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Influence of prostaglandin E_2, D_2 and J_2 on IL-12-related cytokine subunits in murine dendritic cells

Einfluß von Prostaglandin E_2, D_2, J_2 auf die Expression von Untereinheiten der IL-12-verwandten Zytokine in murinen dendritischen Zelllinien

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Abbreviations

15d-PGJ_2	15-Deoxy-Δ-^{12,14}-PGJ_2
αCD40	stimulating antibody specific for CD40
Ab	antibody (pAb-polyclonal, mAb-monoclonal)
AEC	3-Amino-9-ethylcarbazol
Ag	antigen
AP	Alkaline phosphatase
APS	Ammonium persulfate
β-ME	β-mercaptoethanol
BALB/c	inbred homozygotic white-fured mouse strain
bp	base pairs
BSA	bovine serum albumin
C57BL/6	inbred homocygotic black-fured mouse strain
CCR	chemokine receptor
CD	cluster of differentiation
CIA	collagen-induced arthritis
Con A	concanavalin A
CpG	immunostimulatory viral or bacterial DNA motive; **c**ytosine linked to **g**uanine by a **p**hosphate bond
CRTH2	chemoattractant receptor-homologous molecule expressed on Th2
dbcAMP	N-2-O-dibutyryl-cAMP
dest.	distilled
DMSO	dimethylsulfoxide N,N dimethylformamide
DNA	deoxyribonucleic acid
DP	prostaglandin D_2 receptor
DTT	dithiothreitol
EAE	experimental allergic encephalopathy
EBI3	Epstein Barr-virus induced molecule 3
EDTA	ethylendiamine tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EP	prostaglandin E_2 receptor
FACS	fluorescence activated cell sorter
FCS	fetal calf serum
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GM-CSF	granulocyte macrophage-colony stimulating factor
HRP	horseradish peroxidase
IBD	inflammatory bowel disease
IFN	interferon

Ig	immunoglubulin
IL	interleukin
iLC	immature Langerhans cell
iMDC	immature myeloid dendritic cell
LPS	lipopolysaccharide
MΦ	macrophage
MEM	minimum essential medium
MHC	major histocompatibility complex
mo (as prefix)	murine
MyD88	myeloid differentiation primary response gene (88)
NEA	non-essential amino acids
NF-κB	nuclear transcription factor κB
ODN	oligodesoxynucleotide
OVA	ovalbumin
PAA	polyacrylamide
PBMC	peripheral blood mononuclear cells
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PG	prostaglandin
PMN	polymorphal mononuclear cells
PPAR-γ	peroxisome proliferator-activated receptor gamma
PVDF	polyvinylidene fluoride
DNA Pol-γ	mitochondrial DNA polymerase-γ
rec.	recombinant
rpm	rotations per minute
RPMI 1640	Roswell Park Memorial Institute medium 1640
RT	reverse transcriptase
SDS	Sodium dodecyl sulfate
SFC	spot forming cells
STAT	signal transducers and activators of transcription
Taq	Thermophilus aquaticus
T-bet	a transcription factor, pronouncedly expressed in T lymphocytes
TCR	T lymphocyte receptor
TEMED	N,N,N´,N´-Tetra-methyl-ethylenediamine
TGF-β	transforming growth factor-β
Th	helper T lymphocyte
TLR	Toll-like receptor
TNF-α	tumor necrosis factor-α
Tris	Tris(hydroxymethyl)-aminomethan

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1 Introduction

1.1 Components of the immune system

After an extensive research into the adaptive immune system including specific T lymphocytes and antibody secreting B lymphocytes it is only in the last decade that much progress has been made in characterisation of the phylogenetic older innate immune system (formerly known as phagocytic cell system) (Fearon and Locksley, 1996; Hoffmann et al., 1999). The importance of the innate immunity and its interactions with the acquired immune system has been recognised for a long time (Beutler, 2004; Medzhitov and Janeway, Jr., 1997; Mörner and Count K.A.H., 1908) but has been eclipsed by important discoveries in the acquired immunity cell system in the previous decades. Defects of either part but also overreaching activation may lead to severe polymodal immunological and autoimmune diseases with differing phenotypes.

Microbes entering the organisms and cellular and non-cellular residues (as in apoptosis or tissue injury) are in most cases directly eliminated by phagocytic cells such as granulocytes and macrophages (MΦ). Detection of these non-self molecules takes place by a large variety of genetically encoded antigen-specific receptors leading to a constant awareness but usually no alert of the immune system.

1.2 Polarization of the T helper cell response

Surpassing a specific stimulation threshold leads to an additional activation of the adaptive immune system by professional antigen-presenting cells (APC) that process the antigen and present it bound to MHC surface molecules. APC comprise B lymphocytes and MΦ but especially dendritic cells (DC). DC may decend from myeloid or lymphoid precursor cells and during their ontogenesis migrate to peripheral tissues – especially, with respect to the better characterised myeloid DC, immunological barriers such as intestine, skin and lung - and differentiate to take over their tissue-specific tasks. (Banchereau and Steinman, 1998). There in situ as immature DC with high phagocytic capacity, they capture antigen (Ag) and - under appropriately regulated conditions - process it (Fig. 1-1), while migrating to the regional lymph node and presenting it in the proper MHC context to Ag-specific, naïve or memory T lymphocytes. In addition, it is

now accepted that in chronic inflammation antigen presentation may also take place at the site of inflammation.

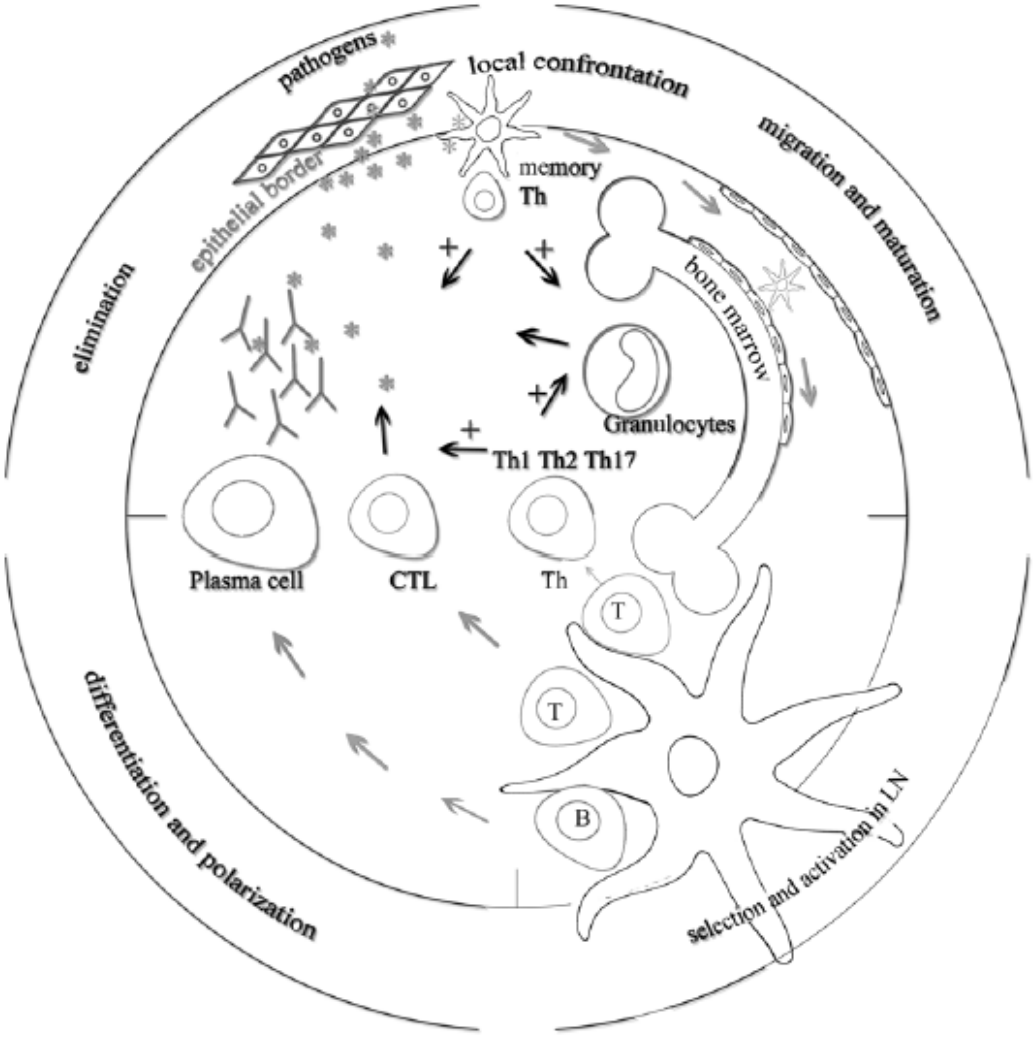

Fig. 1-1: Overview of the interplay of immune system components; modified scheme (Banchereau et al., 2000); to be read clockwise.

Pathogens entering the organism are detected, phagocytised and processed by antigen-presenting cells such as immature DC. DC trigger a local inflammation and activate antigen-specific local memory Th (early response) but also migrate to lymphoid tissue and mature (delayed response), selecting and activating antigen-specific $CD4^+$ T lymphocytes (T) and B lymphocytes (B). B lymphocytes mature to plasma cells, T lymphocytes polarize to either $CD4^+$, $CD8^+$ and NKL (CTL), additionally activating granulocytes. A modulated response is achieved under the action of cytokines and tissue hormones. Myeloid progenitors from the bone marrow constantly renew described immune cells.

The initiation of the adaptive immune response by DC leads to the subsequent activation of effector cells such as B lymphocytes and cytotoxic lymphocytes (CTL) comprising natural killer (NK) and $CD8^+$ T lymphocytes (Abbas and Lichtman, 2003). Tight regulation of the T lymphocyte activation requires two parallel signals: first the MHC-TCR interaction - MHC I-antigen complexes are recognized by T cell receptors

(TCR) of $CD8^+$ T cytotoxic cells, MHC II-antigen complexes are recognized by $CD4^+$ T helper cells (Th) - second costimulating signals through activation via the B7-1 (CD80) and B7-2 (CD86) (Freedman et al., 1987; Greenwald, Freeman, and Sharpe, 2005) and the T lymphocyte CD28 molecule (Lenschow, Walunas, and Bluestone, 1996). The typical morphologic "dendritic" shape by this means favors DC-T lymphocyte contact and in this way directs T lymphocyte response by paracrine cytokine secretion. This initial first contact is crucial for the polarization of the $CD4^+$ Th subsets and is tightly regulated. Naïve and memory Th can differentiate during Ag-presentation into Th1, Th2 and probably Th17. Th1 produce IFN-γ, TNF-α and IL-1 (Mosmann and Coffman, 1986) which in turn promote activation of cytotoxic lymphocytes, lymphocyte recruitment and local inflammation. Inflammation and cytotoxic lymphocyte activation via type I interferon is required for coping with intracellular pathogens (Hertzog, O'Neill, and Hamilton, 2003) but is also associated with chronic inflammation and autoimmune disease. New theories contribute at least parts of auto-aggressive T lymphotoxicity to Th17 cells that may evolve independently of Th1. Th2 products IL-4, IL-5, IL-10 and IL-13 are involved in protection against extracellular pathogens, allergic diseases but also in the promotion of a tolerogenic polarization of the immune system. Both act by regulating immune effectors, including Ag-specific $CD8^+$ cytotoxic T lymphocytes and B lymphocytes as well as non–Ag-specific MΦ, eosinophils and NK cells.

The induction of a Th1 type or a Th2 type response is mediated by a third signal constituted either by a predomination of Th2 type cytokines (especially IL-4, OX40L) – leading to a Th2 response – or by Th1 type cytokines (especially IL-12) that are secreted by DC leading to a Th1-type differentiation. High expression of these polarizing cytokines requires an additional signal assuring the optimal and timely expression of Th-cell-polarizing molecules. For IL-12 it was shown that high expression may involve an effective binding of CD40 by CD40L, which is rapidly expressed by the T cells upon encounter with endogenous danger signals (Cella et al., 1996; Schulz et al., 2000).

Subsequent to polarization, Th1 cytokine secretion including especially IL-2 leads to clonal Th1 expansion and IFN-γ known to influence nearby dendritic cells reinforcing the development of Th1 differentiation.

1.3 Functional states of dendritic cells

Different subsets of DC derived from myeloid as well as lymphoid DC precusors have been characterized as specialized initiators of a specific immune response. These cell subsets differ in morphology and function. Immature tissue-residing DC possess high endocytotic and phagocytotic capacities and after confrontation with antigen and syngeneic T lymphocytes undergo a tightly regulated maturation during migration to secondary lymphoid organs. During this process the DC change their phenotype by upregulating surface molecules such as T lymphocyte costimulating molecules (e.g. CD40, CD80, CD86), intercellular adhesion molecules (CD54, CD58) and integrins (CD11a, CD11b, CD11c, E-cadherin).

The antigen, the DC are confronted with, is either phagocytised (leading directly to an adaptive immune response) or bound to innate pathogen recognition receptors such as Toll-like receptors (TLR) (Rock et al., 1998) or Fc receptors (Geissmann et al., 2001).

Prominent receptor subtypes are TLR9 (Takeda, Kaisho, and Akira, 2003) being highly specific for viral and bacterial DNA (Krieg, 2002) and TLR4 (Poltorak et al., 2000) binding to lipopolysaccharide (LPS) with its immunogenic lipid A as main endotoxin of Gram-negative bacteria (Takada et al., 1985; Beutler and Rietschel, 2003).

It is known that a) different DC subsets express different sets of TLR receptors and b) the mode of TLR-mediated activation shows differences in immune response although presumably common signaling pathways are used. It was demonstrated that in the absence of the signaling adapter molecule MyD88 (Schnare et al., 2000) or the adaptor proteins CD14 (Haziot et al., 1996) and MD-2 (Shimazu et al., 1999) TLR4 but not TLR9-induced activation is severly impaired, suggesting that TLR might mediate a microbe-specific response.

Langerhans cells (LC) constitute a well-characterized tissue-differentiated DC subset derived from myeloid precursors. They are characterized by expression of antigen-presentation markers, different chemokine-recepotors, expression of integrins, absence of the monocyte-marker CD14 but expression of the DC-specific marker CD11c (a iC3b binding integrin) (Larregina et al., 2001). Crucial for the development is the expression of TGF–β_1 receptors (Borkowski et al., 1996). Morphologically LC possess Birbeck granula, intracytoplasmatic vesicles unique for this cell type, presumably involved in the uptake and trafficking of molecules internalised through the C-type lectin langerin (Valladeau et al., 1999). LC express TLR2 (receptor for bacterial lipoproteins and

peptidoglycan), TLR4 and TLR9 (Mitsui et al., 2004); specialized subsets (like XS52 cells) express also the imiquomod-ligand TLR7 (Thatcher et al., 2006).

Plasmacytoid DC (pDC) (Liu, 2005) constitute the major representative of DC derived from lymphoid precursors. They are characterized by the expression of TCR-markers CD4 but not CD3, the expression of CD123, the absence of the expression of antigen-presentation molecule CD1 and the DC marker CD11c. Essential for the development seems the expression of FLT3 ligand. Morphologically, pDC resemble plasma cells. They express mainly TLR7 and TLR9 and produce large amounts of IFN-α upon stimulation, supposedly involved especially in host defence against viral pathogens. pDC are now considered to be responsible for tolerance of the immune system towards auto-antigens (Ochando et al., 2006).

1.4 Characterization of murine dendritic cell lines

XS52 created by S. Xu et al. in 1995 is an immature Langerhans dendritic cell line (iLC) derived from neonatal mouse skin (Xu et al., 1995) differing from freshly isolated Langerhans cells in morphologic and functional features but express comparable cellular differentiation markers as short term-cultured immature Langerhans cells.

They are characterized by a weak expression of the myeloid marker CD34, expression of CD1a (MHC II), weak expression of E-cadherin, a molecule that mediates cell adhesion to keratinocytes by the CLA-1 receptor (Tang et al., 1993), expression of lymph node homing receptors CD11a (binding ICAM-1), CD11b (binding CD54, an endothelial surface molecule), the chemokine receptor CCR6 and the receptor for TGF-β_1 (Radeke et al., 2005) that binds the keratinocyte-released chemokine MIP3α. XS52 have been tested negative for Birbeck granula, whose loss has to be considered as a common feature of Langerhans cells being upheld in cell culture (Schuler and Steinman, 1985).

Under a protocol of T lymphocyte-mediated final maturation (Yamada and Katz, 1999), upregulation of T lymphocyte costimulatory molecules B7-2 (CD86), B7-1 (CD80), CD40, ICAM-1 (CD54) (Bouis et al., 2001), CD11c, MHC II complexes, loss of CD14 and upregulation of CCR7 (interacting with L-selectin ligands in HEV, CCL19 and CCL21) is observed. Although LC generally express prostaglandins (Ruzicka and

Aubock, 1987), it was further demonstrated by the "Foundation Immune Pharmacology" that XS52 cells do not produce significant amounts of neither PGE_2 or PGD_2.

