrogesterone also tended to decrease NK1-R expression on blood lymphocytes, but this did not reach significance.

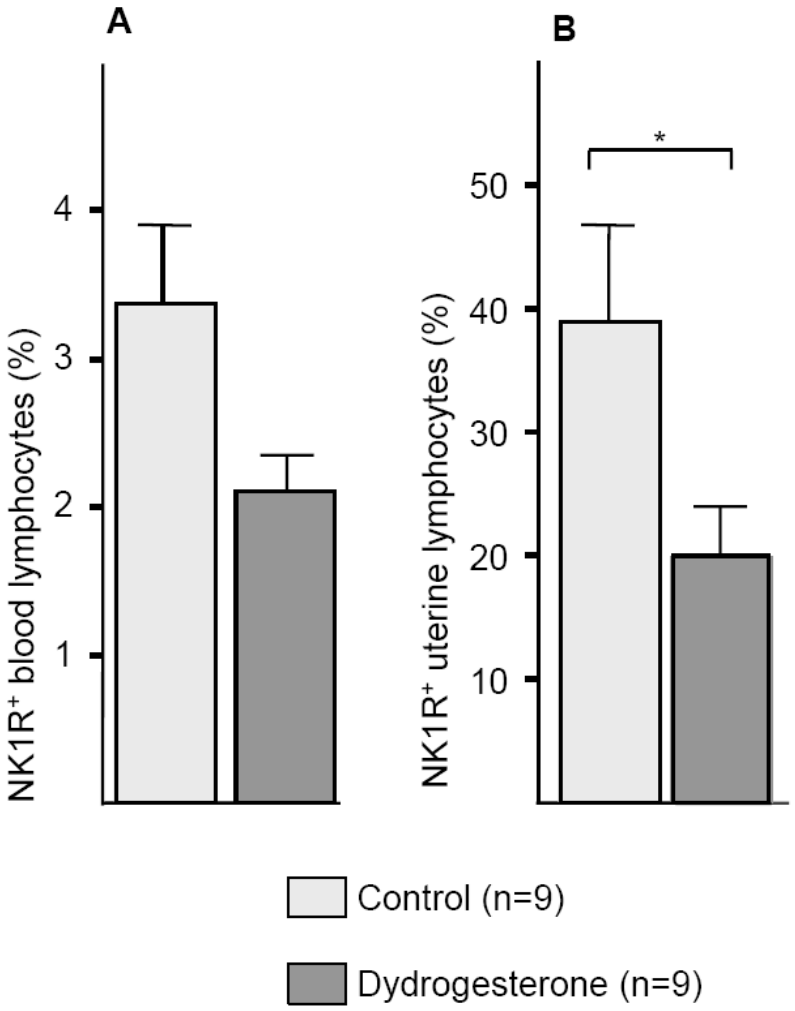

Fig. 5: *In vivo* effects of dydrogesterone on the NK-1R expression on lymphocytes. Dydrogesterone caused a trend towards lower percentages of NK-1R$^+$ blood-derived lymphocytes which did not reach levels of significance (A). In uterine derived lymphocytes, NK-1R expression was significantly reduced (B). Data are means ± SEM. y axis represents the percentage of NK-1R$^+$ lymphocytes, compared with the total number of lymphocytes. *p<0.05, as calculated by the Mann–Whitney U-test.

3.3.4. Uterine and peritoneal mast cells express NK-1R

Employing immunofluorescence staining, the presence of NK-1R$^+$ cells and their tissue distribution in the uterus could be independently confirmed. We observed NK-1R$^+$ epithelial cells (Fig 6A), as well as single resident mucosal cells, which can be identified as mast cells (Fig. 6B). Fig. 6C shows the negative control.

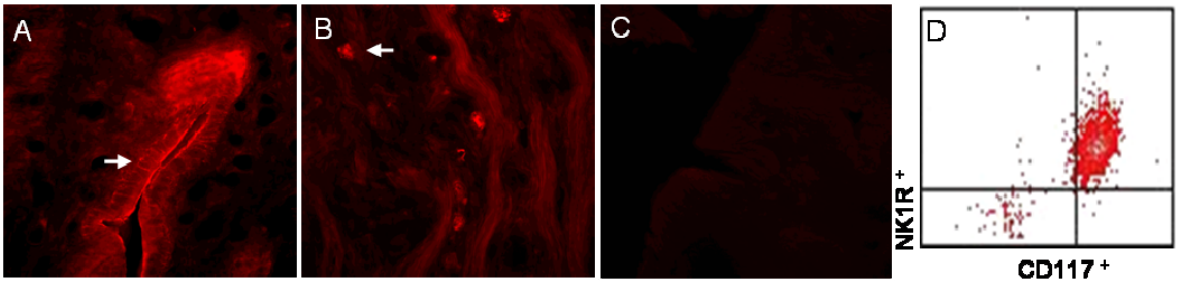

Fig. 6: Phenotype of NK-1R$^+$ cells. A depicts NK-1R expressing epithelial cells (arrow), B shows NK-1R$^+$ mast cells (arrow, further confirmation by streptavidin FITC antibody, data not shown), C is negative control and D reveals peritoneal mast cells expressing the NK-1R, by flow cytometry. (Since isolation and subsequent flow cytometric analysis of mast cells from the uterus is impaired by technical limitations, we decided to obtain mast cells by peritoneal lavage for pilot experiments on regulatory effects of dydrogesterone on mast cell NK-1R expression)

Our observation of NK-1R expression on mast cells compliments published data indicating that the NK-1R expression on mast cells is under the modulation of immune mediators (van der Kleij et al., 2003). Additionally, this prototypic representative population of innate immunity has been suggested to have a functional role when present in the uterus, e.g. in the onset of abortion (Marx et al., 1999; Joachim et al., 2001). Using Avidin-Fluoresceinisothiocyanat (FITC) as a specific marker for mast cells (Tharp et al., 1985), we could further confirm the presence of mast cells in the uterus (data not shown). Since isolation and subsequent flow cytometry analysis of mast cells from the uterus is impaired by technical limitations, we decided to obtain mast cells from peritoneal lavage to investigate the regulatory effects of the dydrogesterone on mast cell NK-1R expression. The majority of these peritoneal mast cells, which were identified by their CD117 positivity (Silverman et al., 2000), also revealed a NK-1R positivity (Fig 6D). We found no significant decrease in the NK-1R expression of CD117$^+$ cells following incubation with dydrogesterone (Fig 7).

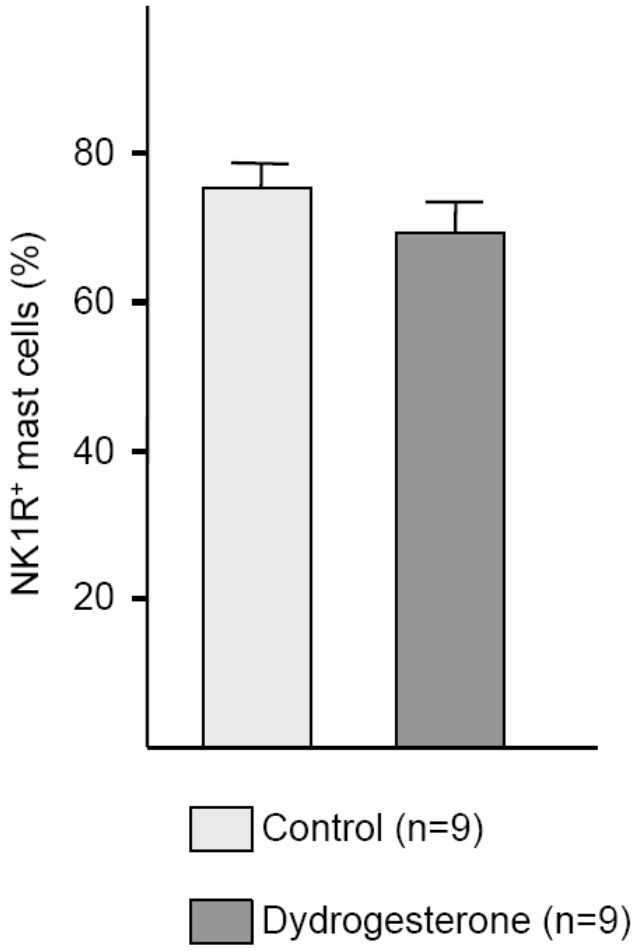

Fig. 7: In vitro effects of dydrogesterone on NK-1R expression on mast cells. Supplementation of dydrogesterone to the culture media did not result in a significant decrease in the percentage of NK-1R$^+$ lymphocytes. Data are means ± SEM. y axis represents the percentage of NK-1R+ mast cells, compared to the total number of mast cells.

3.3.5. Dydrogesterone reduces pain perception

In order to investigate the effect of dydrogesterone on the perception of pain, *in vivo* experiments were performed, employing a standardized and well-established protocol to evaluate nociception, the tail flick test. Strikingly, administration of dydrogesterone induced an increase in tail flick latency time, which reflects a reduced pain perception. As displayed in Fig. 8, this hypoalgesia effect upon dydrogesterone treatment reached levels of significance compared with sham-injected control mice at the time points of 120 and 150 minutes upon injection ($P< 0.05$), (Fig 8).

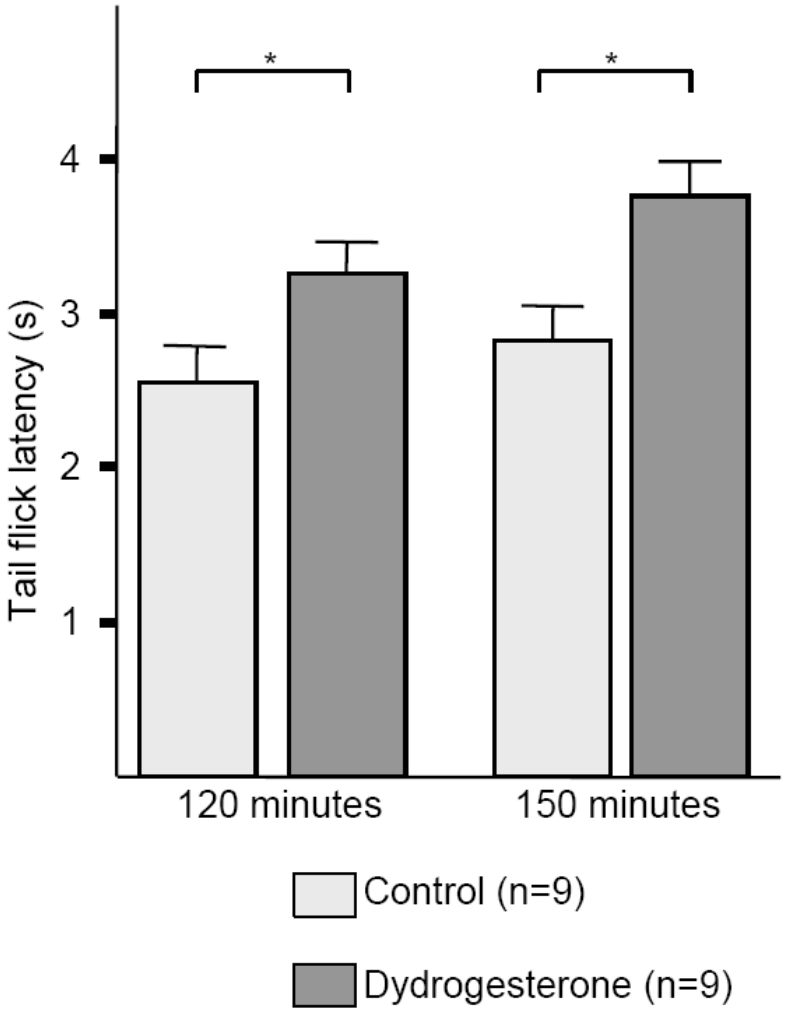

Fig. 8: Effects of dydrogesterone on nociceptive response. Application of 1.25-µg dydrogesterone caused increased tail flick latencies 120 and 150 min upon injections. Data are means ± SEM. y axis represents mean tail flick latencies in seconds (s) in dydrogesterone-treated and control mice. *P<0.05, as calculated by Mann–Whitney U-test.

3.4. Discussion

The present study provides evidence that dydrogesterone, a progesterone derivative, induces an immune bias towards anti-inflammation and results in hypoalgesia accompanied by a decrease in the percentage of lymphocytes expressing NK-1R *in vitro* and *in vivo*.

Here, we showed that dydrogesterone decreased the expression of NK-1R on lymphocytes. This finding on NK-1R protein expression compliments previous studies reporting molecular alterations of NK-1R mRNA expression triggered by corticosteroids, estrogen or progesterone (Ihara and Nakanishi, 1990; Adcock et al., 1993; Pinto et al., 1999). Corticosteroids reduce NK-1R mRNA in rat pancreatic AR42J (Ihara and Nakanishi, 1990) and human lung cells (Adcock et al., 1993). Similarly, NK-1R mRNA was decreased in rat uteri upon treatment with progesterone (Pinto et al., 1999). Moreover, it was shown that progesterone and estrogen regulate the expression of the NK-1R during the course of pregnancy in rat uteri (Candenas et al., 2001). These results suggest a modulatory role of progesterone on NK-1R functions, causing an alteration on SP binding. Additionally, progesterone affects NK-1R at transcriptional levels, changing the affinity and/or the density of SP binding (Bradesi et al., 2003).

Interestingly, we observed that lower concentrations of dydrogesterone decreased the percentage of NK-1R$^+$ lymphocytes more effectively than higher concentrations. This finding, although not reaching levels of significance, may be due to the chemical and pharmacological characteristics of dydrogesterone, since it has a better bioavailability and a lower equivalence dose than progesterone (Schindler et al., 2003). As a consequence, lower concentrations of dydrogesterone might reflect the physiologically effective levels of progesterone, and higher concentrations may be less effective since they may act on a receptor-independent level due to the lipophilic nature of dydrogesterone or trigger counter-regulatory mechanisms. However, additional research is needed to confirm this explanation.