The poorly characterized immature myeloid dendritic cell line JawsII (iMDC) created by V. MacKay is a commercial cell line that is supposed to represent a common precursor of both DC and MΦ (Jorgensen, Haase, and Michelsen, 2002). The CD11b, CD11c, CD14, CD34 expressing iMDC have preserved an immature phenotype (low expression of MHC II) throughout long term culture. Jorgensen et al. further described a deficiency of the cell line for CD40 and IL-12 expression. In contrast, other groups including the "Foundation Immune Pharmacology" could detect the expression of these markers (Awasthi and Cox, 2003; Chen et al., 2004).

1.5 The IL-12 related pro-inflammatory cytokines

1.5.1 Interleukin-12

IL-12, first named "natural killer cell stimulating factor", was identified as a product of EBV-transformed human B-cell lines (Kobayashi et al., 1989) but is physiologically mainly produced by activated DC, monocytes, B lymphocytes, neutrophils and keratinocytes. It is known to exert pleiotrophic effects on B lymphocytes but more prominently on NK cells and also initiates the Th1 response, including the enhancement of cytolytic activity and co-stimulation of proliferation (Trinchieri, 2003).

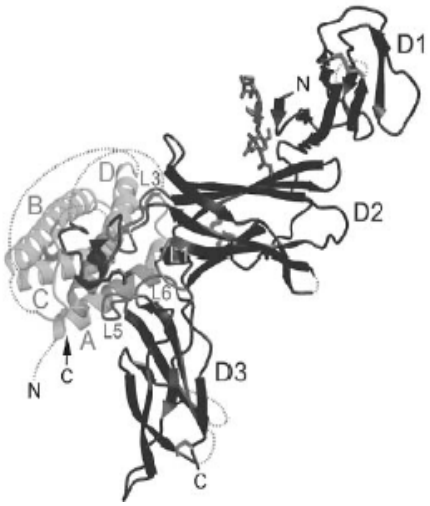

Fig. 1-2: Molecular structure of IL-12 according to Yoon et al. (Yoon et al., 2000).

The figure represents a molecular model of IL-12 based on x-ray crystallography. The IL-12 molecule is composed of the IL-12p40 and IL-12p35 (at the left). The structure of IL-12p40 with its three domains D1, D2, D3 resembles the extracellular domain of class I cytokine receptors. p35 is associated to this unit as a receptor ligand and additionally linked by disulfide-bonds that might be necessary due to a probable instability of the p35-protein.

IL-12 enhances IFN-γ production by these cells which in turn activates DC in a positive feedback mechanism (Ma et al., 1996) crucial for immune response as described in knockout models (Kaplan et al., 1996). The major trigger for the production of IL-12, constitute bacterial, viral and parasitic motives binding to TLR. Further enhancement of bioactive IL-12 is induced by IL-12 itself and in combination with IL-18, TNF-α and as described above by IFN-γ. Inhibition of IL-12 secretion is mediated by TGF-β and IL-10 (Aste-Amezaga et al., 1998; Du and Sriram, 1998).

Structurally IL-12 is a heterodimeric protein composed of two glycoprotein subunits, p35 and p40 linked together by both disulfide-bonds and charge-dependent interactions (Fig. 1-2) binding to a high affinity transmembrane receptor composed of two type-I-transmembrane-glycoprotein subunits (IL-12Rβ_1 and IL-12Rβ_2). IL-12Rβ_2 is expressed on T lymphocytes, NK cells, DC and B lymphocytes and induced during Th1-type immune response (Szabo et al., 1997). STAT4 as downstream signal transducer is considered to be necessary for differentiation of naïve T lymphocytes into IFN-γ producing Th1. IFN-γ then activates the STAT1 transcription factor and subsequent T-bet expression which is required for optimal IL-12Rβ_2 expression.

1.5.2 IL-12-related cytokines

The recently characterized APC-expressed cytokine subunits p19 (Oppmann et al., 2000) that is able to complex with the IL-12p40 subunit and the homologuous p28 (Pflanz et al., 2002) make necessary a revised characterization of IL-12 and its described effects as they partially overlap with these new subunits (Hunter, 2005).

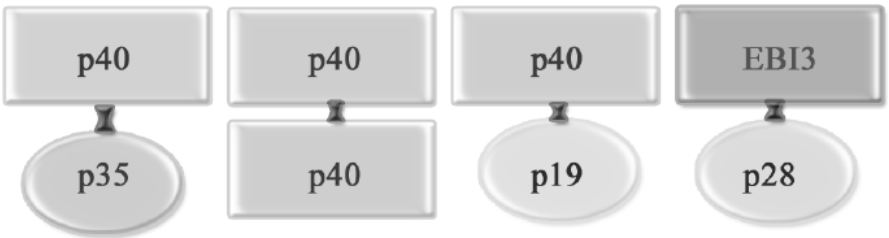

Fig. 1-3: IL-12 subunits according to Brombacher et al. (Brombacher, Kastelein, and Alber, 2003).

From left to right: IL-12 (p70) composed of the p40 subunit bound to the p35 subunit, p80 composed of two p40 monomers, IL-23 composed of the p19 subunit bound to p40 and IL-27 composed of EBI3 – a p40 homologue, bound to p28 (homologue of p35). The subunits are connected by disulfide-bridges.

1.5.2.1 Interleukin-23

IL-23 like IL-12 is a heterodimeric cytokine composed of the promiscuous IL-12p40 subunit and the p19 subunit, connected by a disulfide-bridge and binding to the receptor composed of IL-12Rβ_1 and IL-23R expressed on T lymphocytes, NK cells, monocytes and DC (Parham et al., 2002).

It was shown that IL-12p35-deficient mice develop a severe progression in murine autoimmune disease models such as experimental allergic encephalopathy (EAE) and collagen-induced arthritis (CIA) and presumably inflammatory bowel disease (IBD). These effects are contributed to a more pronounced expression of bioactive IL-23. Aggressive inflammation can also achieved by a transgenic expression of IL-12p40 and more important IL-23p19 leading to premature death while IL-23p19-deficient mice are resistant to the development of EAE (Watford and O'Shea, 2003).

It was shown that IL-23 - especially in the absence of IFN-γ - leads to a pronounced expression of IL-17A and IL-17F by memory and activated Th cells (Aggarwal et al., 2003). IL-17 related cytokines, that are exclusively secreted by $CD4^+$ and $CD8^+$ T lympocytes, are known to provoke tissue inflammation by inducing proinflammatory and neutrophil-mobilizing cytokines and by this constitute major mediators of autoimmune inflammation (Kolls and Linden, 2004). IFN-γ and IL-12 suppress the development of a IL-17 response (McKenzie, Kastelein, and Cua, 2006).

1.5.2.2 Interleukin-27

A further subunit, the Epstein–Barr virus induced gene 3 (EBI3) that is known to be related to IL-12p40 and to be able to engage the p35 subunit was characterized by Devergne et al. (Devergne et al., 1996; Devergne, Birkenbach, and Kieff, 1997). EBI3 further dimerizes the recently described p28 chain to form the heterodimeric APC cytokine IL-27 and binds to the IL-27 receptor composed of WSX1 and gp130, a receptor subunit also used by IL-6. The IL-27 receptor is expressed especially on $CD4^+$, $CD8^+$, NK cells and mast cells.

IL-27 is characterized as a synergistic cytokine to IL-12, inducing proliferation of naive T lymphocytes and IFN-γ production, particularly in synergy with IL-12 and IL-18. It has been demonstrated that IL-27 in early inflammation mediates upregulation of IFN-γ

expression and of the IL-12Rβ_2 (the receptor subunit that is unique for the IL-12 receptor) via STAT1 and T-bet and this way increases the responsiveness of $CD4^+$ to IL-12. It further induces mast cells to secrete proinflammatory cytokines. In the presence of strong polarization, IL-27 acts as limiting cytokine by suppressing IL-2 expression and by this inhibits clonal T cell expansion. EBI3 is highly expressed in human placenta, upregulated in ulcerative colitis and induced during the activation of human monocytes and dendritic cells. Similar to IL-12p35 and IL-23p19, IL-27p28 can be secreted only when associated with IL-12p40 and EBI3, respectively.

1.6 Modulation of immune processes by local tissue cells

In epithelial barriers, bystander tissue cell products such as cytokines, biogen amines (e.g. histamine, VIP), lipidic mediators (e.g. prostaglandins, leukotriens, ceramides and sphingophospholipids), nucleotides and nucleosides (such as ATP and adenosine) morphins, lysophospholipids and PPAR-γ ligands have been reported to modulate diverse immune functions including a) tissue permeability necessary for immune cell migration (Lewis, Berg, and Kleine, 1995) but also b) suppression and initiation of adaptive immune processes. In the skin epithelial barrier specifically keratinocytes and fibroblasts bear these tasks.

Previous to these studies the reciprocal influence of nearby activated T lymphocytes and renal bystander cells (there: mesangial cells) has been studied in detail (Radeke, Schwinzer, and Resch, 1992). In more recent yet unpublished studies, Radeke et al. detected large amounts of PGE_2 and especially PGD_2 in supernatants of LPS-stimulated mesangial cells. Under the assumption that the same paracrinely active substances may modulate T cell function as well as antigen-presentation processes, these observations gave rise to the following studies using a Th1 memory clone and an immature epidermal Langerhans cell clone as APC. Early modulation at the site of inflammation seems to be a principal target insofar as it may suppress early local as well as nodal antigen-presentation of tissue-differentiated DC to Th cells and thus the subsequent polarization of potential harmful subsets of effector T lymphocytes.

1.6.1 Immunomodulatory effects of prostaglandins

Prostaglandins (PG) (Bergstrom and Samuelsson, 1962; Funk, 2001) are ubiquitous tissue hormones, with numerous tissue-specific activities acting paracrinely as well as autocrinely, being present in body fluids in pico- to nanomolar concentrations and reaching concentrations in the micromolar range under inflammation. After cellular activation by mechanical trauma, bacterial peptides, allergens or inflammatory mediators such as cytokines and growth hormones, they are de novo synthesized from arachidonic acid, that is kept esterified to phospholipids of the endoplasmatic reticulum and the nuclear membrane, by the action of isoformes of phospholipase A_2. They are liberated, then converted to an unstable endoperoxide intermediate by cyclooxygenases isoformes and subsequently metabolized to one of several related products including PGD_2, PGE_2, PGF_2, prostacyclin (PGI_2), and thromboxane A_2 through the action of specific PG synthases. Specifically cyclooxygenase 2 (COX 2), competing for the arachinodate with the lipoxygenase but also the specific prostaglandin synthases are induced by proinflammatory stimuli and are preferentially expressed in barrier epithelial tissues (Holtzman et al., 1994).

1.6.2 PGE_2

PGE_2 as potent lipidic mediator derived from arachidonic acid metabolism - produced under the regulation of the microsomal PGE_2 synthase (Jakobsson et al., 1999) - regulates a broad range of physiologic processes in the cardiovascular, endocrine, gastrointestinal, neural, pulmonary, reproductive, visual, and immune systems. Mainly produced by stromal cells and MΦ but also tumor cells, PGE_2 acts by binding to specific receptors belonging to the rhodopsin family of G protein coupled receptors, existing in four subtypes: EP_1, EP_2, EP_3 and EP_4 (Narumiya, Sugimoto, and Ushikubi, 1999).

EP_1 activates phosopholipase C and phosphatidylinositiol turnover and stimulates the release of intracellular Ca^{2+}. While EP_1 generally has low expression, moderate expression is described for kidney and lung epithelial cells.

EP_2 activates adenylate cyclase via a cholera toxin-sensitive, stimulatory G protein coupled pathway in response to butaprost. Although EP_4 also activates adenylate cyclase, it is insensitive to butaprost and has been shown to be responsible for DC

migration. EP_4-deficient mice are resistant to develop CIA (McCoy, Wicks, and Audoly, 2002). EP_2, as well as EP_4 expression is described for immune cells, epithelial cells and smooth muscles.

The EP_3 receptors stimulate the release of intracellular Ca^{2+} and inhibit cAMP metabolism; splicing variants α, β and γ with differing activities are described; the $EP_{3\gamma}$ isoform is also able to stimulate cAMP metabolism. EP_3 is ubiquitously expressed.

Both the cAMP and the calcium cascade fulfill complex "downstream" activations of different kinases, leading finally to the activation of transcription factors and may lead to lasting changes in cellular differentiation. Differential expression of these receptors allows a great variety of response in very different cells to the same stimulant, also reflecting the cellular differentiation state.

Th1 seem to preferentially express the receptors EP_3 and EP_4 (Bloom et al., 1999), PGE_2 seems to inhibit IL-2 and IFN-γ production in Th1 cells but not the production of IL-4 by Th2 cells (Betz and Fox, 1991), effects that can be reproduced by cAMP analogues. It is suggested that Th1 possess more or higher affinity binding sites for PGE_2 that explain differential effects compared to Th2 (Harris et al., 2002).

Contradictory results have been reported regarding T cell polarization induced by DCs that are cultured in the presence of PGE_2: Kalinski et al. (Kalinski et al., 1997; Kalinski et al., 1998; van der Pouw Kraan TC et al., 1995) reported that immature and mature DC, generated in the presence of PGE_2, either initially or during terminal maturation, induce naïve T cells towards a Th2 response by impairing the production of IL-12. Other investigators reported a Th1 type differentiation meditated by an increase in DC IL-12 expression (Morelli and Thomson, 2003; Rieser et al., 1997; Steinbrink et al., 2000). Under costimulation with LPS, PGE_2 depressed the amount of IL-12 in DC supernatants (Jozefowski, Bobek, and Marcinkiewicz, 2003).

This discussion should be understood within the context that the described effects seem to be highly dependent on the source of the DC, the extend of inflammation and the microenvironment. New insights into the biology of the IL-12 cytokine family supplemented these findings: While IL-12p35 - and not IL-12p40 - mRNA expression was observed to be decreased following stimulation with PGE_2 (Kalinski et al., 2001), it was later observed that IL-23p19 and total IL-23 were increased in cell culture supernatants of murine MDC cultured in the presence of PGE_2 (Schnurr et al., 2005; Sheibanie et al., 2004). Sheibanie and Schnurr demonstrated that PGE_2, acting via EP_2

and cAMP, promotes IL-23 but inhibit IL-12 expression in mature murine as well human MDC. Although it is not exactly known how downstream signaling pathways are involved, it was shown that STAT4, T-bet and probably STAT3 take part.

Beside diverse cytokines, cysteinyl leukotriene C_4 and PGE_2 have been demonstrated to potentiate chemokine-driven DC and LC migration by increasing responsiveness to CCR7 that acts as a cofactor on DC maturation (Narumiya, 2003).

Catabolism of PGE_2 takes place intracellularly and is rate-limited by cellular transporters, then oxidized by 15-hydroxy PG dehydrogenase or carbonyl reductase to inactive $PGF_{2\alpha}$. When cells rupture, intracellular PG dehydrogenase is released and acts extracellulary (Ivanov, Scheck, and Romanovsky, 2003). Various effects of PGE_2 on other immune cells are reported.

1.6.3 PGD_2

PGD_2 known to be implicated in platelet aggregation, relaxation of vascular and nonvascular smooth muscles, nerve cell functions and immune response is tightly regulated by the activity of COX2 and PGD_2 synthase existing in two isoformes (Trivedi et al., 2006). The gluthatione-dependent hematopoetic PGD_2 synthase (hPGDS) is expressed in APC, Th2 (but not Th1), megakaryocytes but especially in mast cells (Urade et al., 1990).

PGD_2 acts through its G protein-coupled receptor DP_1 (an activator of adenylate cyclase) and DP_2, a member of the chemoattractant receptors (also named CRTH2 for its selective expression on Th2 and not on Th1) that is an activator of phospholipase C (Nagata et al., 1999; Hirai et al., 2001). CRTH2 is expressed on monocytes, eosinophils and basophil granulocytes mediating chemotaxis and is considered to be implicated as a central player in promoting Th2-related allergic inflammation.

Although Th1 seem not to express DP receptors (Luster and Tager, 2004), increased IFN-γ mRNA levels are reported in skin biopsies sensitized with OVA and BW245C, a specific DP_1 ligand (Angeli et al., 2004).

PGD_2 induces a DP_1-mediated (CRTH2 is not expressed on DC) inhibition of LC trafficking to the draining lymph nodes (Angeli et al., 2001; Hammad et al., 2003).

PGD_2 and its selective agonist BW245C inhibit the IL-12 production in human monocyte derived DC and murine MDC (Faveeuw et al., 2003; Gosset et al., 2003), through DP_1-dependent and independent pathways.

The PGD_2 catabolism involves non-enzymatically intracellular as well as extracellular conversion into the cyclopentenones of the J-series. While some authors describe an albumin-catalized conversion, Shibata et al. (Shibata et al., 2002) describe a stabilization of PGD_2 mediated by human serum albumin.

1.6.4 15d-PGJ_2

PGD_2 can be enzymatically and non-enzymatically converted to the F and J series of prostaglandins beyond which 15d-PGJ_2 has been characterized as a very active metabolite whose effects overlap with PGD_2 (Scher and Pillinger, 2005).

15d-PGJ_2 is also a ligand of CRTH2 and after active resorption, may as a high affinitiy ligand interact with the nuclear peroxisome proliferator-activated receptor γ (PPAR-γ), expressed predominantely in adipose tissue, adrenal gland and spleen but also in MΦ. 15d-PGJ_2-mediated PPAR-γ activation affects the activation of NF-κB, AP1 and MAP kinase pathways, signal transducers and activators of transcription as well as the suppression of inducible nitric oxide synthase and proinflammatory cytokine synthesis (Spiegelman, 1998; Tilley, Coffman, and Koller, 2001). PPAR-γ-independent effects are observed.