A striking result in the present work was the high expression of NK-1R$^+$ lymphocytes in the uterus, compared with blood lymphocytes. Hence, the high percentage of uterine

NK-1R–positive cells and likely SP-positive uterine nerve fibers might indicate the contribution of SP-NK-1R system in inflammatory pathologies such as menstrual pain.

We also demonstrated that dydrogesterone skews an anti-inflammatory environment by increasing the Th2 type cytokines IL-4 and IL-10. Our findings are in accordance with previous works of our group showing that dydrogesterone abrogates stress-triggered abortion in mice, inducing a pregnancy-protective Th2 biased immune response (Pinto et al., 1999; Bradesi et al., 2003; Joachim et al., 2003). In addition, progesterone sustains a Th2 type immunity provoking a gestational attenuation of inflammatory disease (Moro et al., 2001). The NK-1R enhances Th1 pathways, e.g. increases IL-12 (Kincy-Cain and Bost, 1997) and IFN-γ (Blum et al., 2003) production. Notably, Th2 type cytokines have been shown to down regulate NK-1R expression in a model of mucosal inflammation (Weinstock et al., 2003). Further, IL-10 -/- mice express NK-1R mRNA at higher levels. Thus, it is tempting to speculate that Th2 type cytokines help to limit Th1 type immune response through the control of NK-1 R expression on lymphocytes.

The dorsal horn is a complex, multi-synaptic structure having multiple functions. It produces spinal reflexes, relays nociceptive messages to higher structures, and modulates to either inhibit or facilitate nociceptive transmission, depending on information from higher structures or from the periphery. The ligand of NK-1R, SP, is well known to function in the transmission of pain stimuli (Quartara and Maggi, 1998). The dorsal horn of the spinal cord, which is important in this transmission, is also known as a neurosteroidogenic center and enzymes converting progesterone to its neuroactive metabolites are found in this region (Coirini et al., 2002; Patte-Mensah et al., 2003; Patte-Mensah et al., 2004; Kibaly et al., 2005). Such metabolites control functions such as sexual behavior, neuroprotection, stress, anxiety, analgesia, sleep, and locomotion (Melcangi et al., 2005). A recent publication of Patte-Mensah *et al.* reports an inhibitory effect of SP on progesterone conversion into neuroactive metabolites in the dorsal horn of the spinal cord, which was mediated via NK-1R dependent pathways (Patte-Mensah et al., 2005). Hence, anatomical and functional interactions between the SP-NK-1R system and neuroactive steroid-producing cells may modulate the pain threshold. On the one hand, progesterone and/or its derivatives increase the pain threshold by down-regulating the expression of NK-1R. This is complimented by our current observation that dydrogesterone increased the pain threshold which was detected by the tail flick

test. There are additional studies showing steroid modulation of pain sensitivity, e.g., visceral hypersensitivity, induced in rats in response to restraint stress, was abrogated upon application of progesterone (Bradesi et al., 2003). On the other hand, SP decreases the pain threshold by reducing progesterone conversion into neuroactive metabolites. However, it remains to be elucidated if such neuroactive metabolites of progesterone affect the NK-1R expression.

IL-10 is known to reduce inflammation and nociception via an inhibition of COX2 mediated prostaglandin production together with an inhibition of the production and actions of pro-nociceptive cytokines such as a Th1 type cytokine, TNF-α (Mertz et al., 1994; Poole et al., 1995). This is in line with our present findings, since we provide evidence that an increase of Th2 type cytokines –triggered by application of dydrogesterone- results in a hypoalgesic response.

Finally, a hypothetical scenario based on our recent findings is visualized in Fig. 9. After dydrogesterone treatment Th2 type cytokines are up-regulated whereas NK-1R expression is down-regulated leading to an analgesic effect. In contrast, inflammation process or stress induces SP release, increases NK-1R expression and decreases progesterone levels. Consequently, the cytokine balance is skewed towards Th1 predominance, unopposed by Th2 type cytokines, prompting nociception.

Our results clearly point out the importance of neuro-endocrine-immune crosstalk, wherein endocrine system regulates inflammation via neuropeptide/receptor dependent pathways.

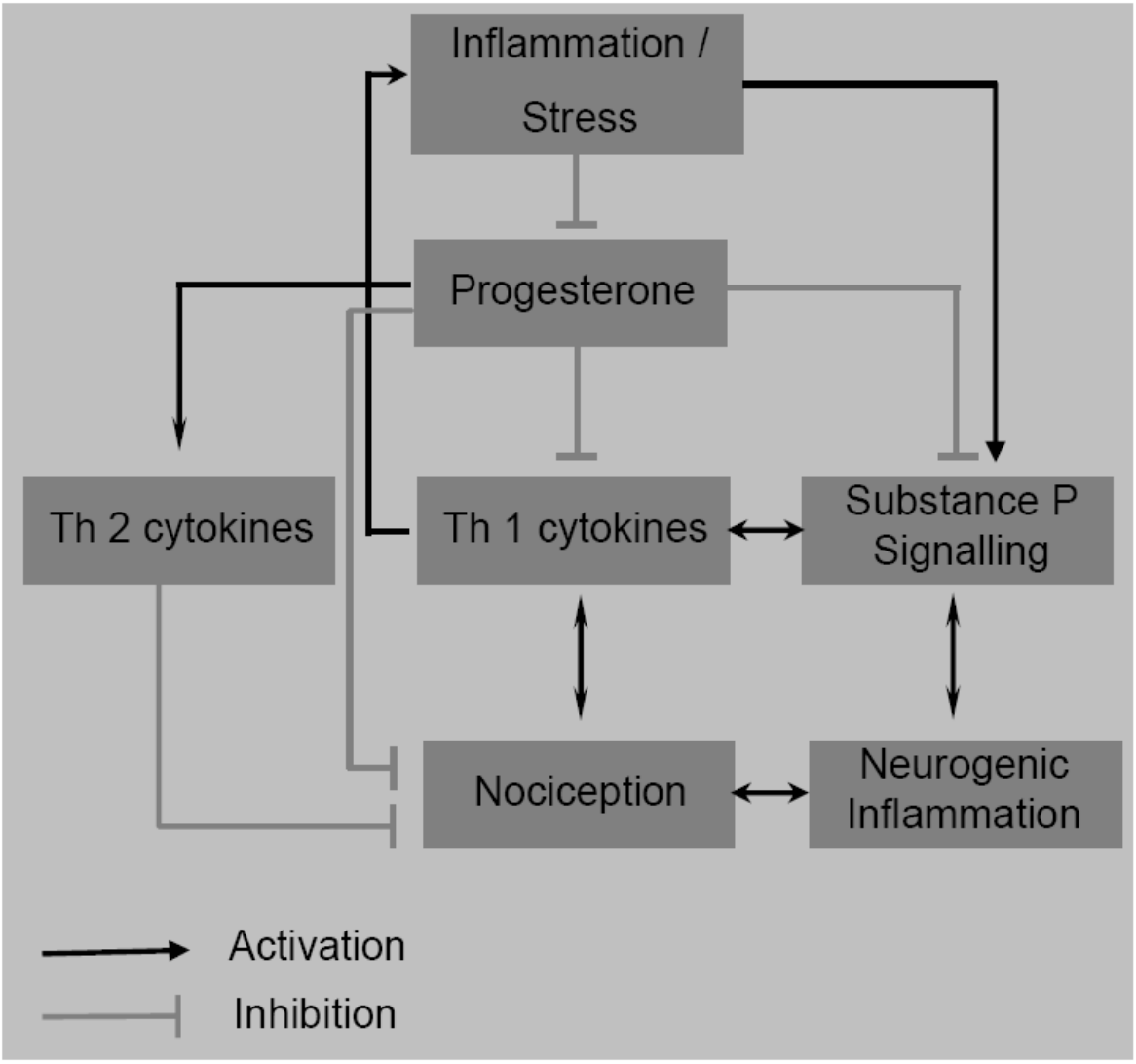

Fig. 9: A hypothetical scenario involving the effects of progesterone on nociception and neurogenic inflammation. Naturally occurring progesterone or its derivative dydrogesterone supports the secretion of Th2-type cytokines, unopposed by Th1- type cytokines, and decreases the expression of NK-1R. Hence, naturally occurring progesterone or the derivative dydrogesterone causes an anti-inflammatory effect and triggers an increased pain threshold. High perception of stress inhibits progesterone secretion and causes the release of neuropeptides, prototypically SP, by afferent nerve fibers. This leads to the activation of inflammatory cells. Secretion of Th1-type cytokines such as TNF-α and additional mediators from inflammatory cells results in an enhanced sensitization of nerve fibers and a decrease in the pain threshold, thus modulating nociception.

4. STUDY 2: INVESTIGATION OF NEURO-IMMUNE INTERACTIONS UNDER THE INFLUENCE OF IFN-α

4.1. Introduction

4.1.1. Interferons

Interferons are a group of cytokines which are known for their interference with virus replication. IFNs comprise many proteins that can be sub-grouped into 2 classes. Type I IFNs are produced mainly by all types of cells in response to viral infections. They consist of still growing cytokines including IFN-α, IFN-β, IFN-κ, IFN-λ, IFN-δ, IFN-ω, IFN-τ and limitin. Type II IFN is produced mainly by T and natural killer (NK) cells in response to mitogenic or antigenic stimuli and is also known as IFN-γ (Corssmit et al., 2000). Phylogenetic analyses indicate that the genes for IFN-α, IFN-β, and IFN-γ evolved from a single ancestral gene about 310 million years ago (Kumar and Hedges, 1998).

The type I interferon, IFN-α is produced in response to viral infections. The antiviral action of IFN-α is carried out by different mechanisms. IFN-α induces an antiviral state inside the cell by inhibition of protein synthesis, and degradation of mRNA. IFN-α also supports an antiviral state in the tissue by stimulating the immune response which results in enhanced recognition of infected cells, activation of NK cells, and increased presentation of antigens on class I MHC molecules (Peters, 1996; Tilg, 1997).

In addition to antiviral effects, IFN-α has antiproliferative effects on tumor cells. It regulates cell growth by increasing the cell multiplication time, slowing the growth of the tumor, and inducing differentiation, and apoptosis of tumor cells. Moreover, it contributes to antiproliferative effects by inhibiting angiogenesis, and by enhancing the immune response against the tumor (Einhorn and Grander, 1996; Strander and Einhorn, 1996; Grander et al., 1997; Jagus et al., 1999).

4.1.2. IFN-α in therapy

Due to its antiviral and antiproliferative effects (Brassard et al., 2002), IFN-α is used for the treatment of numerous viral and malignant diseases (Table 1).

Table 1: IFN-α in therapy (Schaefer and Schwaiger, 2003)

1. Malignant Diseases	**2. Myeloproliferative Diseases**
Hairy Cell Leukemia	Trombocytosis
Multiple Myeloma	Chronic myeloid leukemia
Kaposi's Sarcoma	Polycythemia vera
Cervical Neoplasia	Idiopathic thrombocythemia
Basal Cell Carcinoma	Myeloid Metaplasia
Squamaous Carcinoma	**3. Viral Diseases**
Malign Melanoma	Hepatitis C virus infection
Renal Cell Cancer	Hepatitis B virus infection
Carcinoid	HIV
Cutanoeus T cell Lymphoma	Condyloma Accuminata
Non-Hodgkin Lymphoma	Verruca Vulgaris
Osteosarcoma	Atopic Dermatitis
Laryngeal Papillomatosis	
4. Rhematoid and Immunological Diseases	
Rheumatoid Arthritis	Systemic Lupus Erythematosus
Scleroderma	Behcet's Disease

Besides its efficacy with regard to the therapy of viral and malignant diseases, IFN-α treatment triggers a variety of neuropsychiatric side-effects, including depression, and anxiety (Table 2) (Dieperink et al., 2000; Loftis and Hauser, 2004). These side-effects limit the efficacy of the treatment, sometimes requiring the cessation of the therapy. Depression is the most prevalent and severe neuropsychiatric side-effect of IFN-α treatment (Dieperink et al., 2000; Loftis and Hauser, 2004). In Europe, approximately 20-40 % of the patients treated with IFN-α develop depression (Bonaccorso et al., 2001; Manns et al., 2001; Schaefer et al., 2003b; Lieb et al., 2006). Furthermore, treatment of these patients with antidepressants complicates the situation since most of the antidepressants are metabolized in liver and viral hepatitis alters the pharmacokinetics of these medications. Chronic hepatitis may be associated with lower ability to metabolize antidepressants, therefore blood levels of these drugs may be higher than expected, increasing their side effects and toxicity in these group of patients (Asnis and De La Garza, 2006).

Table 2: Common side-effects during IFN-α therapy (Capuron et al., 2002)

Depressive Symptoms	**Neurovegetative Symptoms**
Depressed Mood	Fatigue/Loss of Energy
Anhedonia	Abnormal Sleep
Suicidal Thoughts	Psychomotor Retardation
Feeling of Guilt	Abnormal Appetite
Anxious symptoms	**Cognitive Symptoms**
Tension/Irritability	Loss of Concentration
Anxious Mood	Memory Disturbances
Fear	Word-Finding Problems
Somatic Symptoms	Episodes of Confusion
Pain	Indecisiveness
Gastrointestinal Symptoms	

In addition to human studies, rodent studies also demonstrate behavioral changes following IFN-α administration. Depression-like behavior as a result of IFN-α treatment was reported in a number of studies based on behavioral analysis in the forced swimming test (FST) (Makino et al., 1998a; Makino et al., 2000b; Makino et al., 2000a), tail suspension test (TST) (Yamano et al., 2000), and sucrose consumption test (Sammut et al., 2002). However, the induction of behavioral changes by IFN-α in rodent models is still controversial, since recent studies failed to show any evidence for depression-like behavior induced by IFN-α. For instance, IFN-α did not change reward behavior in sucrose pellet administration, or depression-like behavior in the FST following IFN-α administration (De La Garza et al., 2005; Loftis et al., 2006a), and also did not induce anhedonia in a brain stimulation reward paradigm (Kentner et al., 2007).