The effects of 15d-PGJ_2 in DC and Th1 are complex and poorly characterized; its role in physiology and pathophysiology is unclear. DC (Jakobsen et al., 2006) as well as Th1 express PPAR-γ but not CRTH2.

PPAR-γ agonists are reported to inhibit NF-κB and a consequent IL-12p40 transcription in DC (Appel et al., 2005; Faveeuw et al., 2000; Nencioni et al., 2002). It was further demonstrated that 15d-PGJ_2 decreases IL-2 in murine Th1 (Clark et al., 2000) and induces apoptosis in murine B and T lymphocytes.

1.7 Aim of the thesis

The following paragraphs describe dendritic cell assays that have been performed between January 2003 and January 2004. The data are based on the immunological and molecular biological tools available at this date. Newer antibodies and findings published since 2004 could not be integrated in these experimental setups.

Three cell lines, the described XS52 cell line, the JawsII cell and the Th1 cell line IF12 were arranged to imitate the in vivo cell contact of specialized tissue-differentiated DC with Th1 cells and the environmental influence of nearby stromal cells at the initiation of inflammation. Therefore I established two different methodical settings. In the first part the direct interactions of Th1 and DC are described through the expression of IFN-γ in Th1 cells as one parameter of the complex cytokine interplay. PGE_2 and PGD_2, known as modulating agents at the site of inflammation and produced by stromal cells were artificially added to the stimulated cells. The influence of these agents on the cytokine production was measured.

The second part of the assays centers on the dendritic cells as initiators of these inflammatory process. The IL-12-related cytokine subunits constitute a cytokine entity yet fragmentarily characterized. The modulation of these subunits by PGE_2 and PGD_2 was analyzed. I specifically addressed to possible differential effects of prostaglandins under differential stimulation with Toll-like receptors 4 and 9.

Moreover, it was my aim to clarify the use of antibodies against IL-12 related cytokine subunits, seeing as for more than ten years IL-12 has been detected in Western blot under non-reducing conditions, yet the p40-linked IL-23 (that shares a common subunit with IL-12) could not be identified. The identification of IL-23 was not possible until 2000 when computer-based homologous search of DNA-sequences led to its detection.

2 Materials and Methods

2.1 Materials

Chemicals were purchased from Merck (Darmstadt), AppliChem GmbH (Darmstadt), Sigma-Aldrich Chemical Co. (St.Louis/MO) and Carl Roth GmbH&CoKG (Karlsruhe) or as indicated in the text.

Concanavalin A, Forskolin, Ionomycin, N-2-O-dibutyryl-cAMP, Escherichia coli-derived (strain 0127:B8) lipopolysaccharide and grade VI ovalbumin were purchased from Sigma-Aldrich Chemical Co. (St.Louis/MO). Prostaglandins were purchased from Cayman Chemicals (Ann Arbor/MI). CpG with the sequence (small "g"s representing phosphothioates): 5'ggGGGACGATCGTCgggggG 3' was purchased from Sigma ARK genosys (Steinheim).

Plastics and cell culture material were purchased from BD Falcon (Bedford/MA), Eppendorf (Westbury/NY) or Greiner (Frickenhausen).

2.2 Cultivation of mammalian cells

All cells were cultivated in a standard cell incubator (*BBD 6220, Kendro Laboratory Products GmbH/ Heraeus, Hanau*) and grown at 37 °C and 5% CO_2 with saturating humidity (95%).

2.2.1 Cultivation and passaging of DC

JawsII (iMDC) medium

500 ml Iscove's medium	*Biochrom, Berlin*
5 ml L-glutamine (final 2 mM)	*Invitrogen Corp., Carlsbad/CA*
5 ml Penicillin/Streptomycin (final 100 IU/100 µg/ml)	*Gibco- Invitrogen, Grand Island/NY*
50 ml FCS (final 10%)	*Bio Whittaker-Cambrex, East Rutherford/NJ*
0.5 ml β-ME	*Gibco- Invitrogen, Grand Island/NY*
5 ng/ml rec. moGM-CSF	*Strathmann, Hamburg*

The commercially available murine C57BL/6 bone marrow-derived immature dendritic cell line JawsII (iMDC) was cultivated in the recommended medium in horizontally stored 75 cm^2 cell culture flasks *(Greiner bio-one, Frickenhausen)*. Cells were split once a week; adherent cells were harvested with cell scrapers, suspended with non-adherent iMDC, centrifuged *(Megafuge 1.0, Heraeus, Hanau)* for 5 min at 1200 rpm in

50 ml tubes *(BD Falcon, Bedford/MA)*. The supernatant was removed and cells were resuspended with unconditioned complete medium. The day before the assay, medium and GM-CSF were renewed and cell number per flask was set at 2.5 x 10^6 cells in 10 ml medium.

XS 52 (iLC) medium

500 ml Iscove's medium	*Biochrom, Berlin*
5 ml L-glutamine (final 2 mM)	*Invitrogen Corp., Carlsbad/CA*
5 ml Penicillin/Streptomycin (final 100 IU/100 µg/ml)	*Gibco- Invitrogen, Grand Island/NY*
5 ml Sodium-Pyruvate (1 mM)	*Gibco- Invitrogen, Grand Island/NY*
5 ml NEA (100 µM)	*Gibco- Invitrogen, Grand Island/NY*
25 ml FCS (final 5%)	*Bio Whittaker-Cambrex, East Rutherford/NJ*
0.5 ml β-ME	*Gibco- Invitrogen, Grand Island/NY*
10 ng/ml moGM-CSF	*Strathmann, Hamburg*

The murine embryonic BALB/c Langerhans cell line XS52 (iLC; kindly provided by *G. Müller, Department of Dermatology, Gutenberg Universität Mainz*) was cultivated in horizontally stored 175 ml cell culture flasks using a modification of the recommended medium; CSF-1 - an active component of fibroblast supernatants (Takashima et al., 1995) – was suggested to be added to the XS52 medium but was replaced by GM-CSF in our laboratories. The cell line tested negative for *Mycoplasma species* contamination in several assays with a commercial mycoplasma kit *(Venor GeM Kit Mycoplasma kit, Minerva biolabs/ Berlin).* iLC were splitted every 3-4 days; adherent cells were harvested with cell scrapers, suspended with non-adherent DC, centrifuged for 5 min at 1200 rpm. One day before the assay the medium was renewed and the cell number was set at 2.5 x 10^6 cells. During the assay no growth hormones were added.

2.2.2 Cultivation and passaging of Th1

IF12 (Th1) medium

500 ml RPMI 1640	*Gibco- Invitrogen, Grand Island/NY*
5 ml L-glutamine (final 2 mM)	*Invitrogen Corp., Carlsbad/CA*
5 ml Penicillin/Streptomycin (final 100 U/100 µl)	*Gibco- Invitrogen, Grand Island/NY*
5 ml NEA	*Gibco- Invitrogen, Grand Island/NY*
5 ng/ml rec. moIL-2	*Roche, Basel/Switzerland*
50 ml FCS (final 10%)	*Bio Whittaker-Cambrex, East Rutherford/NJ*

The $CCR7^+$ (Rubant, 2005), L-$selectin^-$ (unpublished observation) OVA-specific memory T-helper 1 cell line IF12 (Th1) (Radeke et al., 2002; Karulin et al., 2000;

Rubant et al., 2006) was cultivated in vertically stored 75 cm^2 flasks. The medium was renewed every 3 to 4 days, the cell number per flask was set at 250.000 cells/ml and cells were centrifuged at 1000 rpm for 5 min. Th1 were then resuspended in complete medium.

In a period of 4 weeks, a re-stimulation with OVA-loaded dendritic cells was necessary in order to uphold the specificity for the OVA protein. Therefore, 2.5 x 10^6 irradiated spleen-derived APC were added to 6 x 10^5 IF12. After addition of OVA (final concentration: 13.5 µg/ml) the coculture was uphold for 6 days in IL-2-deprived medium leading to the decline of the non-OVA-specific Th1. After a regeneration of 6 to 7 days in complete medium, the surviving Th1 were used again in assays.

2.2.3 Cell freezing and thawing

In a period of approximately twelve weeks the used cell lines were renewed by cell aliquots stored in common stock according to a modified protocol for cryopreservation of eucaryotic cells established by Sherman and Tarkowski (Sherman, 1964).

Approximately 3 x 10^6 cells/ml were pelleted as described above and then solved in freezing medium containing MEM alpha *(Gibco- Invitrogen, Grand Island/NY)*, 40 % heat-inactivated FCS, 20 % DMSO *(Dimethylsulfoxide, Merck, Darmstadt)* and transferred to freezing vials, placed into a Cryo freezing container *(Qualifreeze Cryo-Einfriergeräte, Merck-Qualilab, Darmstadt)* cooled 1°/min in a –80°C refrigerator (*freeze, Heraeus/Kendro, Hanau*) over night, then transferred to a liquid nitrogen tank (-192°C). For thawing, a vial of frozen cells was removed from liquid nitrogen and placed in a 37°C water bath *(W12, Preiss-Daimler, Medingen)* until thawed. To remove DMSO, cells were pipetted into a centrifuge tube containing 5 ml 100% FCS and prewarmed medium. After a centrifugation at 1000 rpm for 5 min, the supernatant was discarded and the cells were resuspended in 10 ml of complete medium and transferred to cell culture flasks. Cells were incubated overnight under usual cultivation conditions; the medium was replaced the next day.

2.3 Immunological methods

2.3.1 Generation of supernatants

Previous to the antigen detection, cultivation methods had to be adjusted due to a 60kDa band that occurred in FCS-containing supernatant showing an interference with the expected 40 to 80 kDa bands of IL-12 subunits. I therefore used FCS-free media for incubation during the assays. FCS contaminations were also observed in the rec. mo$(p40)_2$ *(499-ML, R&D Systems, Minneapolis/MN)* (Fig. 2-1). I standardized a common protocol for the cultivation of DC later using the cells for mRNA detection in RT-PCR and the supernatant proteins for cytokine detection in Western blot.

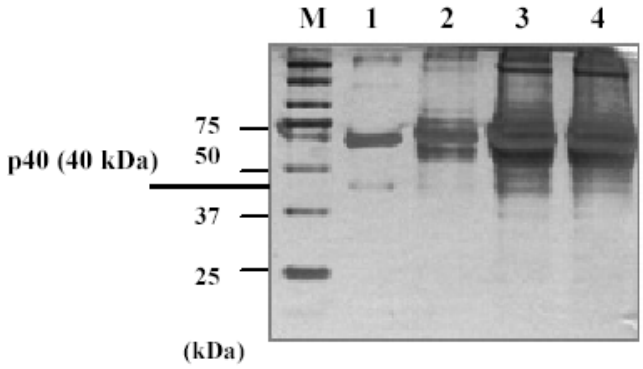

Fig. 2-1: Accompanying proteins in a commercial rec. moIL-12$(p40)_2$ preparation.

From left to right: *M:* 6 µl of protein standard, *lane 1:* 25 ng, *lane 2:* 40 ng, *lane 3:* 250 ng and *lane 4:* 500 ng of recombinant $(p40)_2$ separated under reducing conditions in a 10% polyacrylamide discontinous gel electrophoresis. Protein detection was performed according to the protocol for silver staining. Apart from the suspected 40 kDa band as detected in lane 1, a 60 kDa band with superior intensity could be detected at the molecular weight of approximately 60 kDa, representing contaminations of the commercial rec. protein.

2.3.2 Mass concentration of supernatants

Supernatants were concentrated with filter spin concentrators *(Centriprep YM-10, Millipore Corp., Bedford/MA)* with a molecular weight cut off of 10 kDa for 30 min at 3000 rpm in a centrifuge *(3K30, Sigma, St. Louis/MO)*. Proteins with lower molecular weight than 10 kDa were discarded. The qualitative concentration increased the amount of protein to be separated per lane in SDS-PAGE. The average volume of 9 to 10 ml was reduced to 2–2.5 ml, reflecting a concentration factor of approximately 4.

2.3.3 Measurement of protein concentration

Protein concentration in supernatants was determined with the BCA protein detection assay *(BCA Protein AssayKit, Pierce Biotechnology, Rockford/IL)*. The BCA (bicinchoninic acide) test according to Bredford (Smith et al., 1985) allows the colorimetric determination of protein content based on the biuret-reaction. Spectrometric peak of the reaction product is observed at 562 nm. I diluted the supernatant samples to 1:10, 1:20, 1:50 in dest. water on ELISA plates *(PS microplate 96, Greiner bio-one, Frickenhausen)*, parallelly diluted a protein standard (BSA) with a defined concentration, and incubated it for 45 min with the reagent at 37 °C. Measurement was performed with a spectral-photometer at 562 nm *(Elisa-Reader, SPECTRAFluor Plus, Tecan Maennedorf/Switzerland).*

2.3.4 Western blot assay

Western blot analysis allows the antibody-dependent detection of proteins after their separation according to their molecular weight by polyacrylamide electrophoresis followed by transfer to a PVDF or nitrocellulose membrane. I chose this method to characterize IL-12 subunit detecting antibodies that were later used in ELISPOT assays. Various commercial moIL-12 subunit antibodies with a described specificity for p70 were systematically tested. I used protein supernatants of CpG-stimulated *(Sigma ARK genosys, Steinheim)* or LPS-stimulated murine iLC and rec. mo$(p40)_2$ dimer as target protein. The results further allowed a quantitative analysis of p40 levels.

2.3.5 Discontinuous SDS-polyacrylamide gel electrophoresis

The SDS-polyacrylamide gel electrophoresis was performed with the vertical gel electrophoresis system Mini Protean III *(Bio-Rad, Hercules/CA)* in a 1.5 mm gel at 60 mA for 90 min. By the action of denaturing Sodium dodecylsulfate (SDS), proteins are unfold and unionized, allowing a separation in a polyacrylamide matrix under electricity according to their molecular weight. I chose a discontinuous gel system consisting of two phase of varying concentrations of polyacrylamide allowing a better resolution of the electrophoresis.

The 12.5 % separation gel, first founded in the chamber consists of:

3.3 ml H_20
4.2 ml 30% polyacrylamide
2.7 ml 1.5 M Tris (pH 8.8)
100 µl 10% SDS
100 µl 10% APS
4 µl TEMED
ad 10 ml.

The optimal polyacrylamide concentration depends on the proteins to be resolved; the chosen 12.5% is recommended for proteins < 60 kDa. 1 ml of isopropanol as detergent should be added directly after founding. Polymerization lasts approximately 1 h, then the stacking gel, a thin 4% polyacrylamide gel that should focus the protein bands, is added:

2.1 ml H_2O
0.5 ml 30% polyacrylamide
0.38 ml 1.5 M Tris pH 6.8
30 µl 10% SDS
30 µl 10% APS
3 µl TEMED
ad 3 ml.

5 µl per lane of Precision Plus Protein Standard *(Bio-Rad, Hercules/CA)* and 1 ng per lane of rec. mo$(p40)_2$ *(499-ML, R&D Systems, Minneapolis/MN)* was used as molecular weight marker and protein standard. Protein samples and rec. protein were loaded together with denaturing respectively non-reducing Laemmli-buffer (2X) (Laemmli, 1970). For denaturation, protein samples were heated up to 95 °C (*Heating block, HBT 130-2, HLC, Bovenden)* for 5 minutes in reducing Laemmli buffer.

Laemmli buffer (reducing)
3.8 ml H_2O dest.
1 ml 0.5 M Tris/Cl (pH 6.8)
0.8 ml glycerol
1.6 ml 10% SDS
0.4 ml β̃-ME
0.4 ml 0.005% bromphenol blue

Laemmli buffer (non-reducing)
1 g SDS
3 mg EDTA
0.001 g NaN_3
10 mg bromphenol blue
2.5 ml 0.5 M Tris/Cl (pH 6.8)
ad 100 ml H_2O dest.

2.3.6 Semidry blot

The proteins bound to the polyacrylamide gel were transferred from the gel to a Polyvinylidine fluoride (PVDF) membrane *(polyfiltronics PVDF membrane Immobilon P, Whatman, Kent/UK)* with the semi-dry electrophoresis method (Towbin, Staehelin, and Gordon, 1979).

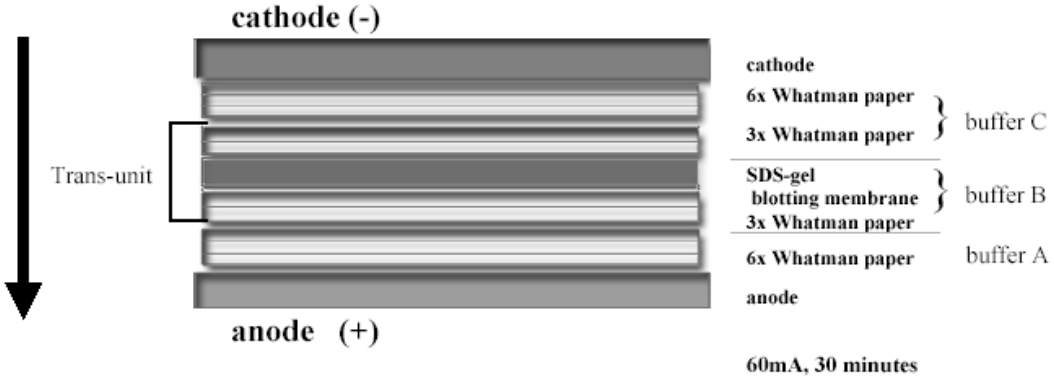

Fig. 2-2: Construction of the Western blotting system.

The Western blot system consists of anode and cathode blades and in-between buffer-wrenched filter papers favoring the transfer of the proteins on the electrophoresis gel to the activated PVDF membrane. Modified according to Westermeier (Westermeier, 1990).

The methanol-sensitized PVDF membrane was buffered with blotting buffer-saturated Whatman-paper sheets (8 x 10 cm) *(Whatman filter paper, Schleicher und Schüll, Dassel)*, the membrane was placed on the side of the gel facing the anode, and transferred during 30 min at 60 mA (0.8 mA/cm^2) in the Consort Western blot system *(V20-SDB, Consort, Turnhout/Belgium)* as described in Fig. 2-2.

Western blot buffers

Blotting buffer A (pH 10.4) anode buffer
0.3 M Tris (36.3 g)
20% methanol (200 ml)
ad 1 l H_2O

Blotting buffer B (ph 10.4) anode buffer
25 mM Tris (3.025 g)
20% methanol (200 ml)
ad 1 l H_2O

Blotting buffer C (pH 9.4) cathode buffer
25 mM Tris (3.025 g)
0.04 % ε-amino-n-capronacid (5.24 g)
20% methanol (200 ml)
ad 1 l H_2O

Electrophoresis buffer (pH 8.3)
2 M Tris (0.025%) 12.5 ml
2 M glycin (0.192%) 95.83 ml
10% SDS (0.1%) 10 ml
ad 1 l H_2O

The membrane was then washed with PBS/TWEEN 0.05% *(Tween 20, Fluka Chemika, Buchs/ Switzerland)* for 45 min and blocked for 1 h with 1% BSA in PBS/TWEEN 0.05%. The membrane was incubated over night on a shaking table. 10 h later the membrane was washed 6 times for 15 min with PBS/TWEEN 0.05% and then incubated with Streptavidin-HRP diluted at 1:200 in 1% BSA *(Bovine Serum Albumine Fraction V, Boehringer, Mannheim)* solved in PBS/TWEEN 0.05% for 1 h, then washed again 6 times for 15 min and then incubated with a ECL Western Blotting Detection Reagent *(RPN 2106, Amersham Biosciences, Little Chalfont/UK)* for 1 min. Luminescense detection was performed with the Hyperfilm ECL system *(Amersham Biosciences, Little Chalfont/UK)*. The gel was silver-stained and dried with the DryEasy system.

Primary antibodies

anti-moIL-12p40	
MAB 499 (capture)	R&D Systems, Minneapolis/MN
BAF 499 (biotinilated)	R&D Systems, Minneapolis/MN
C15.6 (capture)	BD Pharmingen, Franklin Lakes/NJ
C17.8 (biotinilated)	BD Pharmingen, Franklin Lakes/NJ
sc 1283	Santa Cruz, Santa Cruz/CA
anti-moIL-12p70	
9A5 (capture)	BD Pharmingen, Franklin Lakes/NJ
C17.8 (biotinilated)	BD Pharmingen, Franklin Lakes/NJ
C15.6 (capture)	BD Pharmingen, Franklin Lakes/NJ
anti-moCD40	
3/23 (stimulating antibody)	BD Pharmingen, Franklin Lakes/NJ
anti-moIFN-γ	
R3-6A2	BD Pharmingen, Franklin Lakes/NJ
XMG1.2 (biotinilated)	BD Pharmingen, Franklin Lakes/NJ

Secondary antibodies

Biotin-SP-Rabbit Anti-Goat IgG	Dianova, Hamburg/Germany
Rabbit Anti-Rat IgG	DAKO, Glostrup/Danemark

The antibodies BAF499, C17.8, sc-1283 in combination with P 0449 were able to detect rec. mo$(p40)_2$ 499-ML (96 kDa) that - under reducing conditions - could be detected as a 40 kDa double band. Reducing conditions included β-ME-treatment for complete unfolding of the disulfide-linked proteins - necessary for correct contribution of the molecular weight. Alone BAF499 was sensitive enough to detect protein extracted from supernatants in my assays (Fig. 2-3). The other tested antibodies did not lead to successive protein detection under my conditions.

antibody designation	Ab concentration	designated specificity	references (Western blot)
BAF 499	0.2 µg/ml Western blot	goat polyclonal anti- molL-12 p40	***Lazarro (2005) Diss:*** detection of p40 and p40-p35 fusionprotein
C 17.8	0.25 µg/ml Western blot	hybridoma monoclonal anti-CHO-expressed molL-12	***Grumont et al. (2001) JEM:*** p40, IL-12, p80
		specificity for p40, p80 and IL-12	
sc-1283	sc-1283 0.2 µg/ml + rabbit anti-goat IgG 1:4000	goat polyclonal anti-CHO molL-12p40	***Walter et al. (2001) JEM*** : detection of p40, IL-12, p80 ***Becker et al. (2003) JCI:*** detection of IL-23, IL-12, p40 and p80
9A5	used as second Ab in ELISPOT	hybridoma monoclonal anti-CHO-expressed molL-12p70	o
MAB 499	used as second Ab in ELISPOT	hybridoma monoclonal anti-molL-12 p40	o
C 15.6	used as second Ab in ELISPOT	hybridoma monoclonal anti-CHO-expressed molL-12p70	***Heinzel et al. (1997) JI:*** p40 and p80; used in combination with C 15.1 ***Hölscher et al. (2001) JI:*** p40 and p80 in p35-deficient mice
		specificity for p40, p80 and IL-12	

Fig. 2-3: Characterization of commercial murine IL-12 antibodies.

The table provides an overview of the used antibody systems and the concentrations used for detection in Western blot. No specific antibody that specifically detects p40 (with reactivity for the dimerization binding site) has been described yet. p40, p70 and IL-23 Ab target the p40 protein without making difference between dimerized and monomer proteins. Notable is further the producer's description of the antibody specificity. As the IL-12 protein is a dimer of two distinct protein subunits with genes located in the mouse on chromosome 11 and 6, no such "anti CHO-expressed IL-12p70" exists. Especially the specificity of C15.6 is poor. Cross-reactivities (up to 40%) of the other antibodies to different p40 related proteins are described.

2.3.7 Silver staining

After fixation the gel was treated with the destaining solution for 20-30 min, then rinsed with water for 20-60 min in order to remove the resting acid. The gel was sensitized with 0.02% sodium sodium thiosulfate for 1-2 min. Then the solution was discarded and the gel rinsed again with two charges of water. The gel was incubated with 0.1% silver nitrate ($AgNO_3$) solution for 30 min. The solution was discarded and the gel was rinsed with two charges of water. The developing solution was added, shaken and replaced when it turned yellow. The solution was discarded as soon as sufficient staining was achieved, 1% acetic acid solution was added.

Destaining solution
45 ml methanol
5 ml acetic acid
45 ml H_2O dest.
Sodium thiosulfate 0.02%
0.1 g $Na_2S_2O_3$
0.5 l H_2O dest.

Developing solution
150 µl formalin (0.04%)
12.5 g Na_2Co_2 (2%)
0.5 l H_2O dest.
5 ml acetic acid 1%
495 ml H_2O dest.

2.3.8 Easydry gel preservation system

The Easydry system *(Invitrogen Corp., Carlsbad/CA)* is a conservation system for PAGE-gels. The gels were dried between two methanol-wrenched transparent membranes. The membranes adhere and stiffen after contact with methanol and laminate the gel; the gel itself shrinks in the drying process. The quality of the gel-drying system frames is poor.

2.3.9 Densitometry of gel images

Densitometric evaluation of pictures was performed with ImageJ (ImageJ 1997-2004. Online in internet [date: 12-04-2005]: http://rsb.info.nih.gov/ij/). The developed films were scanned with 1200 bpi, converted and normalized to the background. The optical density was determined in grey scales.

2.3.10 ELISPOT assay

The ELISPOT assay is a single cell assay detecting secreted proteins introduced by Sedgwick (Sedgwick and Holt, 1983) and Czerkinsky (Czerkinsky et al., 1983) as a "solid-phase enzyme-linked immunosorbent assay" for the enumeration of cells secreting specific antibodies and later extended for the detection of cytokine-secreting cells (Fig. 2-4). The assay detects cytokine secretion by suspended single cells adhering to an antibody-coated PVDF-membrane. Released cytokines are captured by cytokine-specific antibodies capturing the cytokine directly beside the cell. This allows - after detection with insoluble colors - to contribute single "spot forming cells" (SFC). ELISPOT assay has become an important quantitative assay for cytokine biology.

Cytokines - that are paracrinely secreted - reach high concentrations only in the direct surrounding of an activated cell and have effects only on directly neighboured cells. The measurement of cytokine plasma levels - as it is done in ELISA - does not necessarily correlate with the actual activation. Furthermore, the activation of single cells is the starting point of a signal amplification mechanism: single cells and not the entity of all immunological cells are responsible for the initiation of immune response.

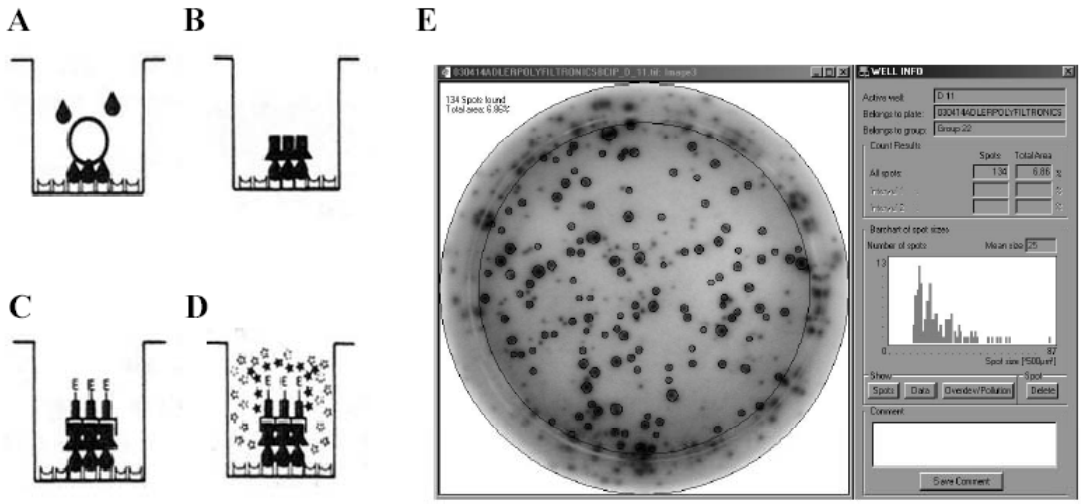

Fig. 2-4: The principle of the ELISPOT assay, modified from Segdwick et al..

After incubation of a 96-well PVDF-Microtiter plate coated with the cytokine-specific coating antibody for at least 6 hours, the plates are blocked with fraction V BSA (Sigma-Aldrich Chemical Co., St.Louis/MO) and washed with sterile PBS. The stimulated cells are suspended and pipetted into the wells. The cells drop to the bottom of the plate and produce cytokines that are bound to the primary antibody directly around the cell (**A**). **B** and **C:** After the incubation period (between 20-50 h), the membrane is washed and a cytokine-specific detection antibody either directly conjugated with a color-reactive enzyme - or as in Fig. **2-4 D** – detected with a secondary antibody coupled to a color-reactive enzyme is added. Insoluble colors precipitate and form spots with typical diameters of 75-400 µm at the bottom of the 96-well microtiter plates (well Ø approx. 5mm), representing single cytokine producing cells. **E.** Spots are macroscoply enumerated with e.g. AELVIS ELISPOT counter.

The advantage of this method compared to the standard ELISA is a higher specifity as only the spots of the cells producing the examined cytokine are colored while sensitivity is impaired compared to ELISA assay (Czerkinsky et al., 1988). Additional information is provided by the quotient of spots per total amount of assayed cells called the responder frequency.

Video-based analysis of ELISPOT plates - such as performed with AELVIS ELISPOT-counter *(ELISPOT analysis, V3.3, A.EL.VIS GmbH, Hannover)* - allows further analysis of the detected SFC such as the distribution of the spot size (Fig. 2-5B). Surpassing the possibilities of visual counting, computer-based evaluation allows based on computer-based graphic recognition a) the definition of a common region of interest preventing a detection of unspecific cell agglomeration e.g. at the well margins; b) the definition of the suspected spot size allows to exclude unspecific small or too big spots, e.g. resulting from an agglomeration of various cells; c) the definition of the circularity indicating e.g. cell migration (differentiation of different cell subtypes) or aggregation; d) the spot intensity indicating e.g. unspecific background effects. Together this makes ELISPOT are more objective cell biology tool.

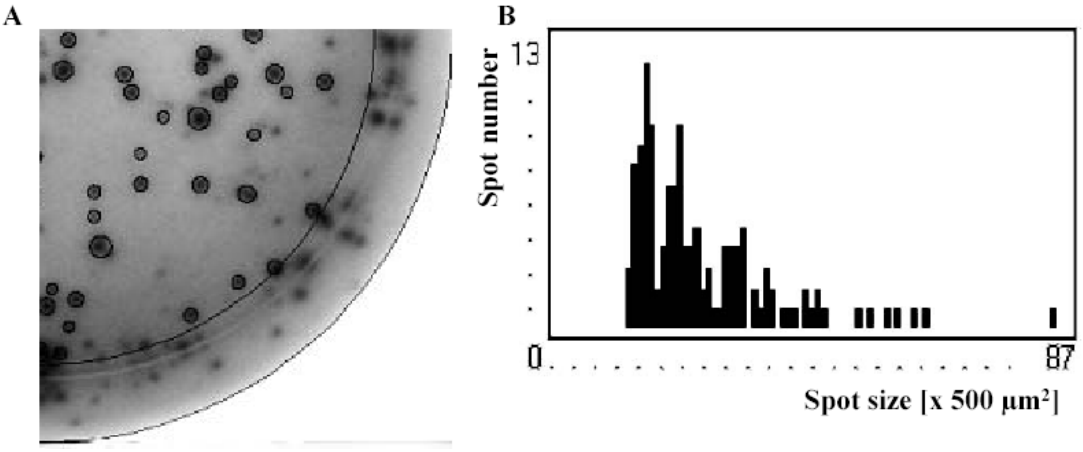

Fig. 2-5: Definition of spot parameters.

2-5 A shows the spots as visualized by the AELVIS ELISPOT counter system. Contured spots represent the spots that are included in the statistical analysis. Spots smaller than 6 x 500 μm^2 were excluded here as "unspecific"– leading to a gap in the corresponding spot distribution curve (**2-5 B**).

2.3.11 IFN-γ ELISPOT

The activation of the murine Th1 cell line IF12 was tested in the ELISPOT assay by measuring the cytokine IFN-γ with an established system of murine αIFN-γ Ab (Taguchi et al., 1990).

Ionized PVDF membrane ELISPOT plates *(unifilter-350, Whatman, Kent/UK)* were incubated over night with the detection antibody R3-6A2 at a concentration of 4 µg/ml in sterile Dulbecco's PBS (1X) *(PAA Laboratories GmbH, Pasching/Austria)*. After overnight incubation with saturated humidity at 4 °C, the plates were washed 4 times with sterile PBS, then blocked in 1% purified sterile-filtered BSA fraction V *(Sigma-Aldrich Chemical Co., St.Louis/MO)* for 2 h, then washed again 4 times with sterile PBS. Cells and additives were solved in a volume of 200 µl per well, reagents were diluted as described. The plates were incubated for 20 hours in a cell incubator. The next day, plates were washed 4 times with PBS, then 4 times with PBS/Tween 0.005%. The biotinilated secondary antibody XMG1.2 was diluted at a concentration of 2 µg/ml in non-sterile PBS/ 1% BSA *(Bovine Serum Albumine, Boehringer, Mannheim)* and incubated over night.

10 h later, the plates were washed again 4 times and incubated with Streptavidin-AP at 1:2000 in PBS/Tween 0.05%/ 1% BSA for 2 h. After washing them 4 times with PBS/TWEEN 0.05%/ 1% BSA, the plates were incubated for 10 min with 50 µl/well of

the precipitating hydrophobic color BCIP/NBT. Plates were photographed with an ELISPOT plate reader *(ELISPOT reader Unit V, A.EL.VIS GmbH, Hannover)* and evaluated (Fig. 2-5).

2.3.12 IL-12p40 ELISPOT

ELISPOT assays are already described for human IL-12p40 and prepared kits for murine IL-12p40 ELISPOT assays have been developed but insufficiently characterized. I established an IL-12p40 ELISPOT with the antibody BAF499 previously characterized for its p40 specificity in Western blot of iLC supernatants. ELISPOT plates *(ELIIP10SSP, Millipore Corp., Bedford/MA)* were incubated over night with the detection antibody MAB499 at a concentration of 6 µg/ml in sterile PBS. After overnight incubation with saturated humidity at 4 °C, plates were washed 4 times with sterile PBS, then blocked in 1% purified BSA fraction V for 2 hours, then washed again 4 times with sterile PBS.