Regardless of these contradictory observations, insights from human and rodent studies suggest that an intricate network of signaling mechanisms is involved in the IFN-α-induced modulation of behavior, which may ultimately lead to depression. For example, IFN-α has been hypothesized to regulate the neurotransmitter systems (Schaefer et al., 2003a), since IFN-α is known to reduce dopamine (Shuto et al., 1997) and serotonin levels (Kamata et al., 2000) in rodent brains, and to increase the uptake activity of the serotonin transporters (Morikawa et al., 1998). Furthermore, IFN-α reduces the availability of tryptophan in the brain by inducing the tryptophan-metabolizing enzyme, indoleamine-2,3-dioxygenase (IDO) (Wichers and Maes, 2004), subsequently resulting

in a decrease in the synthesis of serotonin (5HT) (Taylor and Feng, 1991). Moreover, IFN-α can activate the HPA axis (Gisslinger et al., 1993; Capuron et al., 2003), which has been described to be disrupted in mood disorders and hence may play a role in the etiology of depression (Zobel et al., 1999). Another mechanism by which IFN-α might trigger depression-like behavior is via adult neurogenesis, which entails the generation of new neurons from precursor cells in the brain. It is known that neurogenesis is impaired in depression (Kempermann and Kronenberg, 2003), and IFN-α was recently found to suppress neurogenesis in the hippocampus of rats (Kaneko et al., 2006). Finally, IFN-α may cause behavioral changes in humans and rodents by inducing proinflammatory cytokines and mediators, which are also found elevated in depression (Maes et al., 1991; Maes et al., 1993; Maes et al., 1995; Song et al., 1995) (Table 3).

Table 3: The mechanisms proposed for IFN-α induced behavioral changes

1. Effects on neurotransmitter systems:
a. Reduced dopamine levels
b. Reduced serotonin levels
c. Increased uptake activity of serotonin transporters
d. Reduced availability of tryptophan by induction of IDO
2. Activation of HPA axis
3. Impaired neurogenesis
4. Induction of proinflammatory response

It is unlikely that IFN-α modulates these mechanisms directly in CNS, since IFN-α has a high molecular weight, which prevents its passage across BBB in physiologically relevant concentrations following peripheral administration (Smith et al., 1985; Greig et al., 1988; Greischel et al., 1988). Clearly, this gives rise to the concept of an indirect effect of IFN-α to induce behavioral changes.

Considering immunoregulatory nature of IFN-α, a peripheral immune response following IFN-α administration may signal into the brain and further trigger an immune/inflammatory response. This peripheral response might in turn be responsible for such an indirect effect. Here, lymphocytes provide a good machinery for the indirect effects of IFN-α since they are able to cross the BBB and they can produce ample

amounts of proinflammatory cytokines which might contribute to the pathogenesis in the brain.

BBB is a specialized barrier that limits the transcellular passage of cells and molecules into the brain; however, under physiological conditions lymphocytes enter the healthy CNS across the BBB at low levels. In addition, under inflammatory conditions such as in MS or EAE circulating cells readily get access to the CNS. Meanwhile, during inflammation the phenotype of the BBB endothelium is changed, having increased expression of adhesion molecules and chemokines, leading to enhanced leukocyte traffic into the brain, and consequently resulting in inflammation. However, T lymphocyte entry is limited to the activated T cells which express high amounts of adhesion molecules. Under these circumstances, activated T cells but not resting T cells have the capability to cross the BBB, start inflammation, and contribute to the disease pathology (Hickey et al., 1991)

Adhesion molecules are a group of cellular surface molecules having the ability to accomplish attachment between cells. The major adhesion molecules on leukocytes are β-1 integrins (VLA, very late antigens), and β-2 integrins (LFA-1). LFA-1 is expressed on more than 90 % of thymocytes, mature T cells, B cells, granulocytes and monocytes. The specific ligand for LFA-1 is ICAM-1, which is expressed on a variety of hematopoietic and non-hematopoietic cells, including B and T cells, dendritic cells, macrophages, fibroblasts, keratinocytes, and endothelial cells. Other members of the family include Mac-1 and CD11c which mediate leukocyte attachment to endothelial cells and subsequent extravasation of leukocytes. The β1 integrin subfamily consists of six members with different alpha chains. VLA-4 binds to a protein called vascular cell adhesion molecule-1 (VCAM-1) which is expressed on cytokine-activated endothelial cells. The major functions of T cell integrins are to mediate adhesion to endothelial cells, extracellular matrix proteins, and antigen presenting cells. The avidity of integrins for their ligands is increased rapidly on exposure of the T cells to cytokines and after stimulation of T cells through the T cell receptor (TCR) (Abbas and Lichtman, 2003).

Thus, investigating the involvement of T lymphocyte migration into the CNS in IFN-α treatment might help us to understand the mechanisms of the behavioral effects of IFN-α application. LFA-1 is found important in transendothelial migration of lymphocytes to

the brain (Lyck et al., 2003). In addition, the counterligands of these molecules on lymphocytes, namely LFA-1 (CD11a) and VLA-4 (CD49d), respectively, are particularly important in the migration of lymphocytes to the brain tissue (Engelhardt, 2006).

Microglial cells, the resident macrophages of the brain, proceed important immunoregulatory functions. They search the brain tissue for potential dangers and respond quickly to inflammatory stimuli and alter their phenotype and become antigen presenting cells (Matyszak and Perry, 2002). Consequently, microglial cells may get in touch with the immigrated lymphocytes and modulate the neuro-immune response against IFN-α. Therefore, understanding the interaction between microglia and T cells is crucial for a better insight into the inflammatory processes induced by IFN-α.

Additionally, cytokines are reported to be involved in mood disorders. The levels of anxiety and depression were found to be correlated with the levels of the circulating cytokines (Reichenberg et al., 2001; Reichenberg et al., 2002), and depressed patients show a shift in the Th1/Th2 ratio towards Th1 dominance (Schleifer et al., 1999; Schwarz et al., 2001). Therefore, IFN-α induced alteration in cytokine levels may also play a role in the pathogenesis of IFN-α induced depression.

Taking together all these evidences, we hypothesized that IFN-α acts on the peripheral immune system and subsequently interferes with brain function via secondary mechanisms. This concept is supported by observations indicating that IFN-α increases the levels of the soluble form of ICAM in the serum of patients with Hepatitis C (Schaefer et al., 2004). ICAM is an adhesion molecule expressed on endothelial cells, which is important for the transmigration of lymphocytes into the brain. ICAM and another adhesion molecule, vascular cell adhesion molecule (VCAM), were found to be increased in the brains of depressed individuals in a post-mortem study (Thomas et al., 2003).

Therefore, we aimed to investigate the effects of IFN-α administration on behavior of the mice, leukocyte migration and other immune components. In those experiments we used murine IFN-α instead of human IFN-α since IFN-α is known to have species-specific characteristics (Horisberger and de Staritzky, 1987; Kumaran et al., 2000). Our aim was to investigate the effect of IFN-α on:

1) The behavior of mice in terms of depression-like behavior and anxiety-like behavior
2) The expression of adhesion molecules LFA-1, VLA-4, Mac-1 on leukocytes
3) The percentages of leukocyte subpopulations such as, T lymphocytes, B lymphocytes, NK cells, regulatory T cells, monocytes, granulocytes in blood
4) Secretion of inflammatory, anti-inflammatory cytokines and Th1/Th2 cytokine ratio
5) Presence of lymphocytes in the brain tissue
6) The immune phenotype of microglial cells and brain-derived leukocytes

4.2. Material and Methods

4.2.1. Animals

BALB/c male mice were purchased from Charles River (Sulzfeld, Germany) and housed in community cages in the animal facility of the Charité, Campus Virchow Hospital (Berlin, Germany) with a 12:12 hour light/dark cycle. The mice were 8 weeks old at the time of testing. Animal care and experimental procedures were performed according to institutional guidelines of the respective institutions and approved by local authorities. The detailed information on the chemicals, reagents, antibodies, kits, equipment, and software used in this study can be found in the appendix section.

4.2.2. Administration of IFN-α

Recombinant murine IFN-α (Chemicon, IFN 009) was dissolved in PBS containing 0.1% BSA (Sigma) as recommended by the manufacturer. Mice received daily intraperitoneal (i.p.) injections of 60000 U/kg IFN-α in a volume of 200 µl for for 8 days. Control mice were injected daily i.p. with 0.1 % BSA/PBS solution in a volume of 200 µl. Each mouse was weighed before the first injection and after the last injection.

4.2.3. Behavioral Tests

Behavioral tests were performed after the mice had received seven daily injections of IFN-α, each behavioral test was carried out between 8 am and 4 pm by the same person who was blinded to the experiment groups. The time delay between the last injection and the start of behavioral tests was designed in a manner to minimize possible acute effects of the IFN-α injection. Animals were allowed to adapt to the experimental room for at least 1 hour before testing. The behavioral tests were performed in the following order: tail suspension test (TST), forced swimming test (FST), and Elevated Plus Maze (EPM). The animals were returned to their original housing cages following each test session. All behavioral test sessions were recorded by a video camera. The recorded video tape images were analyzed by a trained observer blinded to the treatment groups. The behavioral data of the male mice in two independent experiments were first normalized by dividing each behavioral score by the mean score of the control group multiplied by 100. After exclusion of significant

differences between the respective control or INF-α treated subgroups, the data from two independent experiments were pooled, and statistical analysis was performed using the t-test.

Tail Suspension Test: TST was performed as described (Steru et al., 1985). Briefly, mice were suspended by the tail by taping the distal part of the tail (1-1.5 cm) to a flat metallic surface 40 cm above the ground. Escape movements were recorded for 8 minutes and the behavior during the last 6 minutes was analyzed. The time spent in an immobile posture was measured (Fig. 10 A).

Modified Forced Swimming Test: Mice were subjected to a modified version of the FST (Porsolt et al., 1977) as described previously (Dulawa et al., 2004). Briefly, mice were placed into a vertical glass cylinder (glass beaker, 2000 ml) filled with 25 cm deep water at 25±2°C (Fig.10 B). Their swimming movements were recorded for 8 minutes and the behavior during the last 6 minutes was analyzed. To distinguish the specific components of active behavior, the predominant behavior was scored every 5 seconds and classified as swimming, immobility or climbing. Immobility was defined as only those movements required to keep the mouse head above the water, swimming was defined as active use of all four paws and moving around the cylinder, climbing was defined as vigorous movements of the mouse forepaws in contact with the cylinder walls. Data from two independent experiments were pooled.

Elevated Plus Maze: EPM was performed according to a published method (Lister, 1987). Briefly, EPM consisted of two open (30 cm X 5 cm) and two closed (30 cm X 5 cm X 15 cm) arms, extending from a common central platform (5 cm X 5 cm). The apparatus was constructed from black plexiglas and elevated to a height of 50 cm above the ground (Fig. 10 C). Each trial lasted for 5 minutes and started with placing the mouse on the central platform facing an open arm. The floor and walls of the maze were cleaned between each trial. An arm entry was defined as entrance into an arm with both forepaws. The following conventional measures were scored: Number of open-arm entries, number of total arm entries, percentage of open and closed arm entries, and percentage of time spent in the open and closed arms.

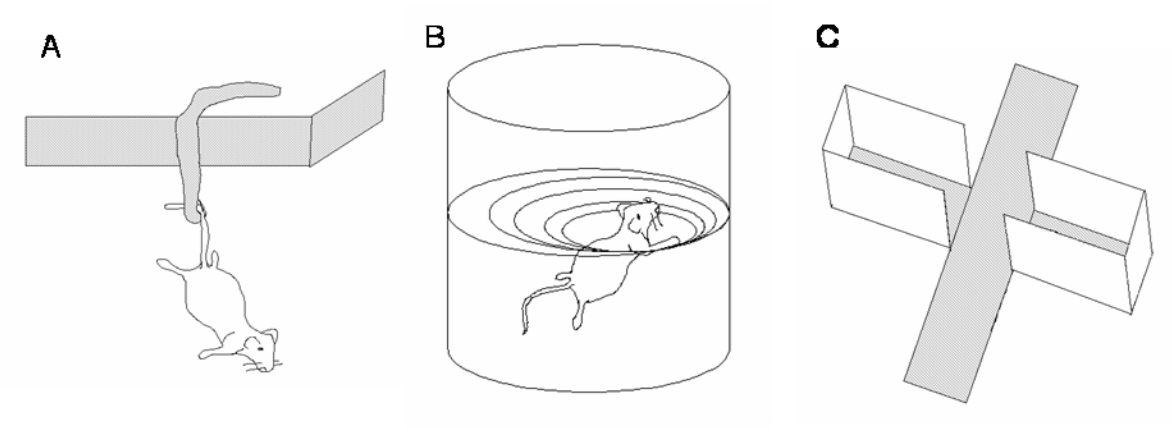

Fig. 10: Illustration of the behavioral tests.
Tail suspension test (a), forced swimming test (b), elevated plus maze (c).