Cells and additives were dissolved in a volume of 200 µl per well, reagents were diluted as described. The plates were incubated for 24 hours in a cell incubator. The next day, plates were washed eight times (four times with PBS, four times with PBS/Tween 0.005%). The biotinilated secondary antibody BAF499 at a concentration of 2 µg/ml was diluted in PBS/ 1% fraction V BSA and incubated over night. 10 h later, the plates were washed again 4 times and incubated with Streptavidin-AP *(Streptavidin-AP, R&D Systems, Minneapolis/MN)* at 1:2000 in 1% BSA/PBS Tween 0.05% for 1 h. After washing them four times with BSA/PBST, the plates were incubated for 5 min with 50 µl/well BCIP/NBT *(Moss Inc., Pasadena/ML)*.

Alternatively to BCIP/NBT, AEC *(Aminopure AEC 34004,Pierce, Rockford/Il*) was used in connection with Streptavidin-HRP *(R&D Systems, Minneapolis/MN*). In order to obtain the AEC solution, AEC was diluted 1:100 in Dimethylformamide, later diluted at 1:30 in 0.1 M acetate buffer and filtered with 45µm filters. H_2O_2 was added 1:2000. 200 µl of AEC solution was applied per well for 15 min. The AP- BCIP/NBT system was superior in the IL-12p40 ELISPOT under the described conditions.

2.4 Molecular Biology

2.4.1 RNA isolation and purification

After centrifugation for 10 min at 1500 rpm *(Sigma 3K30 Heraeus, Hanau)* and isolation of the supernatant, the pellet of 2.5 x 10^6 cells was solved and homogenized in 1 ml of TRIZOL reagent *(Gibco- Invitrogen, Grand Island/NY)* by repetitive pipetting. TRIZOL reagent (Chomczynski and Sacchi, 1987) - a preparation that allows the stabilisation of the RNA during the lysis of the cells - was used according to the supplier's protocol:

Homogenized samples were incubated for 5 min at room temperature. Then 0.2 ml of chloroform were added followed by capping of sample tubes, shaking for 15 s, incubation at room temperature for 3 min, centrifugation at 12000 g for 10 min at 2 to 8°C. The mixture separated into a lower red, phenol-chloroform phase, an inter-phase and a colorless upper aqueous phase containing RNA. The aqueous phase was transferred to a fresh tube and the RNA was precipitated from the aqueous phase by mixing with 0.5 ml isopropyl alcohol. The samples were then incubated for 10 min at room temperature and centrifuged at 12000 g for 10 min at 2 to 8 °C. After the supernatant was removed, the RNA pellet was washed once with 75% ethanol. Therefore 1 ml of 75% ethanol was added, the sample was vortexed and centrifuged at 7500 g for 10 min at 8 °C. After discarding the supernatant the RNA was air-dried for 2 to 5 min and rediluted in an approriate volume of RNAse- free water.

2.4.2 Determination of RNA concentration

As nucleid acids absorb UV light of 250 to 270 nm wavelength, with a maximum at 260nm, DNA and RNA concentrations can be photometrically determined. The absorbance of 1 unit at 260 nm corresponds to approximately 40 µg/ml RNA. The ratio between the readings at 260 nm and 280 nm corresponds with the purity of the nucleic acid. Pure preparations of DNA and RNA have ratios of 1.8 to 1.95, smaller values indicate pollution with phenol or proteins. For determination of RNA concentration I analyzed 1 µl of RNA diluted in sterile distilled water at dilutions of 1:100 and 1:1000 against sterile distilled water in a preset spectrophotometer *(Gene Quant Pro, Amersham Pharmacia, Uppsala/Sweden).*

2.4.3 Semi-quantitative RT-PCR

Reverse-transcribed polymerase chain reaction (RT-PCR) is an in vitro method that allows the amplification of RNA in two steps. The ThermoScript RT-PCR kit *(Invitrogen Life Technologies, Karlsruhe)* uses a RNAse H-deficient mutant of avian myeloblastosis retroviral reverse transcriptase, that transcribes the RNA to complementary DNA – that by its turn can be amplified by the DNA-specific Platinum Taq DNA polymerase *(Invitrogen Corp., Carlsbad/CA)* (Saiki et al., 1988). I used the RT-PCR method in order to detect mRNA of the IL-12 related subunits. The isolated RNA was therefore incubated with the primers for 5 min at 65 °C leading to the denaturation of 2 µg RNA solved in water:

RNA primer mix:	2µg RNA 1 µl Oligo(dT) 1 µl random hexameres ad 10 µl H_2O

The RNA samples were then stored on ice for 4 minutes. In the next step these samples were incubated with 10 µl of RT-master-mix as described below:

RT-master-mix	4 µl 5x cDNA synthase buffer 1 µl of 0.1 M DTT 1 µl H_2O 2 µl 10 mM dNTP-Mix 1 µl of RNAse Out 1 µl of RT

This mixture was then incubated for 10 min at at 25 °C, for 45 min at 50°C, for 5 min at 85 °C. Subsequently 1 µl of RNAse H was added for 20 min at 37°C.
Next, the PCR master mix was incubated for 2 min at 94 °C.

PCR master mix (45.5 µl per sample)	5 µl of 10xPCR buffer 1.5 µl of 50 mM $MgCl_2$ 1 µl of 10 mM dNTP Mix 38 µl H_2O 2 µl cDNA (from RT) 0.5 µl Platinum Taq polymerase

1 µl of 10 µM of forward and 1 µl of 10 µM reverse primers (Tab. 2-1) specific for the analyzed protein were added and then cycled 34-40 times *(Thermocycler, T personal,*

Biometra/Whatman, Kent/UK): 30 s at 94 °C, 30 s at a temperature specific for the oligonucleotide 1 minute at 72 °C.

The primers were determined by M. Schwarz and H. H. Radeke using the program HUSAR (http://genius.embnet.dkfz-heidelberg.de/menu/w2h/w2hdkfz/) according to the published sequences. The primers were synthesized by Invitrogen (Karlsruhe).

Oligodesoxynucleotides

moIL-12**p35**:	5'ATGACCCTGTGCCTTGGTAG3' 5'CCCTGTTGATGGTCACGAC3'
40 cycles, 277 bp	cycle temperature: 94 °C 30 s, 57 °C 30 s,72 °C 60 s.
moIL-12**p40**	5'TCTTTGTTCGAATCCAGCG3' 5'GAAAACTGGAAAAAGCCAACC3'
36 cycles, 316 bp	cycle temp.: 94 °C 30 s, 56 °C 30 s, 72 °C 60 s.
moIL-27**p28**	5'GGCATCACCTCTCTGACTCTG3' 5'AACATTTGAATCCTGCAGCC3'
40 cycles, 198 bp	cycle temp.: 94 °C 30 s, 60 °C 30 s, 72 °C 60 s.
moIL-23**p19**	5'CAGCAGCTCTCTCGGAAT3' 5'ACAACCATCTTCACACTGGATACG3'
34 cycles, 135 bp	cycle temp.: 94 °C 30 s, 57 °C 30 s, 72 °C 60 s.
mo**DNA polymerase-γ**	5'GCACTTCCGCCTCCTGGCCCAGAAGCAGA3' 5'GCTCGGTCAAAGGAAACATTGTGCCCCACCACTAA3'
34 cycles, 419 bp	cycle temp.: 94 °C 30 s, 56 °C 30 s, 72 °C 60 s.
mo**GAPDH**	5'ACCACAGTCCATGCCATCAC3' 5'TCCACCACCCTGTTGCTGTA3'
30 cycles, 452 bp	cycle temp.: 94 °C 30 s, 60 °C 30 s, 72 °C 60s.
mo**β-Tubulin**	5'TTCCCTGGCCAGCTSAANGCNGACCTNCGCAAG3' 5'CATGCCCTCGCCNGTGTACCAGTGNANGAAGGC3'
36 cycles, 484 bp	cycle temp.: 94 °C 30 s, 55 °C 30 s, 72 °C 60 s.

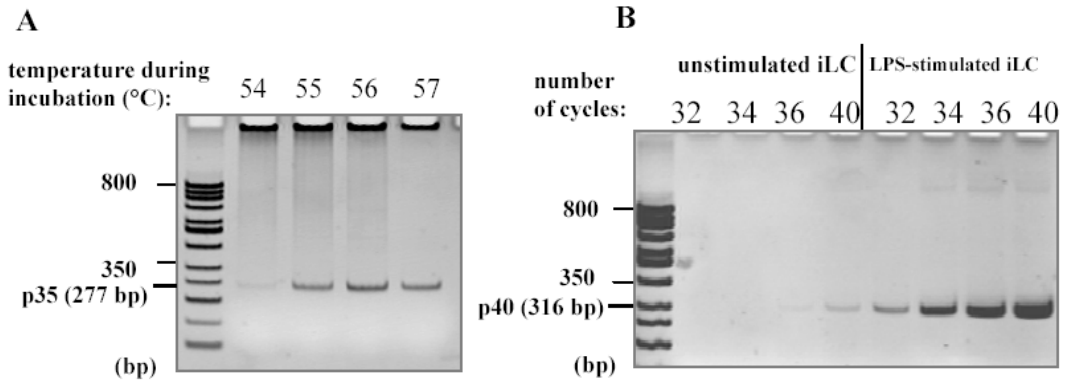

Fig. 2-7: Optimization of PCR amplification conditions.

RT-PCR was performed with 2 µg of total RNA. The amount of input cDNA for PCR was constant. PCR was performed exemplarily with primers for p35 (**2-7A**) and p40 (**2-7B**) and products were detected with a fluorescence camera after Ethidium bromide treatment. Optimal cycling conditions for cytokines and housekeeping genes were performed empirically, as illustrated here for p40 and p35: In order to perform semiquantitative RT-PCR analysis, submaximal band intensity had to be achieved. Three incubation parameters (temperature of second cycle; $MgCl_2$ concentration and cycle number) can be varied in order to configurate band intensities that can be qualitatively distinguished. The temperature during the PCR cycles varies allowing dissociation and aggregation of the used DNA-polymerase to the transcribed molecule. Optimal temperature for the DNA polymerase is molecule-specific and was determined for each subunit. A cycle consists of a heating period (94 °C for 30 s) allowing the dissociation, a transcription period (varying between 54 and 60 °C, according to the molecule; for 30 s) and a third period (72 °C for 60 s). In Fig. **2-7A** I tested the influence of the temperature during the transcription period on the cDNA intensity of the p35 band. Clearly distinguishable bands (277 bp) were reached when transcription was performed at 57 °C. The number of RT-PCR cycles was varied between 32 and 40 cycles (Fig. **2-7B**). The first lane represents the 50 kDa DNA ladder, in the following 4 lanes, cDNA of unstimulated iLC was cycled with the specific primer 32, 34, 36 and 40 times, with no detectable bands. The next 4 lanes of LPS-stimulated iLC represent the products of 32, 34, 36 and 40 cycles. I could detect bands of the suspected 316 bp of the p40 cDNA. Subsequently to these results, I chose 36 cycles for the following p40 RT-PCR. The concentration of $MgCl_2$ (1mM) in the PCR master mix was not altered.

2.4.4 Polyacrylamide gel electrophoresis

12% PAA gel
0.2 ml 50xTAE
4 ml % polyacrylamide
5.8 ml H_20
100 µl 10% APS
4 µl TEMED

50x TAE
242 g TRIS
57.5 ml Ice acid
100 ml 0.5 M EDTA (pH 8.0)
ad 1 l H_2O dest.

10 µl of cDNA of RT-PCR, diluted in 2 µl sample buffer (6X) and 4 µl of 50 bp DNA ladder was pipettted per lane.

The RNA was separated at 140 V for 1 h in a horizontal gel electrophoresis system (*Subcell GT, Bio-Rad, Hercules/CA)*, then detached from electrophoresis apparatus and incubated with a buffered Ethidium bromide solution for 10 minutes:

20 ml 1xTAE
2 µl Ethidium bromide

The fluorescence detection of the cytokine-specific autoradiographic bands was performed with a fluorescence camera *(GelDoc, GelDoc-It, Ltf, Wasserburg)*. Densitometry was performed with ImageJ. Data were normalized to the levels of DNA polymerase-γ. This mitochondrial protein showed constant secretion in stimulated dendritic cells and was recommended as housekeeping gene (Park et al., 2001; Schultz et al., 1998). When CpG was used as a stimulant, the detected mRNA of DNA polymerase-γ was impaired so that I was forced to compare the effectiveness of DNA polymerase-γ with GAPDH and β-Tubulin that were constantly transcribed.

2.5 Statistics

The confidence intervall was defined at 0.95; "*" representing $p<0.05$; "**" representing $p<0.01$ and "***" representing $p<0.001$. Assays in the following were performed at least three times (Fig. 3-2, Fig. 3-5 to 3-9, Fig. 3-11 to 3-13, Fig. 3-17) or considered as representative out of a series of similar experiments. Assays that concluded in the Fig. 3-1A/B were performed 13 times, 4 assays failed (no spots or positive controls); further 3 assays showed spots, but no cell number dependence (possible contamination); 7 assays confirmed the demonstrated graph. Figures 3-3 and 3-10 (n=1) are considered representative based on earlier findings of the "Foundation Immunepharmacology". Assays for figures 3-14, 3-15 and 3-18 were performed two times, figures 3-16 and 3-20 represent experiments that were performed 3 respectively 2 times while two further assays were not valid. The values were given as mean ± one standard deviation (SD). In assays that were repeated less than three times, the standard error of the mean (SEM) was calculated. Unpaired Student t test, paired t test and Whitney-Mann test were performed using GraphPad Prism version 4.00 for windows, GraphPad SoftwareSan Diego/CA, www.graphpad.com.

3 Results

3.1 Interaction of murine epidermal dendritic cells and Th1 cells

The goal of this project was to elucidate and characterize the interaction of two collaborating cell types positioned at the propagation of chronic inflammation: dendritic cells and Th1 cells.

In this project I examined the murine epidermal MHC II I-A^d immature Langerhans dendritic cell line XS52 (iLC), the syngeneic murine MHC-compatible OVA-specific Th1 cell clone IF12 (Th1) and the murine allogeneic MHC II I-A^b immature myeloid dendritic cell line JawsII (iMDC).

The cell types were investigated individually and in their interaction. Therefore prominent cytokines of each cell type - IFN-γ secreted by Th1 cells and IL-12 and IL-12-related cytokines secreted by dendritic cells – were measured in the ELISPOT assay. The ELISPOT assay data were complemented by RT-PCR and Western blot data.

3.1.1 Con A dose-dependent increase of the amount of IFN-γ secreting Th1

The glycopeptide-binding lectin extract of *Canavalia ensiformis* concanavalin A (ConA) circumvents physiological activation mechanisms by capping relevant outer-membrane receptors leading to mitogenic T lymphocyte activation resulting in the release of IFN-γ in Th1 cells (Pilarski, Bretscher, and Baum, 1977; Reeke, Jr. et al., 1974).

Examining the IFN-γ release of Th1 cells, I routinely controlled nonspecific background effects using unstimulated cells as a control. As shown in Fig. 3-1A I tested various concentrations of Con A in order to determine the optimal stimulation conditions of the Th1 cells. Unstimulated Th1 do not produce IFN-γ. High numbers of spot forming cells (SFC) were detected at very different concentrations of Con A. While the number of IFN-γ secreting Th1 cells increased when the Con A concentration was doubled from 1 µg/ml to 2 µg/ml, no further increase was observed when 4 µg/ml of Con A was applied. The spot number even diminished when 5 µg/ml of Con A were added.

The concentration of Con A I used in the following assays was by mistake higher than the optimal 2 µg/ml. Fig. 3-1 B shows the results of six assays performed with a Con A concentration of 5 µg/ml.

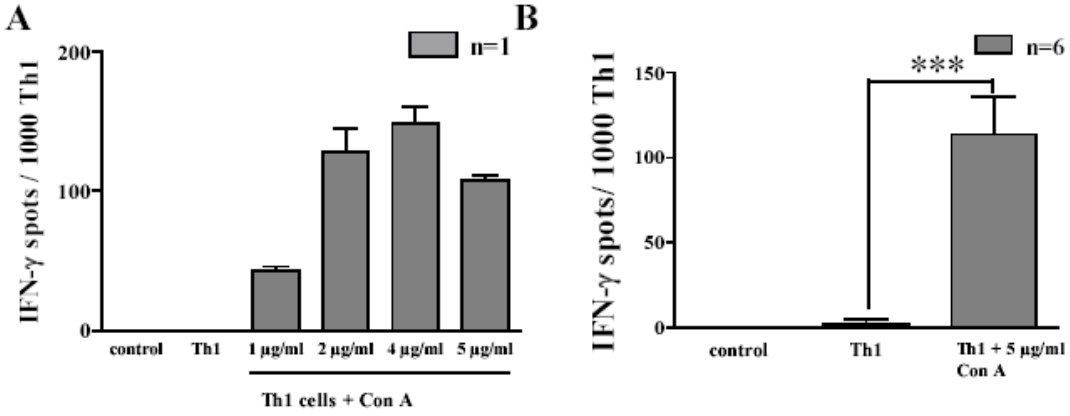

Fig. 3-1: Con A dose-dependent effects on the number of IFN-γ-secreting Th1 cells.

The number of IFN-γ-spots in the Th1-ELISPOT assay (1000 Th1/well; 96 well plates) was measured either in medium (control), in unstimulated Th1 cells (Th1) or Th1 cells stimulated with Con A in concentrations of **A** 1 µg/ml, 2 µg/ml, 4 µg/ml and 5 µg/ml or **B** 5 µg/ml for 24 hours. **A:** Out of a series of six independent experiments one is shown, done in triplicates and expressed as mean ± SEM. Most favourable concentrations of Con A were reached between 2 and 4 µg/ml. This and the following assays demonstrate the excitability of the Th1 cell line IF12. **B** This graph displays the number of SFC after stimulation with 5 µg/ml in mean ± SD. The IFN-γ release of Con A-stimulated Th1 cells is significantly different from medium control (control) and control of unstimulated Th1 with $p<0.0001$ (unpaired Student t test) for all tested concentrations.

An average of 113.8 ± 22.4 (n=6) spots per 1000 T lymphocytes (under stimulation with 5 µg/ml ConA) were detected reflecting the stimulation of 11% of incubated T lymphocytes. These data could be confirmed in a series of more than 10 assays performed in the laboratories of the "Foundation Immune Pharmacology". In comparison to the original characterization of this Th1 clone in 1996 with a IFN-γ responder frequency of 42 % this indicates a decrease in the amount of specifically responding Th1.

3.1.2 Influence of PGE_2 and PGD_2 on Th1 activation

3.1.2.1 Influence of PGE_2 and PGD_2 on Con A-induced Th1 activation

The following assays were designed to examine the effects of the interfering agents PGD_2 and PGE_2 on the number of activated Th1 cells – effects that were previously observed in the laboratories of the "Foundation Immune Pharmacology" in models of autoimmune kidney disease. Especially PGD_2 was surprisingly enriched in supernatants of stimulated mesangial cells.

As a substitute of the formerly applied mesangial cell supernatants I used purified synthetic PGE_2 and PGD_2. Con A and prostaglandins were added simultaneously to the Th1 cells and incubated for 24 h considering that pre-incubation with either substance might lead to different results. The effects of prostaglandins E_2 and D_2 on Con A-stimulated Th1 were measured in ELISPOT assay (Fig.3-2).

Although nonspecific activation of Th1 was observed (n=1), the number of IFN-γ spots was increased under stimulation with Con A to the same extend as in Fig. 3-1. When PGE_2 was added to Con A-stimulated Th1 in tenfold serial dilutions (10^{-9} to 10^{-5} M) the number of SFC was reduced (p=0.07; unpaired Student t test comparing the number of spots of Th1 stimulated with Con A and Th1 stimulated with Con A and 10^{-6} M PGE_2). These results – although with moderate statistical quality (considering that they were the first results of my work) - agree with a series of more than 10 former assays of the "Foundation Immune Pharmacology" where a significant reduction of SFC was observed at concentrations of 10^{-7} M to 10^{-5} M PGE_2.

In the same assays, Th1 cells were stimulated with Con A in the presence of PGD_2 (10^{-9} to 10^{-5} M; tenfold serial dilutions) but no significant effects were observed compared to control.

Subsequently, I analysed the effects of PGE_2 and PGD_2 on Th1 stimulated with OVA-loaded iLC. OVA internalised by iLC, processed in endosomal compartments and complexed to MHC II is supposed to interact with the Th1 TCR-CD3-complex. Complemented by co-stimulating signals it represents the appropriate stimulation for cytokine release of the Th1 cell, leading to equal numbers of activated Th1 as with Con A stimulation.

I could not detect significant influence of prostaglandins on Th1 stimulated with OVA-treated iLC.

By contrast, former assays of the "Foundation Immune Pharmacology" showed an inhibiting effect of PGE_2 on both Th1 stimulated with Con A and Th1 stimulated with OVA-treated iLC (Fig. 3-3; IL-2 ELISPOT). The significant reduction of SFC confirms previous assumptions of PGE_2-induced inhibition of Th1 cell response based on observations performed with ELISA cytokine assays. PGD_2 showed no effects either.

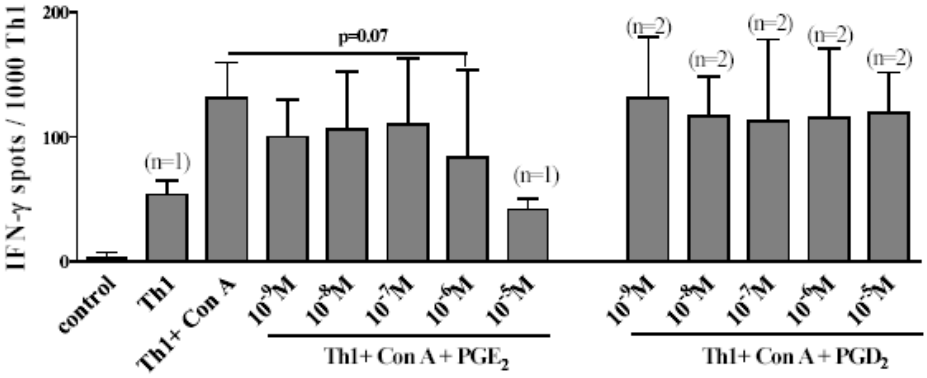

Fig. 3-2: Effects of PGD_2 and PGE_2 on the number of IFN-γ^+ Th1 under Con A–stimulation.

ELISPOT experiment of Th1 cells (1000 /well; 96-well ELISPOT plate) incubated with Con A (5 µg/ml) in the presence of PGE_2 or PGD_2 added in tenfold serial dilutions (10^{-9} to 10^{-5} M). Incubation lasted for 24 h. Plates were prepared as described in „materials". Results represent mean ± SD of three independent experiments done in triplicates. Dose-dependent effects cumulate at a concentration of 10^{-5} M PGE_2 where the amount of Con A-induced activated Th1 cells is reduced from 121.6 ± 9.4 spots/well (absence of PGE_2) to 83.3±70.1 spots (n=3; p=0.07 as determined with unpaired Student t test). PGD_2 effects were not significant.

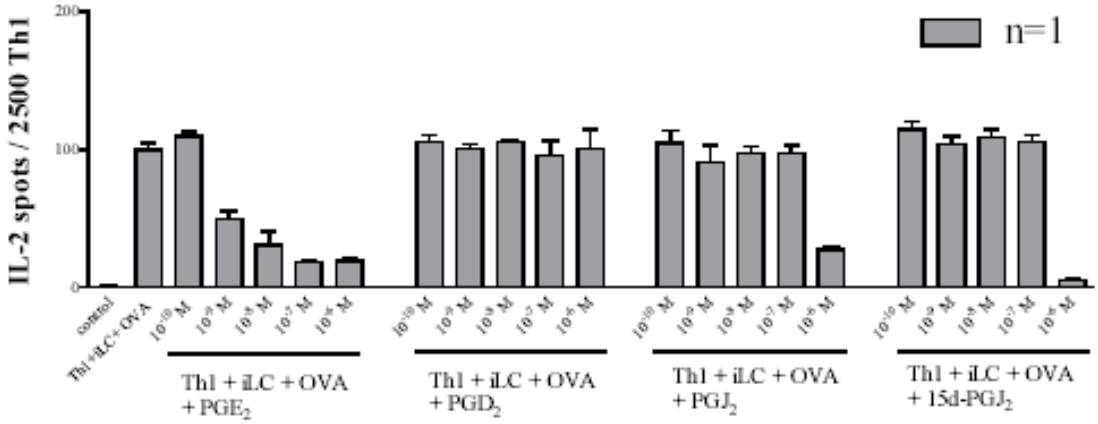

Fig. 3-3: Effects of PGE_2 and PGD_2 on the number of IL-2 secreting Th1 under stimulation with OVA-incubated iLC.

Out of a series of three experiments this is one representative assay analysing the effects of the prostaglandins PGE_2, PGD_2, PGJ_2 and 15d-PGJ_2 on the number of IL-2 secreting Th1 stimulated with OVA-loaded iLC. PGE_2 has pronounced depressing effects on Th1, while PGD_2 and in high dosage, also PGJ_2 and 15d-PGJ_2 are ineffective. This assay was performed by the "Foundation Immune Pharmacology".

3.2 Prostaglandin effects on the mRNA expression of IL-12-related cytokines

IFN-γ, as a key cytokine of the Th1 cell response is promoted by IL-12 among others and is increased upon Th1 IFN-γ-secretion reinforcing a directed inflammatory differentiation towards a Th1 response.

In the following assays I systematically examined the modulating influence of the prostaglandins E_2 and D_2 and stimulating factor αCD40 on the secretion of IL-12-related cytokines by LPS-stimulated and alternatively CpG-stimulated iLC.

Escherichia coli lipopolysaccharide (LPS) - representative for other Gram-negative bacterial endotoxins and differing only in structural details - was used as TLR4-stimulant. The CpG phosphothioate oligodeoxynucleotide 2216 (CpG) (Hartmann et al., 2003), specific for human TLR9 but also known as potent cytokine inductor, especially for IFN-α, in the mouse model (Iho, 2003; Kuramoto et al., 1992) was used as TLR9 stimulant.

3.2.1 mRNA expression of IL-12-related cytokine subunits in iLC and iMDC

The subunits of the IL-12 related molecules were measured semi-quantitatively with reverse-transcribed polymerase chain reaction (RT-PCR) and Western blot assay according to a standardized protocol investigating eight stimulation conditions. The supernatants of the stimulated cells, containing cell culture medium and cellular cytokines were used for protein analysis in Western blot, cellular mRNA was isolated from the pelleted cells for RT-PCR amplification with the described primers against IL-12-related cytokine subunits. Because of that, cellular protein could not be additionally isolated. As control, unstimulated cells were analysed for the same parameters in all assays. Results of the RT-PCR were normalized to the level of DNA polymerase-γ and alternatively GAPDH in assays involving CpG. I repeated the assays under same conditions three and respectively four times - as shown for the p19 subunit detected in LPS-stimulated iLC in Fig. 3-4 - and summed up the densitometrically measured band intensities as shown in Fig. 3-5 to Fig. 3-9.

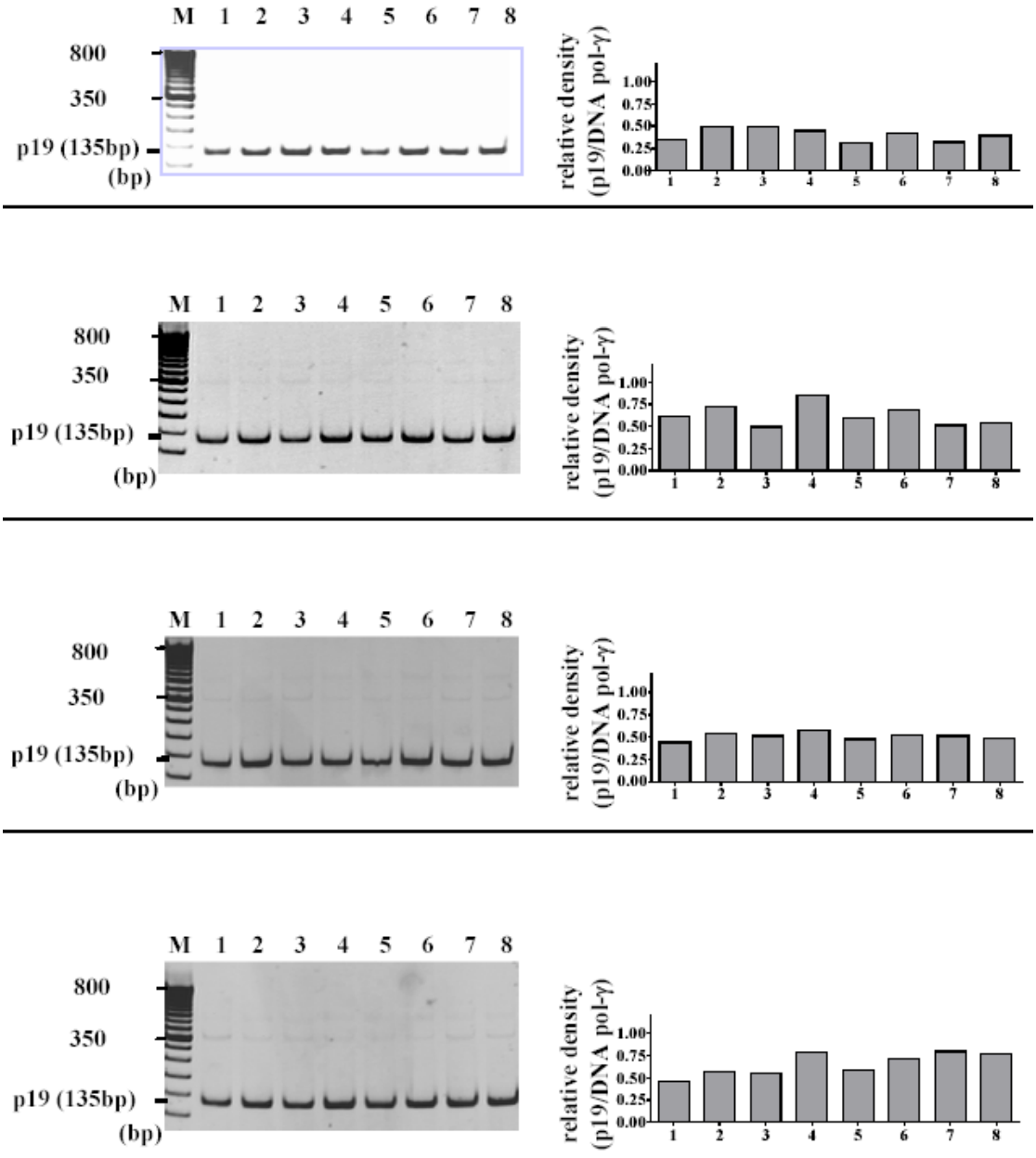

Fig. 3-4: Effects of PGE_2 and PGD_2 on LPS-induced expression of p19 in iLC.

The figure shows 4 gel pictures and 4 densitometrical evaluations (relative density) of a 135 bp cDNA bands - contributed to p19 - in reference to the density of DNA polymerase-γ as standard. 2.5 x 10^6 iLC were incubated for 20 h in 10 ml of the described medium in the absence of growth factors and stimulated as followed: *lane 1:* unstimulated iLC, *lane 2:* iLC + 1 µg/ml LPS *lane 3:* iLC + 10^{-6} M PGE_2, *lane 4:* iLC + 10^{-6} M PGE_2+ 1 µg/ml LPS *lane 5:* iLC +10^{-5} M PGD_2, *lane 6:* iLC +10^{-5} M PGD_2 +1 µg/ml LPS, *lane 7:* iLC + 5 µg/ml αCD40 Ab, *lane 8:* iLC +5 µg/ml αCD40 Ab + 1 µg/ml LPS. *M:* 50 kDa DNA ladder. The cellular RNA was isolated as described in "methods" and separated on a 12% PAA gel. After densitometric evaluation, the results of three, respectively four experiments were summed up (Fig. **3-5A**).

In the following paragraphs RT-PCR results are described and related to the Western blots and ELISPOT assays performed in parallel.

3.2.1.1 IL-23p19 expression of iLC and iMDC

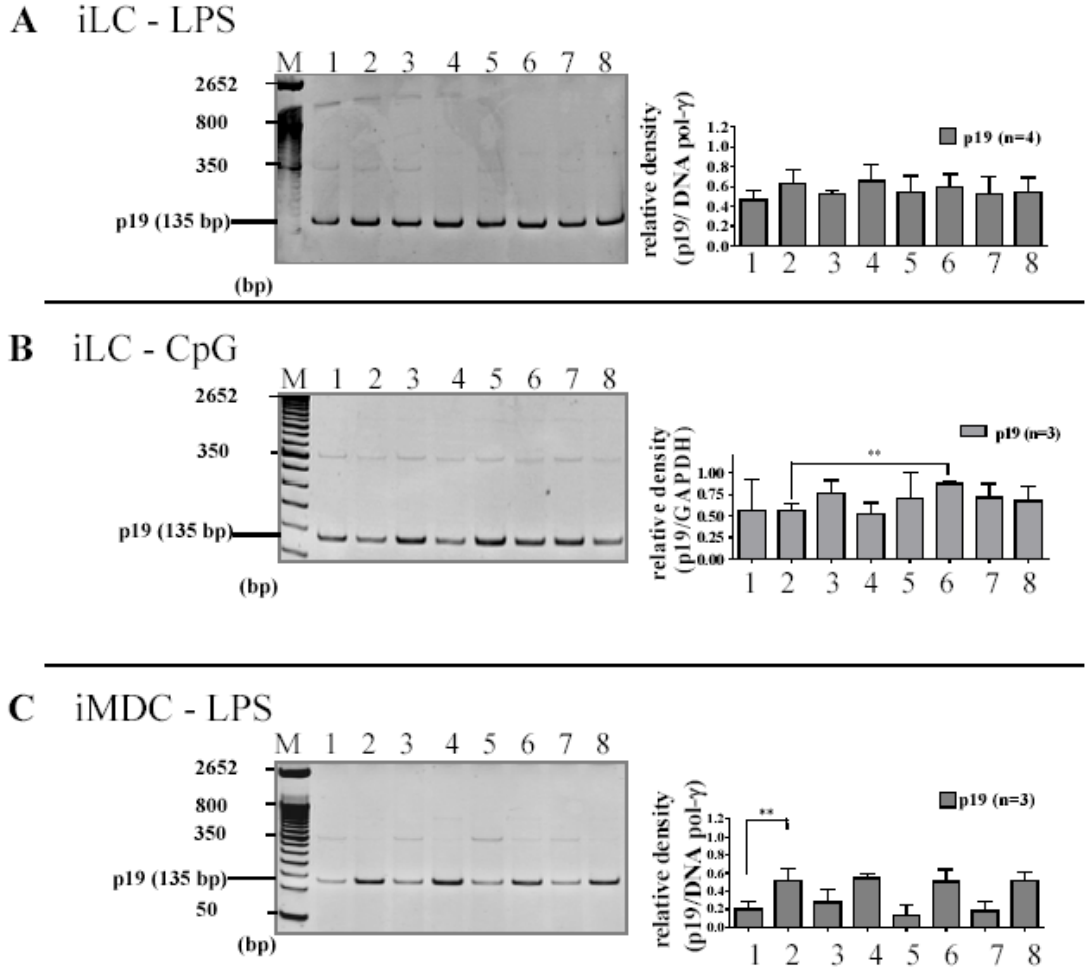

Fig. 3-5: Influence of PGE_2 and PGD_2 on p19 transcription of TLR-stimulated iLC and iMDC.