4.2.4. Isolation of peripheral blood cells

One day after the behavioral tests, mice were anesthetized and blood samples were obtained by retro-orbital puncture and collected into tubes containing heparin. After treatment with ammonium chloride lysis buffer for 10 minutes to deplete erythrocytes, the white blood cells were centrifuged at 1100 rpm for 10 minutes. Then, the cell pellets were dissolved with PBS and centrifuged again. The cell pellets were dissolved in 500µl of FACS buffer. Cell numbers were determined by an automated cell counter (CASY, Schaerfe Systems, Germany), and remaining cells were used for flow cytometric analysis.

4.2.5. Preparation of leukocytes and microglia from brain tissue

In our experiments, we used the density gradient Percoll (Amersham) to separate leukocytes and microglia from other cells of the brain cell suspensions. For this purpose, mice were first perfused transcardially with 30 ml of ice-cold PBS in order to wash out the remaining leukocytes inside the blood vessels, and then the brains of mice were removed, minced with a scalpel and passed through a nylon cell-strainer (100 µm, BD Falcon). The cell suspensions were centrifuged at 1100 rpm for 10 minutes at 4 °C. The cell pellet was resuspended in 3 ml of 30 % Percoll solution and overlaid on top of 3 ml of 70 % Percoll solution. Following centrifugation at 1,300 g for 30 minutes at room temperature with slow acceleration and without brake, the cell layer in the interphase

was collected in another tube, FACS buffer were added, and then centrifuged again. The supernatants were discarded and cell pellets were resuspended with FACS buffer. An aliquot of the cell suspensions were used for cell counting by an automated cell counter (CASY, Schaerfe Systems, Germany), and remaining cells were used for flow cytometry. Staining of the cell suspension for flow cytometric analysis was performed as described.

4.2.6. Preparation of peritoneal mast cells

In order to evaluate the expression of NK-1R on mast cells, peritoneal lavage was performed. Two hundred µl of cell suspension (10^4 cells /ml) were incubated per well under conditions of 5% CO_2, and 37 °C. After 24 hours of incubation, cells were harvested from the wells and used for the flow cytometric analysis.

4.2.7. **Flow cytometric analysis**

All antibodies were purchased from BD biosciences (See appendix) except for the NK-1R antibodies (goat anti NK-1R (1/50), APC conjugated donkey anti goat antibody (1/100), Santa Cruz Biotechnology, Inc, Heidelberg Germany). Flow cytometry was performed according to a published protocol (Blois et al., 2004b). Briefly, purified cell suspensions were incubated with 2 % normal mouse serum (Sigma) to avoid nonspecific binding of Fc receptors. Cells were incubated for 30 minutes at 4°C with the antibodies against surface antigens. Controls consisted of the following; a) isotype controls, cells stained with the corresponding isotype-matched monoclonal antibodies, b) negative controls, unstained cells for instrument settings c) compensation controls, 4 different tubes containing cells stained with single antibodies conjugated with FITC, Phyoerythrin (PE), Peridinin chlorophyll protein (PerCP) and APC for the purpose of compensation of fluorescence channels of FACS. Following the incubation with antibodies, FACS buffer was added to the cells and they were centrifuged at 1100 rpm at 4°C for 10 minutes. Data acquisition was performed using the FACS calibur system (BD Biosciences), and analyzed using the Cell Quest software. The results are expressed as percentage of cells positive for the surface marker evaluated.

4.2.8. Determination of cytokine levels

For the detection of cytokine secretion (IL-10, IFN-γ, TNF-α, IL-6 and IL-2), lymphocytes isolated from peripheral blood were plated in 96-well plates at a concentration of 1x 10^6 cells/ml in RPMI supplemented with 10% FCS (Biochrom) with or without 10 ng/ml phorbol-myristate acetate (PMA, Sigma) and 1 μg/ml ionomycin (Calbiochem). Following 24 hours of incubation, supernatants were collected and the concentrations of the cytokines were measured by flow cytometry using the BD TM Cytometric Bead Array Flex Set Assay according to the manufacturer's instructions. Briefly, cytokine-specific antibody-coated beads were incubated with cell culture supernatants or standard solutions. Then, samples were incubated with the corresponding detection antibodies and were measured by flow cytometry. Analysis of the data was performed using Flow Cytometric Analyses Program (FCAP) Array^{TM} software (Becton Dickinson) on the basis of corresponding standard curves.

4.2.9. Determination of endotoxin levels

Endotoxin levels in the IFN-α preparation were measured using limulus amebocyte lysate assay (LAL-QCL-1000, Cambrex) according to the manufacturer's instructions. A standard curve of known amounts of endotoxins was used to determine endotoxin concentrations in the specimens. Endotoxin levels in samples are expressed as endotoxin unit (EU) per ml of sample.

4.2.10. Statistical analysis

All data are means ± standard error of the mean. The Kolmogorov-Smirnov test was used to test for normal distribution. For the normal distributed data, groups were compared by t-test, otherwise non-parametric Mann-Whitney U test was used to compare groups. For the statistical analyses, SPSS 14 software was used. The significance level was set to 0.05.

4.3. Results

4.3.1. Determination of endotoxin levels in IFN-α solutions

It was recently shown that carrier proteins might interfere with the effects of IFN-α (Wang et al., 2007), possibly through contaminated bacterial products. To exclude this potential confounder of our results, we tested both IFN-α and vehicle solutions for contamination with endotoxin by using the limulus amebocyte lysate test (LAL-QCL-1000 Cambrex). The results revealed no differences in endotoxin levels between IFN-α solution and vehicle solution (0.1% BSA/PBS). The endotoxin limit for a parenteral product is defined by the Food and Drug Administration (FDA) as the maximum allowable endotoxin concentration administered in one hour which is equal to 5 EU/kg (FDA, 1987). Our test results revealed that the endotoxin levels of both IFN-α and vehicle solutions were within the FDA norm for sterile fluids (0.14 ± 0.03 EU/ml for IFN-α solution; 0.20± 0.02 EU/ml for vehicle solution).

4.3.2. IFN-α treatment induced behavioral changes in FST, but not in TST, and EPM

Our first aim was to analyze the effect of IFN-α on the behavior of mice. Therefore, in our experiments we incorporated a battery of behavioral tests. We started our behavioral experiments first with TST, and then followed by FST, and at last we performed EPM test.

In the FST, the behavior of the mice was classified into three categories: immobility, climbing or active swimming. Increased immobility is suggestive of depression-like behavior. For the entire 2-6 minutes of the test period, IFN-α treated mice showed a tendency towards increased immobility compared with the control mice ($p=0.082$). Climbing and swimming scores were not different between groups (Fig. 11). To reveal time-dependent behavioral changes during the test, we analyzed the data in two-minute intervals. We found that during the last 2 minutes of the FST, IFN-α treated mice showed significantly increased immobility and decreased climbing behavior compared with the controls (Fig. 11). Interestingly, immobility during the last 4 minutes of the test period (4-8 minutes) was also significantly increased in the IFN-α treated group compared to the control group. No changes were observed for swimming scores during

the last 2 minutes of the test period, and for climbing and swimming scores during the last 4 minutes of the test period.

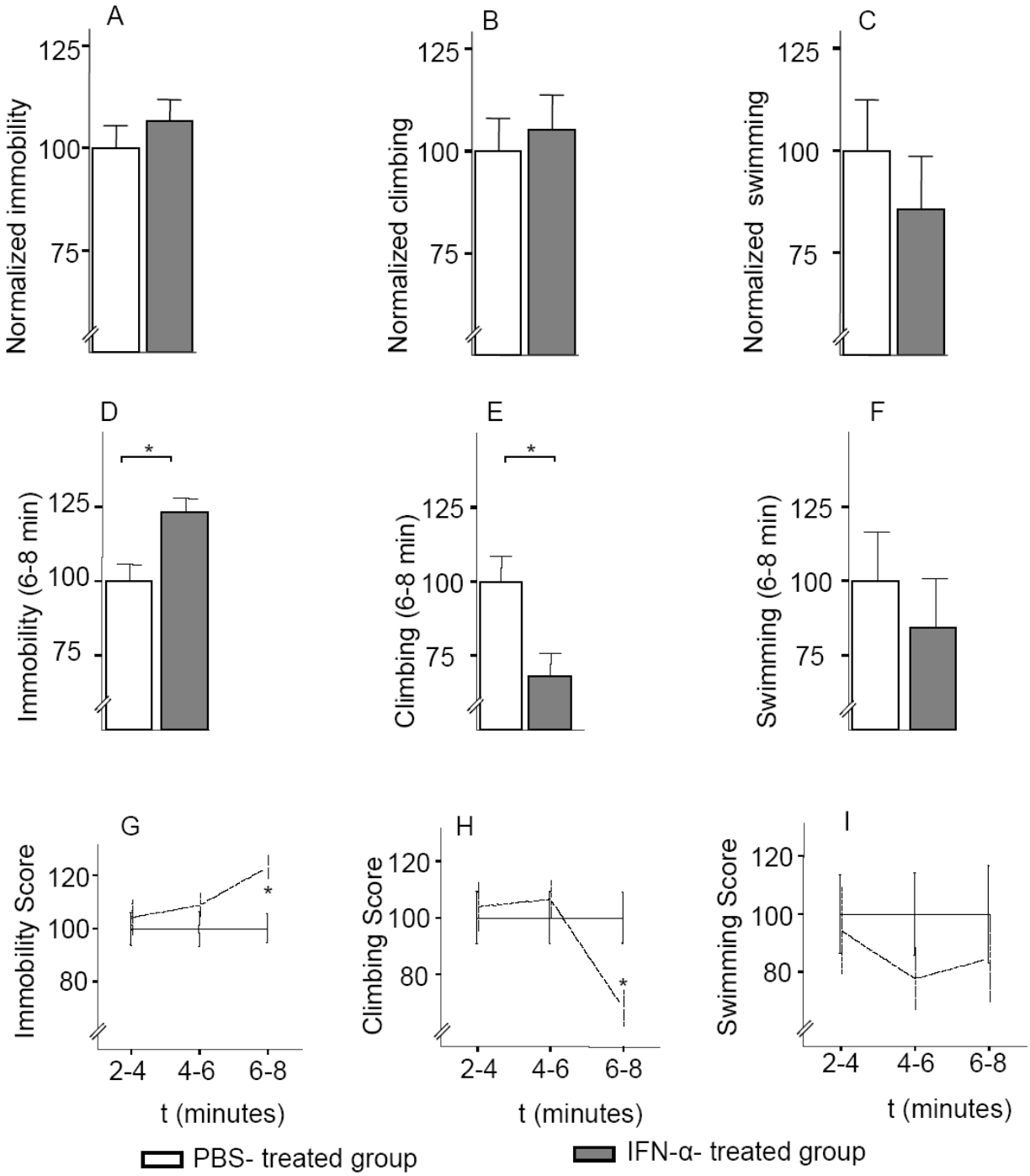

Fig. 11: Effects of IFN-α on behavior in the FST. FST was performed after the mice had received seven daily injections of IFN-α. The behavioral data from two independent experiments were normalized, and pooled for statistical analysis using t-test (*p<0.05). Data are means ± SEM. Immobility (A), climbing (B) and swimming (C) scores during 2 to 6 minutes. Immobility (D), climbing (E), and swimming (F) scores during 6 to 8 minutes. The behavioral scores for each 2 minute interval are visualized in G, H and I.

In the TST, total immobility time during the last 6 minutes of the test was not different between IFN-α treated mice and the control group (Fig. 12A).We re-analyzed the TST data in two minute intervals. However, there were no significant differences between IFN-α and control groups in any test interval (Fig. 12B).

The parameters, which are used for detecting anxiety-like behavior in the EPM, namely the percentage of time spent in the open arms and the number of open arm entries, were not different between IFN-α treated mice and the control group (Fig. 12C-D). The number of total arm entries, which reflects locomotor activity, was not different between the groups (Fig. 12E).

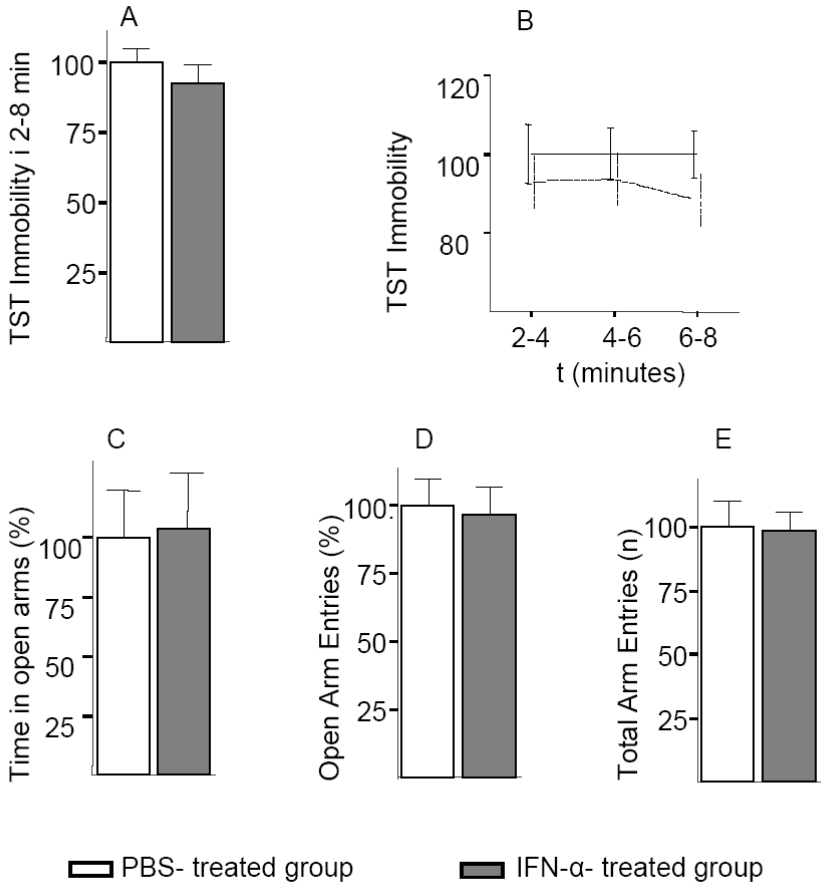

Fig. 12: Results of the TST and EPM test. Data from two experiments were normalized, and pooled for statistical analysis using t-test. Data are means ± SEM. Immobility time in the TST (A), immobility time during 2 minute intervals (B), percentages of time spent in open arms (C), open arm entries (D), number of total arm entries (E) were evaluated.