The RT-PCR products of the p19 subunit of **A** iLC stimulated with LPS, **B** iLC stimulated with CpG, **C** iMDC stimulated with LPS were genererated as described in "methods" and separated on a 12% PAA gel. 2.5 x 10^6 iLC or iMDC were incubated for 20 h in 10 ml of the described medium in the absence of growth factors with the following stimulants: *lane 1:* unstimulated DC, *lane 2:* DC + 1 µg/ml LPS respectively 2,5 µM CpG, *lane 3:* DC + 10^{-6} M PGE_2, *lane 4:* DC + 10^{-6} M PGE_2 + 1 µg/ml LPS respectively 2,5 µM CpG, *lane 5:* DC +10^{-5} M PGD_2, *lane 6:* DC +10^{-5} M PGD_2 +1 µg/ml LPS respectively 2,5 µM CpG, *lane 7:* DC + 5 µg/ml αCD40 Ab, *lane 8:* DC +5 µg/ml αCD40 Ab + 1 µg/ml LPS respectively 2,5 µM CpG. *M:* 50 kDa DNA ladder. The gel pictures represent one representative out of 3 respectively 4 assays. Data were normalized to DNA polymerase-γ (**A** and **C**) or GAPDH (**B**) (standard figures: Fig. 3-9). The results represent mean ± SD of 3 respectively 4 assays under the same conditions. While a constant high transcription is observed in **A**, p19 cDNA levels are impaired under stimulation with PGD_2 in **B** (p=0.005 as determined by unpaired Student t test comparing iLC stimulated with CpG in the presence or absence of 10^{-5} M PGD_2). p19 cDNA levels in iMDC (**C**) are increased in all lanes where LPS is applied to the cells (p=0.0278 comparing unstimulated in the presence or absence of 1 µg/ml LPS as determined by unpaired Student t test).

IL-23p19 mRNA - but not the mRNA of p28, p35 and p40 - was constitutively expressed in both iLC and iMDC as shown in lane 1 of figures 3-5. While a constantly high transcription of p19 mRNA without modulation under addition of LPS,

prostaglandins E_2 or D_2 or αCD40 Ab was observed in iLC stimulated with LPS, the transcription of the p19 subunit was inconstant under stimulation with CpG. A significant increase can be distinguished in iLC stimulated with CpG in combination with PGD_2 compared to stimulation with CpG alone ($p=0.005$; unpaired Student t test, n=3). These results should be interpreted with care as I observed an irregular transcription of DNA polymerase-γ while GAPDH levels were constant. Contrary to the iLC I demonstrated in iMDC that basal transcription was significantly induced under stimulation with LPS. Bands of cells stimulated with LPS have an increased intensity [$p=0.0278$ (comparison of unstimulated cells to cells stimulated with LPS alone)]. No additional effect of prostaglandins was detected.

Remarkably, I constantly detected a band of 350 bp in all PCRs in addition to the p19 band of 135 bp.

Western blot assays performed later in the laboratory of the "Foundation Immune Pharmacology" with the p19-antibodies detected the p19 protein in supernatants and cell lysates of unstimulated iLC.

3.2.1.2 IL-12p35 expression of iLC and iMDC

Fig. 3-6 demonstrates that IL-12p35 was transcribed at a very low level in iLC, without influence of stimulating agents. No differences between LPS and CpG are observed here. In Western blots later performed in the laboratory of the "Foundation Immune Pharmacology" the p35 subunit could not be detected in either iLC supernatants or cell lysates. Contrary, the p35 subunit was detected in iMDC under stimulation with LPS while no basal transcription was observed and other additives were ineffective. This test failed once (n=3); it should be cautiously interpreted.

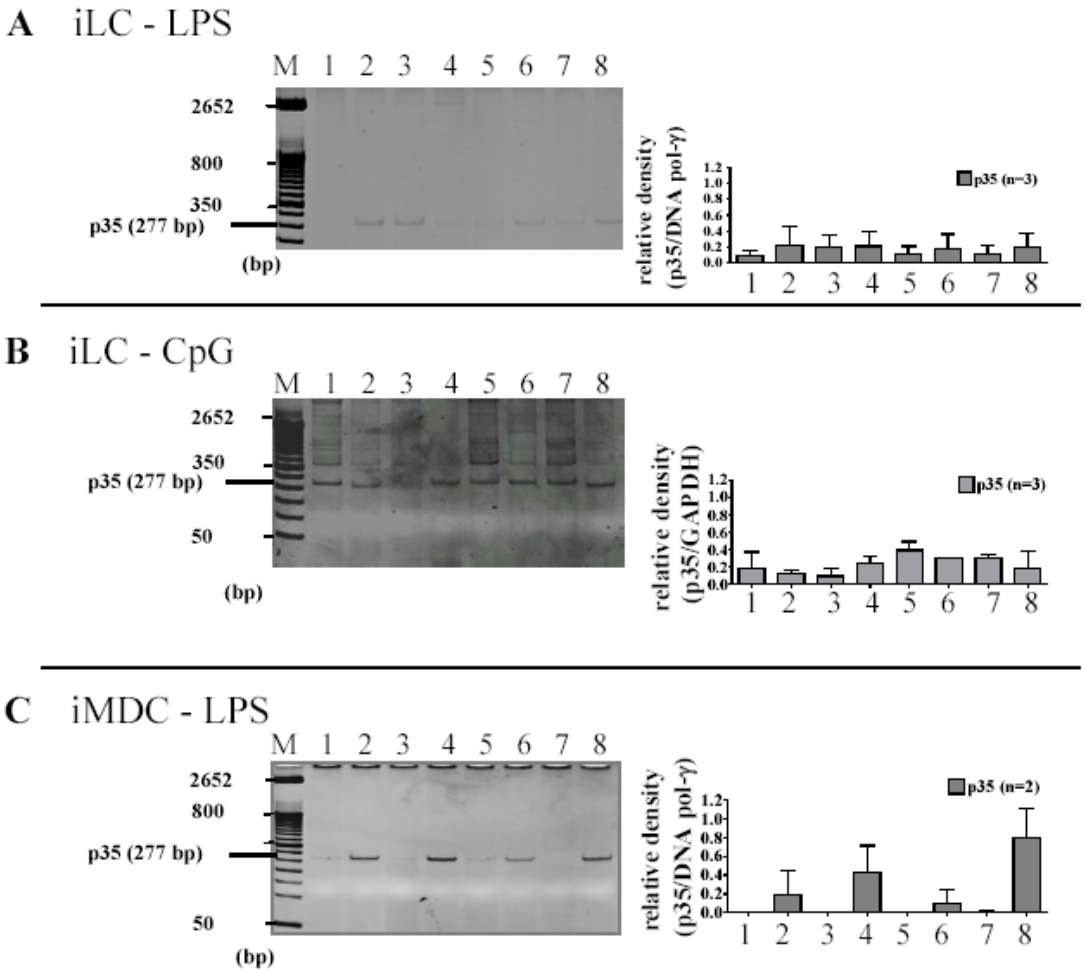

Fig. 3-6: Influence of PGE_2, PGD_2 and αCD40 Ab on p35 transcription of iLC and iMDC.

The RT-PCR products of the p35 subunit of **A** iLC stimulated with LPS, **B** iLC stimulated with CpG, **C** iMDC stimulated with LPS were genererated as described in "methods" and separated on a 12% PAA gel. 2.5 x 10^6 iLC or iMDC were incubated for 20 h in 10 ml of the described medium in the absence of growth factors with the following stimulants: *lane 1:* unstimulated DC, *lane 2:* DC + 1 µg/ml LPS respectively 2,5 µM CpG, *lane 3:* DC + 10^{-6} M PGE_2, *lane 4:* DC + 10^{-6} M PGE_2 + 1 µg/ml LPS respectively 2,5 µM CpG, *lane 5:* DC +10^{-5} M PGD_2, *lane 6:* DC +10^{-5} M PGD_2 +1 µg/ml LPS respectively 2,5 µM CpG, *lane 7:* DC + 5 µg/ml αCD40 Ab, *lane 8:* DC +5 µg/ml αCD40 Ab + 1 µg/ml LPS respectively 2,5 µM CpG. *M:* 50 kDa DNA ladder. The gel pictures represent one representative out of 3 respectively 2 assays. Data were normalized to DNA polymerase-γ (**A** and **C**) or GAPDH (**B**) (standard figures: 3-9). The results represent mean ± SD of 3 respectively 2 assays (± SEM) under the same conditions. While a constant low p35 transcription is observed in iLC under stimulation with LPS or CpG (**A** and **B)** p35 cDNA levels in iMDC (**C**) are increased in all lanes where LPS is applied to the cells. This assay was performed thrice, in one assay, p35 could not be detected.

3.2.1.3 IL-12- and IL-23p40 expression of iLC and iMDC

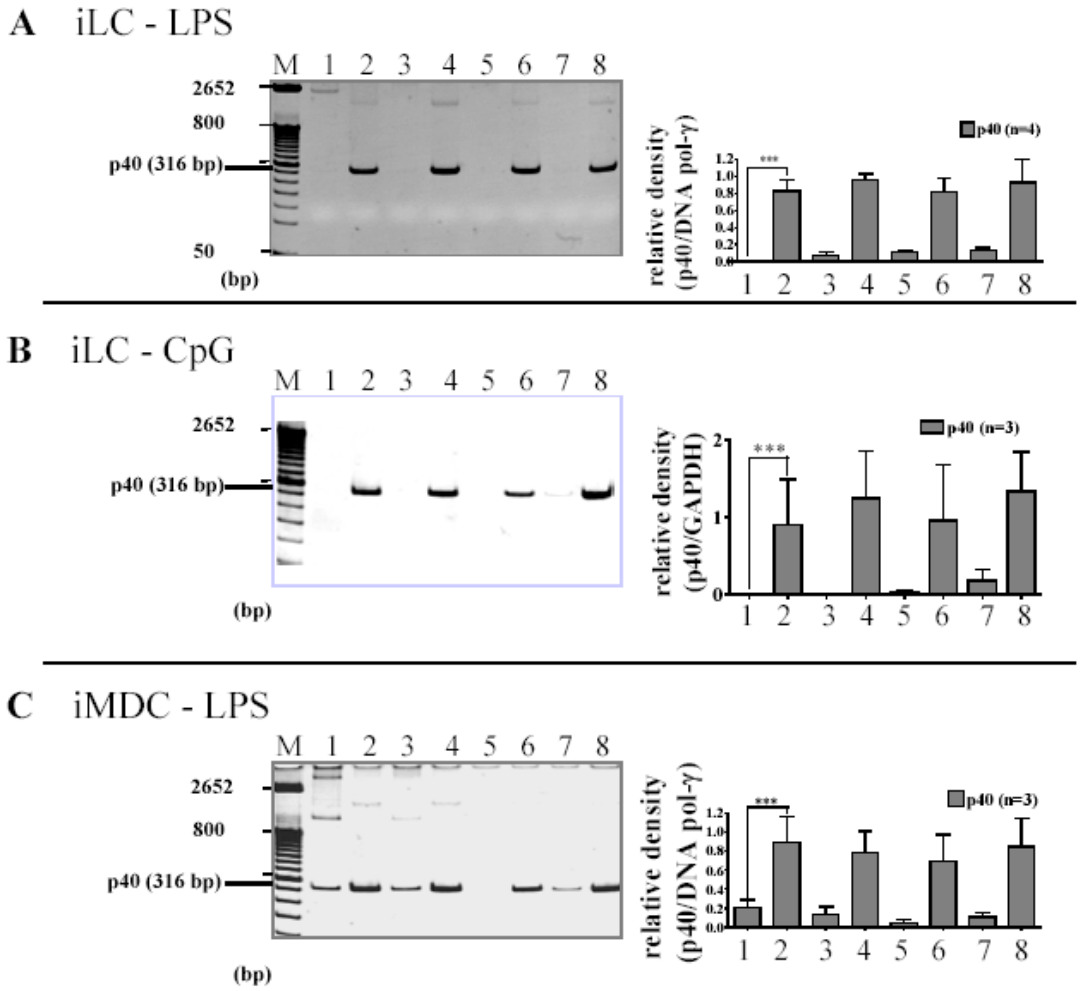

Fig. 3-7: Influence of PGE_2 and PGD_2 on p40 transcription of TLR-stimulated iLC and iMDC.

The RT-PCR products of the p40 subunit of **A** iLC stimulated with LPS, **B** iLC stimulated with CpG, **C** iMDC stimulated with LPS were genererated as described in "methods" and separated on a 12% PAA gel. 2.5 x 10^6 iLC or iMDC were incubated for 20 h in 10 ml of the described medium in the absence of growth factors with the following stimulants: *lane 1:* unstimulated DC, *lane 2:* DC + 1 µg/ml LPS respectively 2,5 µM CpG, *lane 3:* DC + 10^{-6} M PGE_2, *lane 4:* DC + 10^{-6} M PGE_2 + 1 µg/ml LPS respectively 2,5 µM CpG, *lane 5:* DC +10^{-5} M PGD_2, *lane 6:* DC +10^{-5} M PGD_2 +1 µg/ml LPS respectively 2,5 µM CpG, *lane 7:* DC + 5 µg/ml αCD40 Ab, *lane 8:* DC +5 µg/ml αCD40 Ab + 1 µg/ml LPS respectively 2,5 µM CpG. *M:* 50 kDa DNA ladder. The gel pictures represent one representative out of 3 respectively 4 assays. Data were normalized to DNA polymerase-γ (**A** and **C**) or GAPDH (**B**) (standard figures: 3-9). The results represent mean ± SD of 3 respectively 4 assays under the same conditions. Increase of cDNA levels in all lanes were LPS or CpG was added ($p<0.0001$; unpaired Student t test) was observed for **A-C.**

While no detectable levels of p40 is transcribed in the absence of LPS, the appearance of the p40 bands under stimulation with LPS respectively CpG is specific ($p<0.0001$; n=3; unpaired Student t test) (Fig.3-7). Further distinction between the p40 band density of cell preparations containing LPS is not possible due to the semi-quantitative assay design of the RT-PCR. Western blot assays of the p40 subunit (Fig. 3-10) indicate a

regulation of the p40 subunit by PGE_2 and PGD_2.Massive induction of p40 transcription in the presence of LPS was observed to the same extend in iMDC. Additional to iLC, a basal transcription of the p40 subunit could be detected here.

3.2.1.4 IL-27p28 expression in iLC and iMDC

IL-27 is a sequence homologue to IL-12 consisting of p28 and EBI3 with p28 as a homologue to p35. In my assays I isolatedly respected the p28 subunit.

I could detect a low basal transcription in both cell lines (Fig. 3-8). The amount of mRNA differed from the control when additional to the TLR agonist αCD40 Ab was added. Stimulation of αCD40 as necessary condition for effective IL-27p28 secretion has been suggested by Planz et al.. Additional effects of PGE_2 and PGD_2 could not be detected.