4.3.3. IFN-α did not change body weight of the mice

We weighed the mice before and after the treatment (Table 4). We detected that IFN-α does not affect the weights of the mice.

4.3.4. IFN-α did not change total leukocyte concentrations in blood and brain

We first analyzed the effect of IFN-α on the general concentration of leukocytes derived from blood and brain. We found that IFN-α treatment did not induce significant alterations with regard to the total number of leukocytes derived from blood and brain tissue (Table 4). Using flow cytometry, the percentages of $CD4^+$ and $CD8^+$ T lymphocytes were determined in blood. IFN-α treatment did not result in any changes in the percentages of these lymphocyte subpopulations (Table 4).

Table 4: Change of body weight and leukocyte parameters in brain and blood of mice

Variable	*Control*	*IFN-α*	*P*
Change of weight (g)	1.23±0.15	1.25±0.19	0.925
Blood leukocytes(10^6/ml)	4.21±0.31	5.10±1.11	0.449
Brain leukocytes (10^6/ml)	1.55 ±0.29	2.28±0.52	0.237
$CD3^+CD4^+$ in blood (%)	43.42±1.55	41.89±2.09	0.554
$CD3^+CD8^+$ in blood (%)	10.51±.051	9.99±0.61	0.512
Ratio CD4/CD8 in blood	4.22±.021	4.25±0.15	0.920

Data are means ± SEM. t-test was used for statistical analysis, 15 mice per group

4.3.5. IFN-α treatment increased the expression of adhesion molecules on lymphocytes

Next, we analyzed the expression of adhesion molecules on lymphocytes and other leukocyte populations. For this purpose, we investigated the expression of LFA-1, Mac-1 and VLA-4 on lymphocytes (Fig. 13A and 13B shows a representative example of the analysis of adhesion molecule expression). IFN-α-treated mice showed a higher percentage of blood LFA-1^{high} lymphocytes compared to the control group (Fig. 13C). Moreover, the percentage of $CD4^+$LFA-1^{high} lymphocytes was increased in the IFN-α-treated group (Fig. 13D). However, no difference was found for the percentages of $CD8^+$ LFA-1^{high} lymphocytes between the two groups (Fig. 13E). Whereas the percentages of VLA-4^+ lymphocytes were not different between the groups (Fig. 13F),

IFN-α-treated mice had a higher percentage of Mac-1^+ cells in blood compared to the controls (Fig. 13G). Moreover, IFN-α treatment increased the percentages of Mac-1^+LFA-1^{high} lymphocytes (Fig. 13H) and Mac-1^+VLA-4^+ lymphocytes (Fig. 13I) in blood.

The changes in the expression of adhesion molecules following IFN-α administration were specific to the lymphocyte cell population since the expression of LFA-1, Mac-1 and VLA-4 on non-lymphocyte leukocyte populations, including monocytes and granulocytes were not different between groups (Table 5). The percentages of LFA-1+, Mac-1+, VLA-4+ and ICAM-1^+ cells were not different between the groups.

Table 5: Expression of adhesion molecules on non-lymphoid leukocyte populations of blood (gated on R2 in Fig.13A).

Percentage	***Control***	***IFN-α***	***P***
LFA-1^+	70.12±3.23	71.57±2.45	0.722
Mac-1^+	71.32±3.15	76.22±2.52	0.235
LFA-1 Mac-1^+	63.95±3.60	64.81±2.88	0.853
VLA4^+	16.22±1.37	14.21±1.17	0.273
CD11c^+	3.80±0.34	4.54±0.51	0.238
ICAM$^+$	37.57±3.15	35.45±2.41	0.597

Data are means ± SEM. t-test was used for statistical analysis,15 mice per group

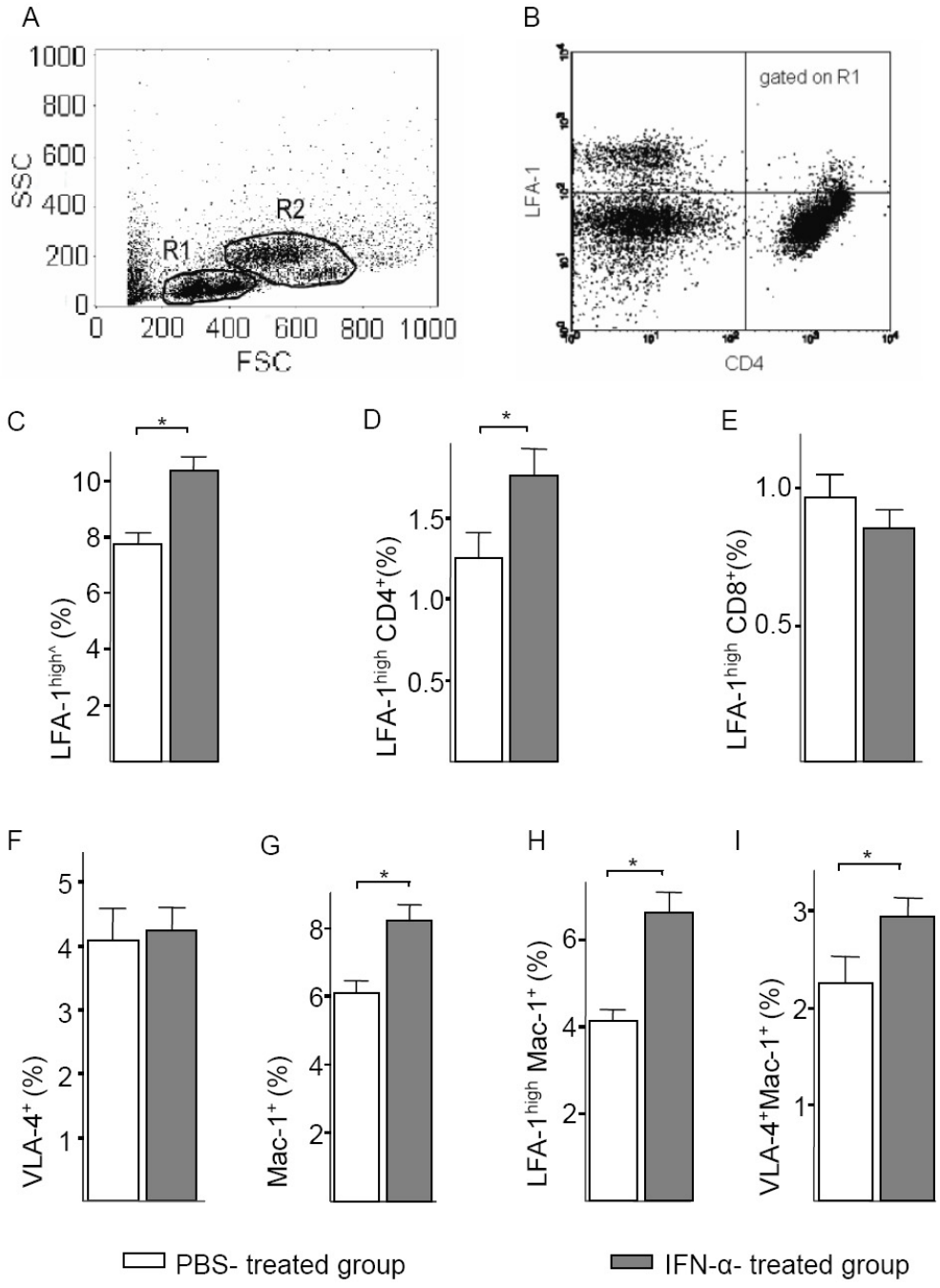

Fig. 13: Effects of IFN-α on the expression of adhesion molecules on lymphocytes.

Data are expressed as means ± SEM and analyzed by t-test (*p<0.05), 15 mice per group. Blood leukocytes were isolated (A); Lymphocyte (R1) and non-lymphocyte (R2) gates were drawn for further flow cytometric analyses (B). Further analysis was performed to detect the expression of stained molecules on the gated cell population. Percentage of LFA-1^{high} lymphocytes (C). Percentage of $CD4^+$ LFA-1high lymphocytes (D). Percentage of $CD8^+$ LFA-1high lymphocytes (E). Percentages of $VLA4^+$ (F), $Mac\text{-}1^+$ (G), LFA-$1^{high}$$Mac\text{-}1^+$ (H), $VLA4^+$ $Mac\text{-}1^+$ (I) lymphocytes.

4.3.6. IFN-α treatment increased the percentage of NK cells in blood

NK cells have important regulatory roles in innate immunity, and their concentration and activity are altered in depressed patients (Seidel et al., 1996; Jozuka et al., 2003). Therefore, we aimed to investigate the effects of IFN-α administration on the percentage of NK cells in blood (Table 6). IFN-α administration significantly increased the percentages of NK cells ($CD49b^+CD3^-$), but not of NK-T cells ($CD49b^+CD3^+$) in blood (Table 6). The percentages of $CD25^+$ cells and of $CD4^+CD25^+$ cells were slightly higher in IFN-α treated mice, but the changes did not reach statistical significance (Table 6).

Table 6: Effects of IFN-α treatment on NK cell populations and $CD25^+$ cells in blood.

Percentage	***Control***	***IFN-α***	***P***
$CD25^+$	1.58±0.18	2.07±0.23	0.102
$CD4^+CD25^+$	1.11±0.17	1.68±0.24	0.066
$CD49b^+$	**8.60±0.49**	**11.62±0.58**	**0.0004***
$CD3^-CD49b^+$	**5.43±0.24**	**7.23±0.40**	**0.001***
$CD3^+Cd49b^+$	3.18±0.43	4.40±0.55	0.094

Data are means ± SEM. t-test was used for statistical analysis (*$p<0.05$), 15 mice per group.

4.3.7. IFN-α treated mice had decreased IL-6 levels after stimulation in vitro

We investigated whether IFN-α had effects on the production of Th1 and Th2 type cytokines. Cytokine production by lymphocytes isolated from blood of the mice was analyzed *in vitro* (Table 7). Under basal conditions, the production of IFN-γ, IL-2, TNF-α, IL-10, IL-6 and the Th1/Th2 ratio calculated as (IFN-γ+ TNF-α /2)/IL-10 were not different between both experimental groups. However, after stimulation with PMA/ionomycin, lymphocytes isolated from IFN-α-treated mice showed a significantly decreased production of IL-6 compared to lymphocytes from control mice (Table 7). Lymphocytes from IFN-α-treated mice also showed a tendency towards increased levels of IFN-γ and IL-2 cytokines upon stimulation with PMA/ ionomycin, but this did not reach statistical significance.

Table 7: Effects of IFN-α treatment on cytokine secretion levels *ex vivo*.

Cytokines	***PMA/Ionomycine***	***Control (pg/ml)***	***IFN-α (pg/ml)***	***P***
IFN-γ	+	541.31±158.85	787.29±197.11	0.341
IL-2	+	951.04±220.15	1175.95±312.3	0.562
TNF-α	+	425.01±57.63	451.02±85.77	0.803
IL-10	+	64.50±12.75	52.38±11.12	0.480
IL-6	+	**209.74±31.15**	**122.80±26.85**	**0.045***
Th1/Th2 ratio	+	15.13±7.25	11.72±0.86	0.650
IFN- γ	-	1.73±0.22	1.44±0.46	0.585
IL-2	-	2.02±0.27	1.67±0.59	0.611
TNF-α	-	30.43±8.50	28.40±9.19	0.876
IL-10	-	10.85±3.30	9.93±5.22	0.885
IL-6	-	3.17±0.81	2.92±0.85	0.841
Th1/Th2 ratio	-	1.68±0.51	1.45±0.34	0.770

Lymphocytes were incubated in the presence or absence of PMA and ionomycin. After 24 hours, cytokine expression levels were detected by flow cytometry using the CBA Flex Set Assay. Data are means ± SEM. A t-test was used for statistical analysis (*$p<0.05$), 15 mice per group

4.3.8. IFN-α did not change the expression of molecules involved in antigen presentation

To investigate the effects of IFN-α on the antigen presentation markers, we analyzed the expression of several molecules involved in antigen presentation. Flow cytometric analyses revealed that there were no differences between the groups in terms of the percentages of cells expressing CD11c, ICAM-1, and MHC-II (Table 8).

Next, we analyzed the expression of LFA-1, Mac-1 and VLA-4 on non-lymphoid leukocyte populations, including monocytes and granulocytes, in both experimental groups (Table 8). The percentages of LFA-1^+, Mac-1^+, VLA-4^+ and ICAM-1^+ cells were not different between the groups.

Table 8: Expression of adhesion molecules on non-lymphoid leukocyte populations in blood.

Percentage	*Control*	*IFN-α*	*P*
LFA-1$^+$ granulocytes	81.73±2.96	82.24±2.75	0.901
LFA-1$^+$ monocytes	46.10±2.24	50.94±1.70	0.102
Mac-1$^+$ granulocytes	80.20±3.77	82.57±3.37	0.645
Mac-1$^+$ monocytes	12.95±1.45	14.11±1.45	0.896
LFA-1$^+$Mac-1$^+$ granulocytes	75.05±4.03	75.62±4.04	0.922
LFA-1$^+$Mac-1$^+$ monocytes	10.92±1.41	12.29±1.27	0.480
VLA4$^+$ granulocytes	11.24±0.55	10.86±0.73	0.677
VLA4$^+$ monocytes	14.77±1.71	11.11±1.01	0.082
CD11c$^+$ cells	3.80±0.34	4.54±0.51	0.238
ICAM-1$^+$ cells	37.57±3.15	35.45±2.41	0.597
MHC-II$^+$ cells	36.45±9.81	33.18±7.90	0.298

Data are means ± SEM. t-test was used for statistical analysis,15 mice per group.

4.3.9. IFN-α did not change the expression of NK-1R on leukocytes

SP-NK-1R system is an important mediator of neuro-immune-endocrine interaction. It has been suggested to have a role in behavior, therefore we investigated whether IFN-α alters the expression of NK-1R. We detected that IFN-α did not change the expression of NK-1R on leukocytes (Table 9).

Table 9: NK-1R expression on leukocytes.

Percentage	*Control*	*IFN-α*	*P*
NK-1R$^+$ lymphocytes	12.45±1.64	13.24±2.15	0.776
NK-1R$^+$ granulocytes	6.81±0.74	7.78±0.91	0.421
NK-1R$^+$ monocytes	6.40±0.62	7.62±0.83	0.257

Data are means ± SEM. A t-test was used for statistical analysis, 16 mice for control group, 18 mice for IFN-α group.

4.3.10. IFN-α treatment increased the percentages of CD4$^+$ and CD8$^+$ cells in the brain

In order to investigate whether the functional changes in the peripheral immune system induced by IFN-α administration also resulted in changes in the brain, mice were perfused transcardially with PBS to wash out residual blood cells from brain tissue and leukocytes were isolated by percoll density centrifugation (Fig. 14A). Flow cytometric analyses revealed that IFN-α-treated mice had increased percentages of CD3$^+$ CD4$^+$ lymphocytes (Fig. 14B), and CD3$^+$ CD8$^+$ lymphocytes (Fig. 14C) in the brain. The percentage of LFA-1^{high} cells in the total leukocyte population derived from brain tissue was increased in the IFN-α-treated group (Fig. 14D). Moreover, the percentages of CD4$^+$ LFA-1^{high} cells (Fig. 14E) and of CD8$^+$ LFA-1^{high} cells (Fig. 14F) were increased in the brains of IFN-α-treated mice compared with controls.

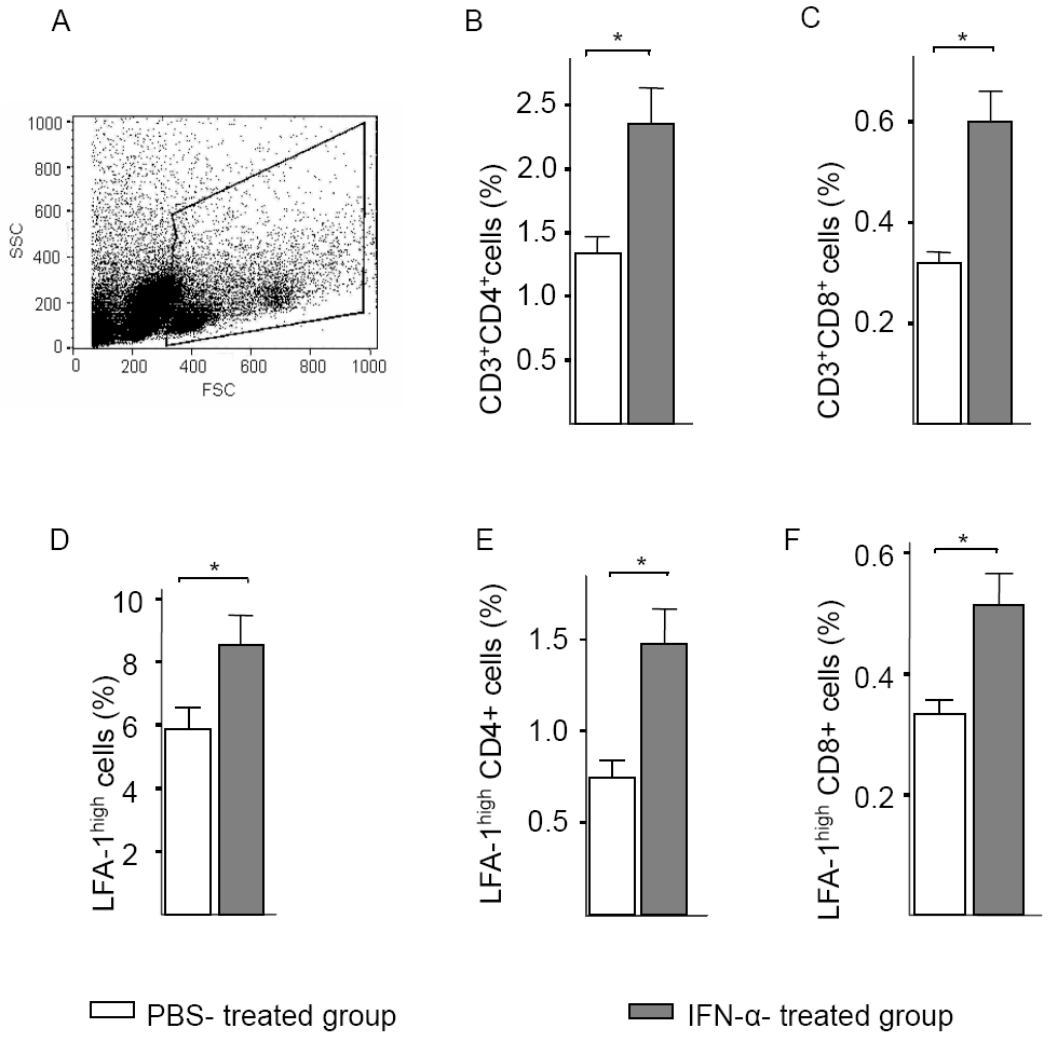

Fig. 14: Effects of IFN-α on the percentages of lymphocytes in the brain.
Brain leukocytes were isolated using the Percoll separation method. Presence of lymphocyte subpopulations were investigated by flow cytometric gating (A). Percentages of $CD3^+CD4^+$cells (B), $CD3^+CD8^+$cells (C), LFA-1high cells (D), LFA-1high $CD4^+$cells (E), LFA-1high CD8+cells (F) were calculated. Data are expressed as means ± SEM and analyzed by t-test (*$p<0.05$), 15 mice per group.

4.3.11. Microglial cells and brain derived leukocytes

Since microglial cells are important modulators in the neuro-immune interaction, we investigated whether IFN-α effects the immune phenotype of microglial cells. We isolated brain derived leukocytes and microglia with the help of density gradient centrifugation. The recovered cell suspension contained microglia, macrophages, and lymphocytes. We further identified each cell population with the help of flow cytometry staining. Microglial cells and macrophages are Mac-1^+ cells. Additionally macrophages express high levels of CD45 and microglia express low levels of CD45. These cell populations were identified as follows: microglial cells Mac-1^+CD45^{low}, macrophages Mac-1^+CD45^{high}, lymphocytes Mac-1^-CD45^+. Following gating of these cell populations, we investigated the expression of the markers involved in antigen presentation such as MHC-II, CD11c, and ICAM-1.

CD11c expression on microglia and macrophages was not different between groups (Fig. 15A, G). Percentage of microglial cells expressing MHC-II was significantly higher in IFN-α treated group compared with the control group (Fig. 15B). Similarly, the percentages of MHC-II^+ macrophages were also increased in the IFN-α treated group compared to the controls (Fig. 15F). Almost all microglial cells expressed ICAM-1 in both groups (Fig. 15C).

We also investigated the expression of adhesion molecules on microglial cells. We found that LFA-1 and VLA-4 levels were not different between the groups (Fig.15D, E).

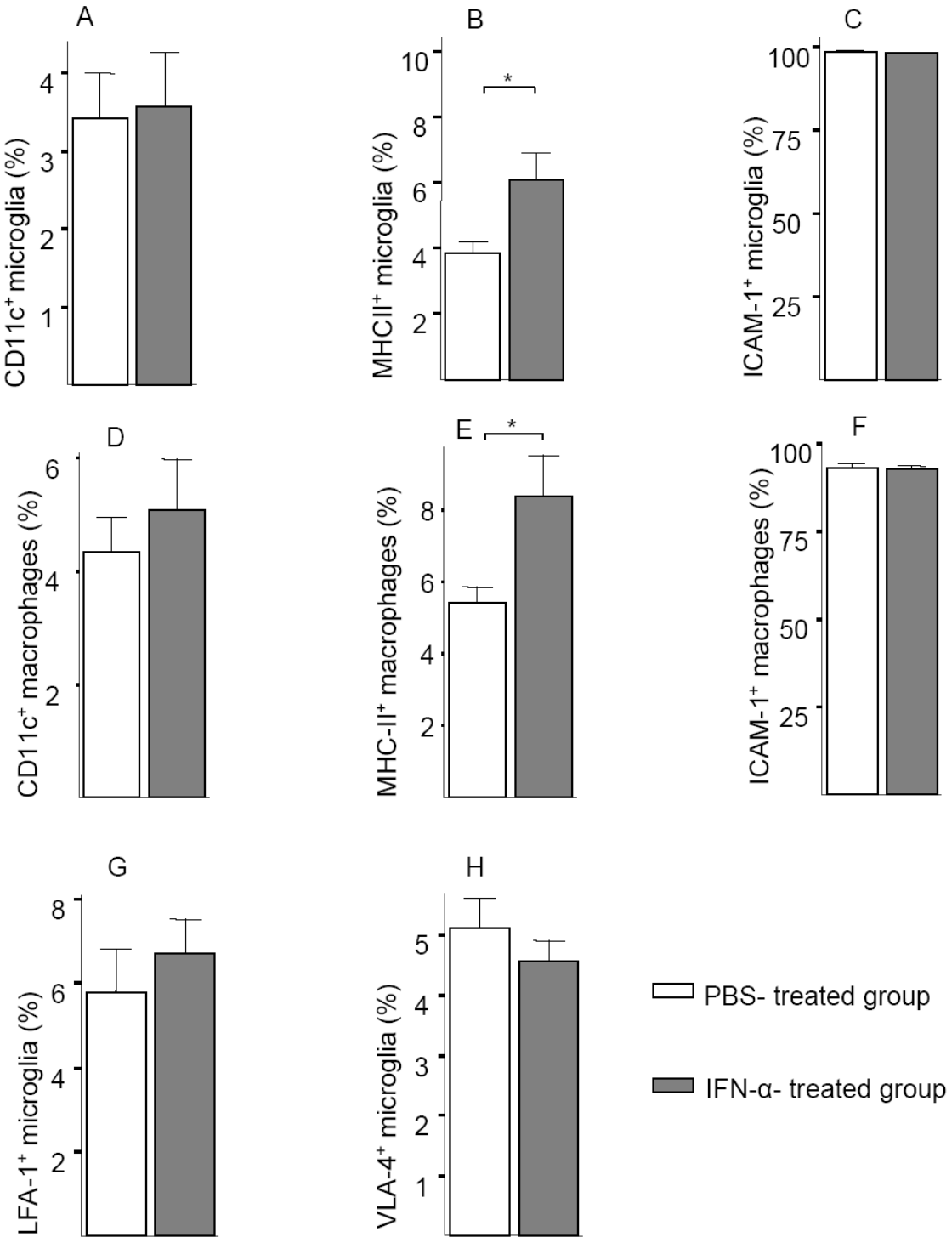

Fig. 15: Effects of IFN-α on the expression of CD11c, MHC-II, ICAM-1, LFA-1 and VLA-4 on microglia and brain derived macrophages. Data are expressed as means ± SEM and analyzed by t-test (*p<0.05), 15 mice per group.

4.4. Discussion

In this study, we investigated behavioral and immunological effects of murine IFN-α administration in mice. IFN-α is known to have species-specificity, i.e. IFN-α proteins from one species are most effective in the same species and have limited effects in other species (Crnic and Segall, 1992; De La Garza II et al., 2005; Loftis et al., 2006b). Therefore, in order to overcome the species-specific effect of IFN-α, we employed murine IFN-α in our experiments.

Recombinant human and murine IFN-α proteins are produced from genetically engineered Escherichia coli bacteria. Escherichia coli is a gram negative bacillus which contains, similar to other members of the gram negative bacteria, lipopolysaccharide molecules (also called endotoxins) on their cell walls. Endotoxins are potent stimulators of innate and adaptive immunity (Abbas and Lichtman, 2003). Therefore, any contaminant endotoxin in the IFN-α preparation might interfere with immune and behavioral effects of IFN-α. For that reason, we measured endotoxin levels in IFN-α protein solutions using LAL test, and we found that the endotoxin levels in the IFN-α products were below the accepted limits.

The mouse is used frequently to model human diseases. The main reason for this selection is the shared similarities with humans in terms of anatomical, cellular, biochemical and molecular characteristics. Additionally, the practical reasons such as being easy to handle, and accommodate, and financial advantages made mice the preferable species in animal experiments (Van Meer and Raber, 2005). The rationale for choosing the BALB/c strain of mice was their reported sensitivity to the effects of IFN-α and their suitability for the behavioral tests used (Crnic and Segall, 1992). In our experiments we aimed to have a rodent model of IFN-α administration in order to investigate the possible underlying neuro-immunological mechanisms of IFN-α induced behavioral changes.

The similarities of murine brain structure and functions with humans resulted in the use of mice in behavioral investigations. However, modeling depression has its difficulties since depression is a heterogeneous, multifaceted disorder with symptoms manifested

at psychological, behavioral, and physiological levels. Many of the symptoms of depression, including suicidal ideation and feeling of guilt seem impossible to model in mice. Nevertheless, from an evolutionary point of view, animal species might have similar behavioral patterns as humans (Jones and Blackshaw, 2000). For instance, several characteristic symptoms of depression, such as behavioral despair, anhedonia, insomnia, and fatigue, can be modeled in mice. With the emergence of antidepressant drugs in the second half of the 20th century, the need for animal models of depression has emerged, and since then various behavioral models that document different aspects of depression have been developed (Petit-Demouliere et al., 2005)

Among all of the experimental procedures used in depression research, FST is probably the most widely and most frequently used test (Porsolt et al., 1977; Cryan et al., 2002). Porsolt et al. (1977) described “a new behavioral method for inducing a depressed state in mice”. The test is developed from the water maze experiments with rats where they discovered that most rats found the exit within a 10 minute period. However some rats ceased struggling and remained immobile. In the FST, initial escape-orientated behaviors such as swimming and climbing are followed by immobile posture indicating behavioral despair. In this context, increased immobility provides a correlate with the clinical observation that depressed patients show psychomotor impairments, particularly in those tests requiring sustained expenditure of effort (Cryan and Holmes, 2005). In our experiments, we used a modified version of FST (Dulawa et al., 2004), in which we used a time-sampling technique to rate the predominant behavior over 5 second intervals. The behavior was analyzed during 8 minutes, and the last 6 minutes were evaluated. We observed that IFN-α treated mice showed increased immobility, which progressed with time during the FST and reached significance during the last 4-8 minutes of the test. Our results are in accordance with previous reports indicating increased immobility in the FST following 7 days of IFN-α injections (Makino et al., 1998b; Makino et al., 2000a). In contrast to our study, in these studies, human IFN-α was used and it was administered intravenously at a dose of 6,000 U/kg. On the other hand, it was shown that 3 weeks of administration of recombinant pegilated IFN-α did not increase immobility in the FST in rats (Loftis et al., 2006a). The absence of behavioral changes was explained by the possibility that pegilated IFN-α does not bind to rat IFN-α receptors or is unable to cross the BBB (Loftis et al., 2006a). Another recent

study found that 14 days of i.p. administration of human IFN-α (100,000 U/kg) did not change immobility in the FST in rats (De La Garza II et al., 2005).

Like the FST, the TST is based on the observation that, when placed in an inescapable stressful situation after initial escape-oriented movements, rodents develop an immobile posture (Chermat et al., 1986; Varty et al., 2003). In TST the stressful situation involves the stress of being hung up by the tail under inescapable conditions. The immobility may be analogous to the clinical observations that depressed patients often lack sustained expenditure of effort reflected in a pronounced psychomotor impairment (Weingartner and Silberman, 1982). In our experiments, we did not observe any behavioral effect of IFN-α administration in TST. However, Yamano et al. found increased immobility in the TST following treatment of mice with human IFN-α (Yamano et al., 2000).

The EPM is used widely to detect anxiety-like behavior in rodents. The test is based on the concept that mice prefer the safety of the closed arms, but on the other hand they also like to explore the open arms. Less anxious mice venture onto the open arms and anxious mice will prefer to stay in the closed arms (Van Meer and Raber, 2005). Thus, percentages of time spent in open arms, percentage of open arm entries, percentage of time spent in closed arms, and numbers of open arm entries constitute the parameters reflecting anxiety-like behavior. However, a decrease in the arm entries might also result from a decrease in the locomotor activity. Therefore, total arm entries and closed arm entries are used to evaluate the locomotor activity in the EPM test. In our experiments, IFN-α administration did not change the behavior of mice in the EPM. In humans, a coexistence of anxiety with depression has been reported in IFN-α treated patients (Renault et al., 1987; Bonaccorso et al., 2002b). Anxiogenic effects of acute administration of mouse IFN-α have been also reported (Nakamura et al., 2003). One reason for the discrepancy of our results with previous studies might be the different duration of IFN-α treatment.

In our study, the increased immobility that we found in our FST was not accompanied by changes in TST or EPM tests. Although, TST, similar to FST, detects behavioral despair in mice, the type of stress, the response of mice, and setup of the experiment

are not the same in both of these paradigms. Moreover, EPM investigates another aspect of behavior, anxiety-like behavior.

Thus, the behavioral studies, which have attempted to model IFN-α-induced changes of behavior in rodents, have yielded conflicting results. The discrepancies may result from variations in experimental design, including the type of IFN-α used, the duration of treatment, the dosage, the rodent species employed, and the behavioral tests performed. For example, IFN-α was found to induce anhedonia in mice in the sucrose consumption test (Sammut et al., 2002). On the other hand, acute administration of 100,000 U/kg of human IFN-α did not change reward behavior in the sucrose pellet administration test (De La Garza II et al., 2005). Moreover, IFN-α did not induce anhedonia in a brain stimulation reward paradigm in rats (Kentner et al., 2007). Further, variation of the behavioral testing approaches may account for conflicting data. However, since emerging research aims to improve and validate behavioral testing approaches in rodents, i.e. automated testing or individual phenotyping, future research may provide a better understanding of mechanisms at work.

Because of its high molecular weight, IFN-α is unable to cross the BBB in significant concentrations. Rodent studies showed that only very low (0.02-0.18%) or undetectable amounts of IFN-α crossed the BBB following peripheral administration (Smith et al., 1982; Greig et al., 1988; Greischel et al., 1988). In primates including humans, only high dosages of IFN-α were associated with the appearance of IFN-α in the cerebrospinal fluid (Habif et al., 1975; Rohatiner et al., 1983). Thus, indirect effects of IFN-α rather than direct effects are likely to be responsible for the behavioral changes. Immune cells may be candidates for the indirect effects of IFN-α, because they produce proinflammatory cytokines and are capable of crossing the BBB.

During their life cycle, lymphocytes continuously circulate the body via blood and lymphatic vessels. Searching for their specific antigens, lymphocytes exit the circulation and migrate into the tissues. The migration of lymphocytes into the tissues in the presence of blood flow requires interaction with endothelial cells. In the first step, lymphocytes make a transient contact with the vascular endothelium. This step initiates rolling of the lymphocyte along the surface of the endothelial cells. The rolling lymphocytes decrease their velocity and chemokines expressed on the surface of the

endothelial cells get the opportunity to bind to their receptors on the lymphocytes. Binding of the chemokines to their receptors on lymphocytes initiates conformational changes of the integrins on lymphocytes. This conformational change initiates adhesion of integrins to their ligands on endothelial cells which results in firm attachment of these cells. Therefore, only activated integrins can mediate firm adhesion, and further lymphocytes can migrate into the tissues through the endothelial cell layer (Engelhardt, 2006). The same mechanisms are involved in the transendothelial migration of lymphocytes into the brain tissue. Lymphocytes use adhesion molecules expressed on their surface to attach to the endothelium and further migrate into the brain tissue (Mazzone and Ricevuti, 1995). Particularly, LFA-1 and VLA-4 on lymphocytes, and their ligands ICAM-1 and VCAM on brain endothelial cells are relevant for the migration of leukocytes into the CNS (Engelhardt, 2006). Furthermore, the expression of these molecules was found to be altered in depressed patients. For instance, ICAM-1 and VCAM levels were increased in the brains of depressed patients (Thomas et al., 2000; Thomas et al., 2002; Thomas et al., 2003). In addition, serum ICAM-1 levels were found to be related to depression (Empana et al., 2005). Interestingly, IFN-α has effects on both adhesion molecules. IFN-α enhanced T cell migration in an *in vitro* model, and induced the expression of the adhesion molecules LFA-1 and VLA-4 on T cell lines (Foster et al., 2004). Additionally, the soluble form of ICAM-1 (sICAM) was elevated in blood following IFN-α administration, and the levels of sICAM were found to correlate with depression scores (Schaefer et al., 2004).

Considering these data, we investigated the effects of IFN-α on the expression of adhesion molecules on lymphocytes and the presence of these cells in the brains of IFN-α treated mice. We found that IFN-α administration increased the percentage of LFA-1^{high} cells in mice. Moreover, the percentages of LFA-1^{high} $CD4^{+}$ cells, Mac-1^{+} cells and LFA-1^{high} Mac-1^{+} lymphocytes were increased in the IFN-α treatment group compared with control. In contrast to the changes in the expression of adhesion molecules on lymphocytes as a result of IFN-α treatment, we did not find any changes in the expression of the adhesion molecules LFA-1, Mac-1, VLA-4, CD11c, and ICAM-1 on non-lymphocyte leukocyte populations. However, we detected increased percentages of NK cells after IFN-α treatment, which is in line with previous studies suggesting a role of this cell population in depression (Seidel et al., 1996; Jozuka et al., 2003).

In our study, we did not investigate the expression of the adhesion molecules on endothelial cells. Future studies investigating the expression of ICAM-1 on brain endothelial cells and the level of the soluble form of ICAM-1 in rodent models might contribute to our knowledge of the effects of IFN-α on adhesion molecules.

Homeostasis of the CNS microenvironment is essential for its proper functioning and is maintained mainly by the BBB. It is known that activated $CD4^+$ lymphocytes can enter the brain and perform immune surveillance functions even under normal conditions, although in low numbers and in a tightly controlled process (Hickey et al., 1991). Under inflammatory conditions, circulating lymphocytes readily cross the BBB and gain access to the CNS, leading to inflammation (Engelhardt, 2006). In our study, we detected that IFN-α increased the percentages of $CD4^+$ and $CD8^+$ lymphocytes in the brain tissue. A similar effect of IFN-α administration on lymphocyte recruitment to the CNS was reported in transgenic mice, in which astrocytes chronically secrete IFN-α (Campbell et al., 1999). In that model, lymphocyte infiltration was dominated by $CD4^+$ cells, and ICAM levels were upregulated on the cerebrovascular endothelium, suggesting that endothelial cells are important targets for IFN-α and contributors to CNS inflammation. Moreover, it has been reported that $CD4^+$ T cells activated *in vitro* are able to migrate across the healthy BBB into the CNS and start molecular events leading to inflammation (Wekerle et al., 1986; Hickey et al., 1991). Therefore, in IFN-α treated patients, activated lymphocytes might gain access to the brain and become sources of inflammatory stimulants which might contribute to the behavioral changes. Here, the neural components that lymphocytes modulate become important in order to understand the behavioral effects of inflammatory stimuli.

SP is a potent inflammatory molecule with neurotransmitter and neuromediator functions. SP is present in the CNS, PNS and ENS (Black and Garbutt, 2002). The receptor of SP is found in the brain in the localizations related to behavior and stress response such as amygdala, hypothalamus, hippocampus, frontal cortex, and the midbrain monoaminergic nuclei. SP is also involved in nociceptive systems, in the dorsal horn of the spinal cord (Black and Garbutt, 2002). SP-NK-1R system has important roles in perception and response to noxious and stressful stimuli. Based on these findings it has been suggested that SP-NK1R system may be also involved in

affective behavior. This led to the pharmacological investigations which are focused on SP-NK1R system in affective disorders especially in depression and anxiety (Santarelli et al., 2002). Administration of SP or its agonists increased anxiety-like behavior in rodents (Elliott, 1988; Aguiar and Brandao, 1996; Teixeira et al., 1996). On the other hand, stress exposure resulted in changes in SP levels (Bannon et al., 1986; Brodin et al., 1994).Further, antidepressant and anxiolytic drugs have been shown to reduce SP synthesis (Walker et al., 1991; Shirayama et al., 1996).

SP also have important roles in the inflammation and cell trafficking. It is known that several immune cells including lymphocytes, macrophages, mast cells and microglia express receptors for SP, and further, they can be stimulated by SP to produce inflammatory cytokines (Ho et al., 1997; De Giorgio et al., 1998; Tripp et al., 2002) (Giulian et al., 1996; Maeda et al., 1997; Marriott and Bost, 2001b). SP increases vascular permeability and increases the migration of leukocytes into the inflamed tissue (Pernow, 1983). SP also induces the expression of adhesion molecules on leukocytes. SP is found to increase Mac-1 levels on neutrophils, ICAM-1 and LFA-1 levels on endothelial cells and lymphocytes, respectively (Matis et al., 1990; DeRose et al., 1994; Vishwanath and Mukherjee, 1996) which results in the migration of lymphocytes (Saban et al., 1997). Moreover, SP enhances the production of proinflammatory cytokines (Lotz et al., 1988; Lieb et al., 1996; Kincy-Cain and Bost, 1997). SP can modulate the function of immune cells via NK-1 receptors (Kincy-Cain and Bost, 1997; Marriott and Bost, 2000; Marriott et al., 2000; Marriott and Bost, 2001a). Cytokines are also able to modulate the SP-NK1R system. Both inflammatory cytokine IFN-γ and anti-inflammatory cytokine IL-4 are found to modulate NK-1R expression on peritoneal macrophages (Marriott and Bost, 2000).

Therefore, we investigated the effects of IFN-α on the expression of SP receptor on leukocytes. We aimed to find out whether IFN-α alters NK-1R expression on different populations of leukocytes. However, in our experiments we found that IFN-α did not change the expression of NK-1R on lymphocytes or other leukocyte populations. We investigated the expression of NK-1R on tissue macrophages; however we could not detect any changes. These finding suggests that the increase of adhesion molecules on lymphocytes following IFN-α administration is not modulated by SP-NK-1R system.

The mechanisms, by which immune changes modify behavior, might include cells linking the immune system to the brain. Microglial cells, the resident macrophages of the brain, make up to 10 % of the brain population and might have important roles in cytokine induced depression. Being very sensitive to the changes in the brain environment, microglial cells respond quickly to inflammatory stimuli and alter their phenotype and morphology. It was also shown that activated microglia can present antigens to T cells (Matyszak and Perry, 2002). The binding sites for IFN-α are found in the brain tissue, where specific receptors for IFN-α are detected on neurons, but also constitutively expressed on microglial cells (Janicki, 1992). In our experiments, we detected that the percentages of MHC-II$^+$ microglia and brain macrophages were increased compared with the control group. Activated microglial cells might get in touch with the presently migrated activated lymphocytes and modulate the immune response. Therefore, understanding the interaction between microglia and T cells is crucial for a better insight into the inflammatory processes induced by IFN-α.

Cytokines are known to be involved in the communication between the CNS and the neuroendocrine system. The levels of anxiety and depression were found to be correlated with the levels of the circulating cytokines (Reichenberg et al., 2001; Reichenberg et al., 2002). Depressed patients show a shift in the Th1/Th2 ratio towards Th1 dominance (Schleifer et al., 1999; Schwarz et al., 2001). However, in our study we did not find significant changes in the Th1/Th2 ratio. Instead, we detected a trend towards increased TNF-α and IFN-γ levels, and significantly reduced IL-6 levels. Further studies are needed to uncover the role of cytokines in IFN-α-induced changes in behavior and the permeability of the BBB.

In summary, we investigated the behavioral and immune effects of IFN-α administration in mice. We focused on lymphocyte trafficking into the brain as a possible mechanism providing neuro-immune cross-talk and contributing to IFN-α-induced behavioral changes. Our data demonstrate that mouse IFN-α can cause behavioral changes in the FST. Moreover, IFN-α affected lymphocyte trafficking into the brain via increasing the expression of adhesion molecules, especially LFA-1 on lymphocytes and enhancing the presence of the lymphocyte subsets CD4$^+$ and CD8$^+$ in the brain. Our results suggest a relationship between the immune system and behavior in terms of IFN-α administration. Further rodent studies are required to investigate the effects of IFN-α administration in

mice on the functions of lymphocytes in brain. The cross-talk of lymphocytes with resident brain cells, such as microglia, will contribute to our understanding of IFN-α-induced depression.

5. Conclusions

In this work, we investigated the neuro-immune-endocrine interactions. For this purpose, we used two different animal models both of which helped us to investigate different aspects of immune and nervous responses depending on the stimulus. We can conclude our data as follows:

Regarding dydrogesterone,

1) Injection of 1.25 µg of the progesterone derivative dydrogesterone decreased the expression of SP receptor NK-1R on blood derived and tissue derived (uterine) lymphocytes.

2) Culture of splenocytes in the presence of dydrogesterone at 10^{-8} and 10^{-9} M decreased the percentage of $NK1R^{+}$ cells *in vitro*.

3) Dydrogesterone increased the expression of Th2 type cytokines IL-4 and IL-10 on splenocytes *in vitro.*

4) Dydrogesterone reduced the pain perception in the tail flick test 120 minutes upon injection *in vivo.*

With regard to IFN-α,

1) We detected that repeated administration of recombinant mouse IFN-α resulted in increased immobility of male mice in the late phase of the forced swimming test, without significant effects in the tail suspension test and the elevated plus maze test, thus inducing depression-like behavior without effecting anxiety-like behavior in mice.

2) IFN-α increased the expression of adhesion molecules LFA-1, Mac-1 on blood derived lymphocytes but not on granulocytes or monocytes.

3) IFN-α increased the percentage of NK cells in the peripheral blood

4) The percentages of $CD4^+$ and $CD8^+$ lymphocytes and the percentages of LFA-1-expressing $CD4^+$ and $CD8^+$ lymphocytes were increased in the brains of IFN-α treated mice compared with control mice.

5) *In vitro* stimulation of blood-derived lymphocytes showed a trend towards increased TNF-α and IFN-γ levels, and decreased IL-6 levels. However the Th1/Th2 ratio was not altered.

6) IFN-α did not change the NK-1R expression on lymphocytes, monocytes and granulocytes.

Thus, our data support the notion of reciprocal functional interactions between the nervous and immune systems.

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7. Appendix

7.1. Reagents and Chemicals

Product Name	***Catalog Number***	***Supplier***
Mouse interferon alpha	IFN009	Chemicon
Phosphate buffered saline	D5652	Dulbecco
Bovine serum albumine	A7030-50G	Sigma Aldrich
Ketanest S	0041116	Pfizer
Rhompuan 2%	KPD2XXXN	Bayer
Normal Mouse Serum	X0910	Dako Cytomation
RPMI	FG1385	Biochrom
Fetal Calves Serum	S0615	Biochrom
Percoll	17-0891-02	Amersham
Sodium azide	K32996788	Merck
phorbol-myristate acetate	P148	Sigma
ionomycin	407951	Calbiochem
Ethylenediaminetetraacetic acid	1.08418.0250	Merck
Ammonium chloride	1.01145.500	Merck

7.2. Kits

Product Name	***Catalog Number***	***Supplier***
Cytometric Bead Array Flex Set	CBA Flex Set	BD Biosciences
Limulus Amebocyte Lysate Assay	LAL-QCL-1000	Cambrex

7.3. Antibodies

Antibody	*Conjugated*	*Catalog Number*	*Working dilution*
Rat anti- CD3	Biotin	553060	1/200
Rat anti- CD4	PE	553049	1/200
Rat anti- CD8	APC	553035	1/200
Rat anti- CD19	Biotin	553784	1/200
Rat anti- CD25	PE	553075	1/200
Ar. Ham anti- CD11c	Biotin	553800	1/200
Rat anti- Mac-1	PerCP	550993	1/200
Rat anti- CD49b	Biotin	553856	1/200
Rat anti- LFA-1	FITC	553120	1/100
Rat anti- VLA-4	FITC	553156	1/100
Ham anti- ICAM-1	PE	553253	1/200
Ms anti- MHCII	PE	553544	1/200
Streptavidin	PerCP	554064	1/200
Streptavidin	APC	554067	1/200

7.4. Isotype controls

Antibody	*Conjugated*	*Catalog Number*	*Working dilution*
Rat IgG2a,k	FITC	554688	1/100
	PE	559317	1/200
Rat IgG2b,k	FITC	556923	1/100
	PE	553989	1/200
	biotin	553987	1/200
Ar Hamster IgG1,k	biotin	553970	1/200
Ms IgG2a,k	PE	555574	1/200

7.5. Equipment

Product Name	***Name/Catalog Number***	***Supplier***
Flow Cytometer	FACS calibur system	BD Biosciences
Automated cell counter	CASY, cell counter	Schaerfe Systems,
Centrifuge	5415C Labofuge 400R	Eppendorf,Germany
		Heraeus Instruments,
		Germany
Incubator	9140.000	Binder GmBH
Heat Plate	MR3001	Heidolph, Germany
Heat Bath	1052	GFL Germany
Light Microscope	Primostar	Zeiss, Germany
pH meter	Microprocessor pH meter	WTW, Germany
	pH 537	
Sterile Bench	HBB2448	Heraueus Instruments
Vortex	Vibrofix VF1 IKA labortechnik	Jahnke and Kunkel
		Germany
Cell Strainer(40,100	352340,352360	BD labware
Conical tubes	352070	BD Falcon
Eppendorf tubes	352096	BD Falcon
Plastic Pasteur Pipettes	EF20365H	BD Falcon
Falcon Cell culture	Cell Culture Plates	BD labware
plates (6,24 wells)		

7.6. Buffers

Ammonium chloride lysis buffer: Ammonium chloride 82.9 g, potassium bicarbonate 10.0g, EDTA disodium salt 0.37g, distilled water *ad* 1.0 liter, pH adjusted to 7.2 to 7.4 using 1 M NaOH or 1 M HCl, and stored at 4°C.

FACS buffer: 1% BSA in PBS containing 0.1 % Na-acid, pH is adjusted to 7.2-7.4 using 1 M NaOH or 1 M HCl and stored at 4 °C.

Ketanest-Rhompun Solution: Stock solution: a mixture of 2 ml Ketanest S (25 mg/ml) and 0.332 ml Rompuan (2 %). Working solution: a dilution of 1:4 of stock solution with 0.9 % NaCl

Stock isotonic percoll solution*:* Prepared by adding 9 parts (v/v) of Percoll to 1 part of x10 concentrated PBS solution. Further, 30 % and 70 % of Percoll solutions were prepared by necessary amount of adding X1 PBS solution.

7.7. Softwares

Product Name	***Supplier***
FCAP ArrayTM software	Becton Dickinson
Cell Quest	Becton Dickinson
SPSS version 14	SPSS inc

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9. *Curriculum Vitae*

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht

List of publications related to the thesis

1. **Orsal AS**, Blois SM, Bermpohl D, Schaefer M, Coquery N. Administration of Interferon-Alpha in Mice Provokes Peripheral and Central Modulation of Immune Cells, Accompanied by Behavioral Effects. Neuropsychobiology 2009 Feb 12;58(3-4):211-222.
2. Blois SM, Barrientos G, Garcia MG, **Orsal AS**, Tometten M, Cordo-Russo RI, Klapp BF, Santoni A, Fernández N, Terness P, Arck PC. Interaction between dendritic cells and natural killer cells during pregnancy in mice. J Mol Med. 2008 Jul;86(7):837-52.
3. Blois SM, Ilarregui JM, Tometten M, Garcia M, **Orsal AS**, Toscano MA, Handjiski B, Tirado I, Markert UR, Poirier F, Szekeres-Bartho J, Rabinovich GA & Arck PC. A pivotal role for galectin-1 in fetomaternal tolerance. Nat Med. 2007 Dec;13(12):1450-7.
4. Pincus-Knackstedt MK, Joachim RA, Blois SM, Douglas AJ, **Orsal AS**, Klapp BF,Wahn U, Hamelmann E, Arck PC.Prenatal stress enhances susceptibility of murine adult offspring toward airway inflammation. J Immunol. 2006 Dec 15;177(12):8484-92.
5. Garcia MG, Tirado-Gonzalez I, Handjiski B, Tometten M, **Orsal AS**, Hajos SE, Fernandez N, Arck PC, Blois SM. High Expression of Survivin and Down-Regulation of Stat Characterize the Feto-Maternal Interface in Failing Murine Pregnancies During the Implantation Period. Placenta. 2007 Jul; 28(7):650-657. Epub 2006 Nov 16.
6. **Orsal AS**, Blois S, Labuz D, Peters EM, Schaefer M, Arck PC. The progesterone derivative dydrogesterone down-regulates neurokinin 1 receptor expression on lymphocytes, induces a Th2 skew and exerts hypoalgesic effects in mice. J Mol Med. 2006 Feb;84(2):159-67.

Meetings with poster /oral presentations related to the thesis

1. Behavioral and immune effects of chronic interferon alpha administration in mice.Society for Neuroscience Meeting, San Diego, USA, 2007
2. Behavioral and immune effects of chronic interferon alpha administration in mice. Turkish National Neuroscience Congress, Safranbolu, Turkey, 2007
3. Effect of chronic interferon alpha application in mice on behavior and immune response. 6th meeting of the German Endocrine Brain Immune Network, Freiburg, Germany, 2007
4. Progesterone supplimentation abrogates stress-triggered foetal rejection in a mouse model via upregulation of Galectin-1, Th2 cytokines and CD8+ cell-dependent pathways. 6th meeting of the German Endocrine Brain Immune Network, Freiburg, Germany, 2007
5. Allergic disease reduces strss-coping skills: indications from a mouse model. 6th meeting of the German Endocrine Brain Immune Network, Freiburg, Germany, 2007
6. Behavioral and –immune effects of chronic interferon alpha administration in mice. Berlin Brain Days, 3rd International PhD Student Symposium & Seminar on Imaging in Neuroscience, Berlin, Germany, 2006
7. The progesterone derivative dydrogesterone down-regulates neurokinin 1 receptor expression on lymphocytes, induces a Th2 skew and exerts hypoalgesic effects in mice. Forum of European Neuroscience, 2006, Vienna, Austria
8. Behavioral changes as a response to stress exposure in a mouse model of experimental allergic dermatitis.Annual Conference of the German Dermatological Research Society, Aachen, Germany, 2006
9. The progesterone derivative dydrogesterone downregulates Neurokinin 1 receptor expression on uterine lymphocytes, and induces a Th2 skew in mice. Joint Annual Meeting of the German and Scandinavian Societies for Immunology, Kiel, Germany 2005
10. Pregnancy protective asymmetricantibodies (Aab) are down-regulated in failing mammalian pregnancies. Joint Annual Meeting of the German and Scandinavian Societies for Immunology, Kiel, Germany 2005

11. Effects of stress on the ICAM-1/LFA-1 cross talk during pregnancy. 4th symposium of German Brain Endocrine Immune Network, Bayreuth, Germany, 2005
12. Progesterone derivative dydrogesterone down-regulates the Neurokinin 1 receptor on lymphocytes, induces a Th2 skew and exerts hypoalgesic effects in mice. 4th symposium of German Brain Endocrine Immune Network, Bayreuth, Germany, 2005
13. Progesterone derivative dydrogsterone has hypoalgesic effect and modulates NK1 receptor expression. International Alfried Krupp Stress, Behavior, and Immune Response Symposium, Greifswald,Germany, 2004.

10. Eidesstattliche Erklärung

„Ich, Arif Suphi Örsal, erkläre, dass ich die vorgelegte Dissertationsschrift mit dem Thema: „Investigation of neuro-immune interactions under the influence of dydrogesterone and interferon alpha“ selbst verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt, ohne die (unzulässige) Hilfe Dritter verfasst und auch in Teilen keine Kopien anderer Arbeiten dargestellt habe.“

Berlin, den 09. März 2009 Arif Suphi Örsal