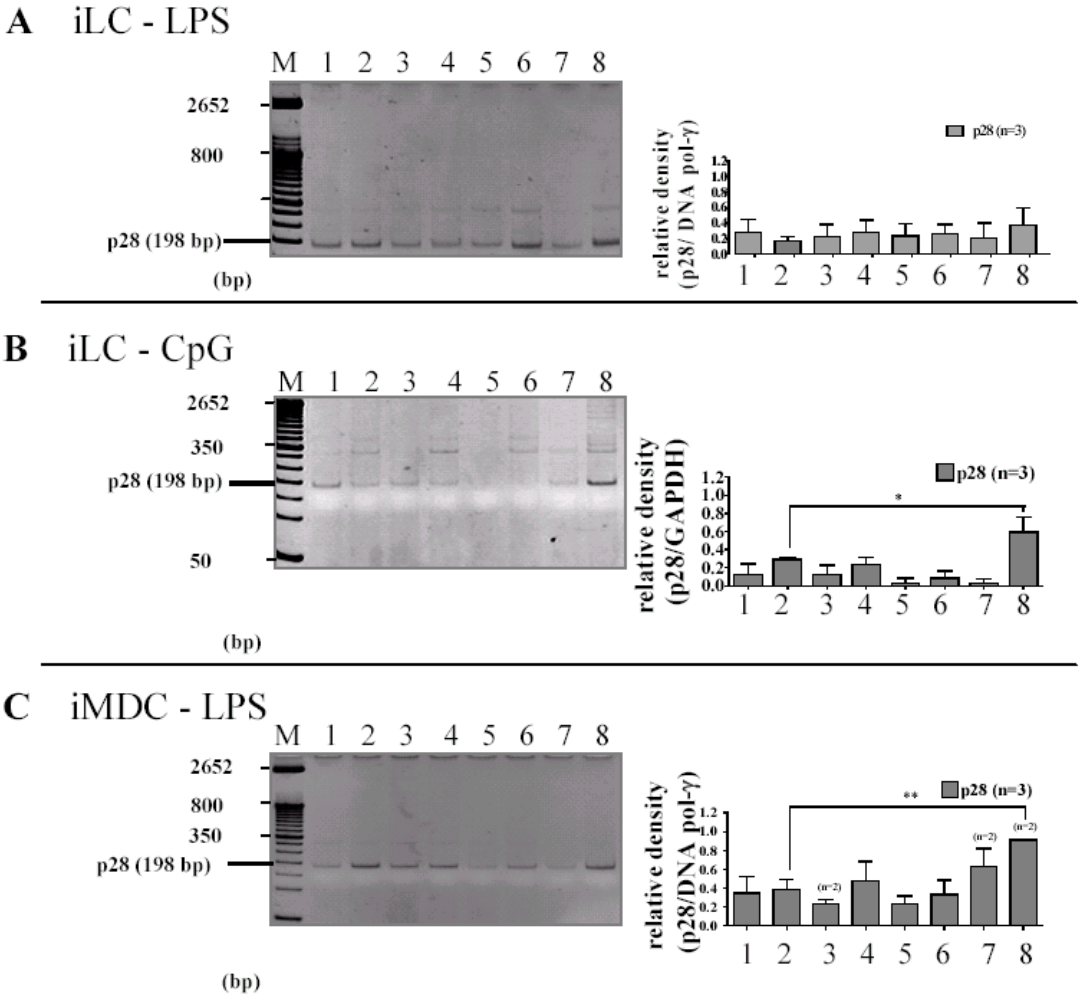

Fig. 3-8: Influence of PGE_2 and PGD_2 on p28 transcription of TLR-stimulated iLC and iMDC.

The RT-PCR products of the p28 subunit of **A** iLC stimulated with LPS, **B** iLC stimulated with CpG, **C** iMDC stimulated with LPS were genererated as described in "methods" and separated on a 12% PAA gel. 2.5 x 10^6 iLC or iMDC were incubated for 20 h in 10 ml of the described medium in the absence of growth factors with the following stimulants: *lane 1:* unstimulated DC, *lane 2:* DC + 1 µg/ml LPS respectively 2,5 µM CpG, *lane 3:* DC + 10^{-6} M PGE_2, *lane 4:* DC + 10^{-6} M PGE_2 + 1 µg/ml LPS respectively 2,5 µM CpG, *lane 5:* DC +10^{-5} M PGD_2, *lane 6:* DC +10^{-5} M PGD_2 +1 µg/ml LPS respectively 2,5 µM CpG, *lane 7:* DC + 5 µg/ml αCD40 Ab, *lane 8:* DC +5 µg/ml αCD40 Ab + 1 µg/ml LPS respectively 2,5 µM CpG. *M:* 50 kDa DNA ladder. The gel pictures represent one representative out of 3 assays. Data were normalized to DNA polymerase-γ (**A** and **C**) or GAPDH (**B**) (standard figures: 3-9). The results represent mean ± SD of 3 under the same conditions. p28 cDNA levels are low in **A-C**. Alone the addition of stimulating αCD40 Ab seems to increase the transcription levels of this subunit (**B**: p=0.035 comparing iLC stimulated with CpG and iLC stimulated with CpG and αCD40 Ab as determined by unpaired Student t test; **C**: p=0.0061 comparing iMDC stimulated with LPS compared to iMDC stimulated with LPS and αCD40 determined by unpaired Student t test).

3.2.1.5 mRNA expression of standards in iLC and iMDC

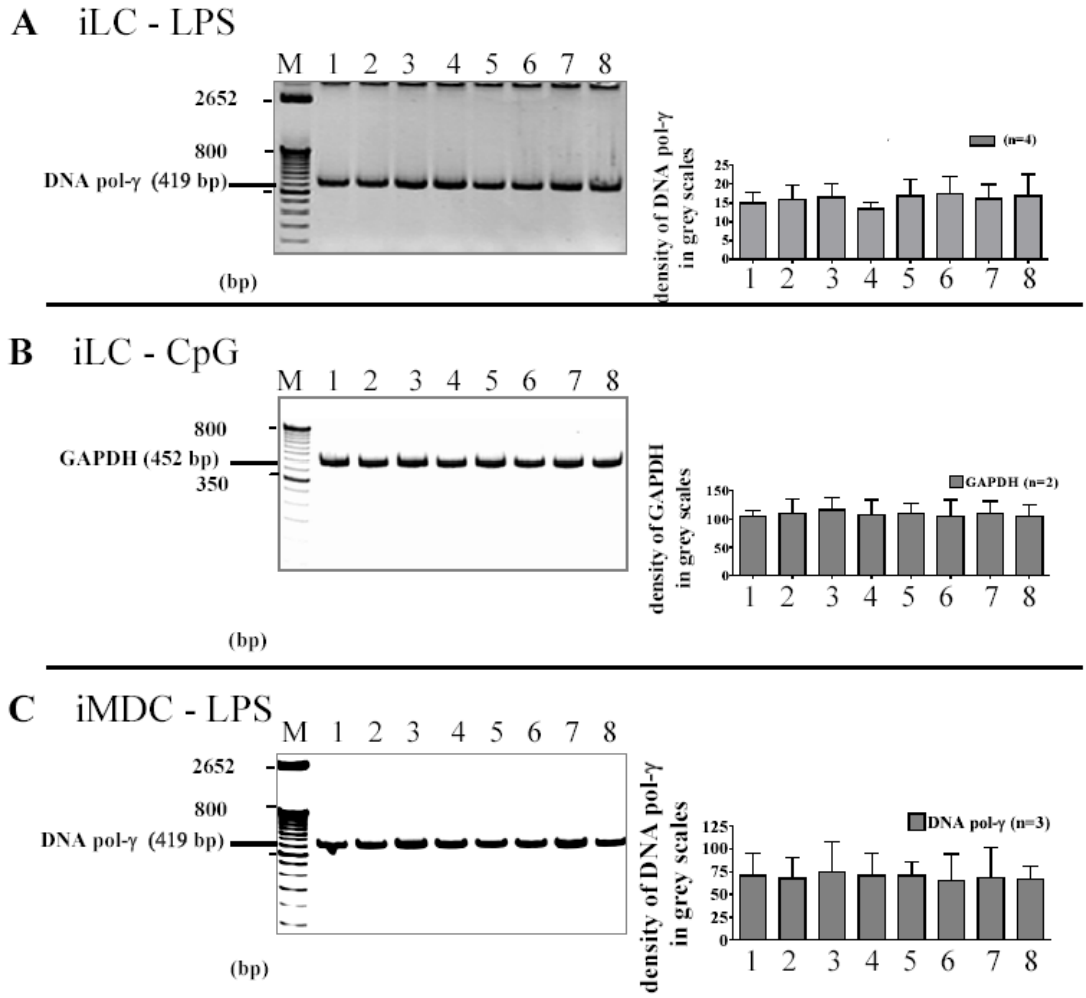

Fig. 3-9: Influence of PGE_2 and PGD_2 on standard transcription of TLR-stimulated iLC and iMDC.

RT-PCR products of standards of **A** iLC stimulated with LPS, **B** iLC stimulated with CpG, **C** iMDC stimulated with LPS were genererated as described in "methods" and separated on a 12% PAA gel. 2.5 x 10^6 iLC or iMDC were incubated for 20 h in 10 ml of the described medium in the absence of growth factors with the following stimulants: *lane 1:* unstimulated DC, *lane 2:* DC + 1 µg/ml LPS respectively 2,5 µM CpG, *lane 3:* DC + 10^{-6} M PGE_2, *lane 4:* DC + 10^{-6} M PGE_2 + 1 µg/ml LPS respectively 2,5 µM CpG, *lane 5:* DC +10^{-5} M PGD_2, *lane 6:* DC +10^{-5} M PGD_2 +1 µg/ml LPS respectively 2,5µ M CpG, *lane 7:* DC + 5 µg/ml αCD40 Ab, *lane 8:* DC +5 µg/ml αCD40 Ab + 1 µg/ml LPS respectively 2,5 µM CpG. *M:* 50 kDa DNA ladder. cDNA levels for DNA polymerase-γ were constant in all assays where cells were stimulated with LPS (**A** and **C**). Under stimulation with CpG (**B**), cDNA levels of DNA polymerase-γ were impaired but not the levels of β-tubulin (not shown) or GAPDH. I therefore used GAPDH as standard for these assays.

DNA polymerase-γ - the enzyme responsible for the transcription of mitochondrial DNA in human and in murine cells - was used as mRNA standard as inspired by Park et al.. Under incubation of iLC with CpG, I observed an isolated impaired expression of DNA polymerase-γ and for this reason I chose to use GAPDH as standard for these assays which, same as β-tubulin, was constantly expressed.

3.3 IL-12p40 expression in iLC supernatants

While the stimulated cells of the previously described assays were used for mRNA analysis in RT-PCR, the supernatants were concentrated with the help of 10 kDa centriprep® tubes to 25% of their previous volumes, aliquoted and stored at –80 °C.

In order to assign the molecular weight of the proteins detected with the examined antibodies I used reducing conditions in SDS-PAGE (including heating for 5 min at 95°C, Laemmli buffer containing 5% β-ME and 2% SDS).The use of reducing Laemmli buffer leads to the complete dissociation of the dimeric proteins as can be seen in the lane of the recombinant protein (Fig. 3-10). This according to the product description - containes pure p40 homodimeric molecules.

Because of the subunit dissociation under reducing conditions, it was not possible to distinguish between the p40 dimer, the p40 monomer or the p40 possibly coupled to p19 or to p35. In the running front of the described rec. $(p40)_2$ -fragments two bands of approximately 40 kDa were detected that also appeared in the lanes where supernatants of LPS-stimulated iLC were separated. No other band supposedly detectable at 80 kDa, 70 kDa or 60 kDa could be detected. As the supernatant of unstimulated dendritic cells did not show any protein band, the LPS-dependent induction of this protein band and its molecular weight confirmed the specifity of the antibody for a 40 kDa protein and the used recombinant protein. Lane 2 of Fig. 3-10 displays the amount of detected IL-12p40 in LPS-stimulated iLC. When PGE_2 was added the density of the band increased overadditively compared to LPS alone (lane 4). Lane 6 demonstrates that PGD_2 added to cells that were simultaneously stimulated with LPS diminished the density of the p40 band that was observed under LPS-stimulation alone. PGD_2 alone (lane 5) had no effect.

The bands in lane 7 and 8 represent the p40-levels in iLC stimulated with either αCD40 alone or with αCD40 in combination with LPS. αCD40 added alone to the iLC did not lead to detectable levels of p40. In combination with LPS a possible moderate increase compared to LPS-stimulated cells could be observed.

In a further Western blot that I performed under non-reducing conditions, I could detect an intact 80 kDa (respectively 96k Da) band in the lane of the recombinant protein, but only the p40 kDa band could be detected in the supernatant of the LPS-stimulated iLC.

This Western blot assay was only performed once. Although not statistically meaningful, these results are in agreement with the following quantitative cytokine

ELISPOT assays. The confirmation of the antibody specifity in this Western assay should be considered as important quality control for the following assays.

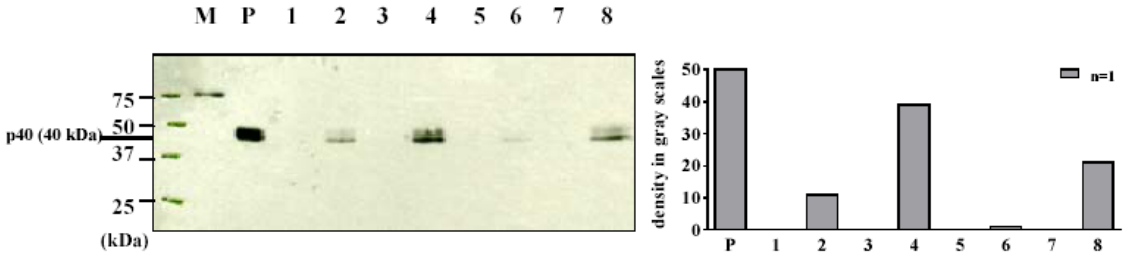

Fig. 3-10: Effects of prostaglandins, LPS and αCD40 Ab on IL-12p40 protein release in iLC.

The Western blot was performed with the biotinilated antibody BAF499 at a concentration of 0.2 µg/ml. Supernatants were isolated from 2.5 x10^6 iLC incubated for 20 h with the following stimulants solved in iLC medium in the absence of growth factors: *lane 1:* unstimulated iLC, *lane 2:* iLC + 1 µg/ml LPS, *lane 3:* iLC + 10^{-6} M PGE_2, *lane 4:* iLC + 10^{-6} M PGE_2+ 1 µg/ml LPS, *lane 5:* iLC +10^{-5} M PGD_2, *lane 6:* iLC +10^{-5} M PGD_2 +1 µg/ml LPS, *lane 7:* iLC + 5 µg/ml αCD40 Ab, *lane 8:* iLC + 5 µg/ml αCD40 Ab + 1 µg/ml LPS solved in 10 ml of total volume. Concentration was performed with 10 kDa Centriprep®-filters to one-fourth of their original volume. Protein amount was normalised to a constant amount of 25 µg/lane, protein mass was determined with BCA-assay. Protein samples were unpicked using a 12.5% SDS-PAGE. Precision plus® marker was used with auto-luminescent protein bands at 37 kDa and 50 kDa (M). 50 ng of rec. $(p40)_2$ was applied in the first lane (P). The semi-quantitative Western blot – regarded as antibody control - was performed once. The intensity of the bands reflects the amount of detected supernatant protein and was estimated by densitometrical analysis and then normalized to the recombinant protein. The band intensity of the double band of the p40 protein is increased in the supernatants where LPS is added to the cells. The presence of PGE_2 in cell culture increases the band intensity overadditively while PGD_2 seems to diminish the amount of this protein.

3.3.1 Influence of PGD_2 and PGE_2 on the number of IL-12p40 SFC

Western blot assay and RT-PCR allow qualitative and quantitative statements about mRNA or protein levels, but no information about actual biological processes such as cell activation and therefore ELISPOT data are a good complement. In the following ELISPOT assay I quantified the effectively LPS-stimulated iLC and the interfering effects of PGE_2 and PGD_2.

3.3.2 LPS dose-dependent effects on the number of IL-12p40 secreting cells

The ELISPOT was carried out with an antibody pair including the antibody BAF499 that was previously characterized in Western blot assay. Polyclonal BAF499 used as secondary antibody in ELISPOT is specific for p40 and also binds to $(p40)_2$. MAB499 used as primary purified antibody is a monoclonal antibody with a declared specificity against rec. moIL-12p40.

Previously to the following assays I performed tests to empirically determine the appropriate cell amount for the following assays at 50 000 cells/well.

It could be shown that LPS dose-dependently increases the amount of IL-12p40 SFC excluding unspecific background effects (Fig. 3-11).

In order to examine LPS-dose-dependent effects on the amount of IL-12p40 SFC LPS concentrations between 1 ng/ml and 10 µg/ml were tested. While 1 ng/ml did not induce significant increase of the amount of IL-12p40 SFC ($p=0.1442$; unpaired Student t test in reference to unstimulated conditions), the concentration of 100 ng/ml to 1 µg/ml LPS led to a peak in the number of detected SFC ($p<0.0001$; unpaired Student t test, examined value compared to unstimulated conditions). Higher concentrations of LPS led to a reduced number of spots.

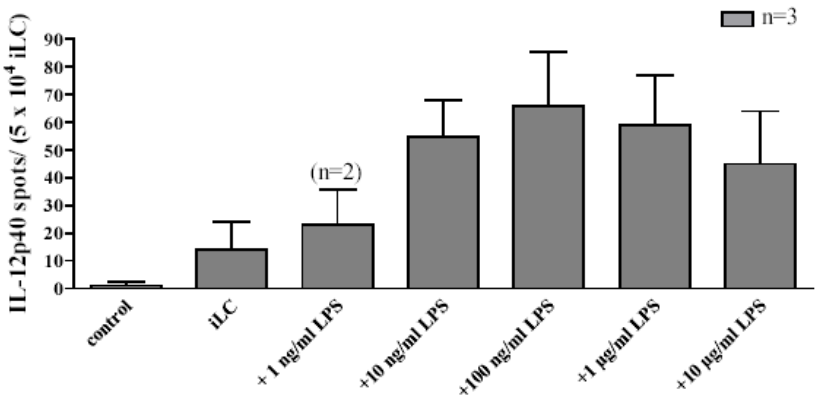

Fig. 3-11: LPS concentration-dependent effects on the frequency of IL-12p40 secreting iLC.

ELISPOT assay. iLC (50 000 /well in 96-well ELISPOT plates) were incubated with different doses of LPS solved in iLC medium in the absence of growth factors. LPS was added in tenfold serial dilutions between 1 ng/ml and 10 µg/ml. Cells were incubated with the reagent for 24 h. Results represent mean ± SD of three independent experiments with triplicates. Maximal amount of spots is measured at 1 µg/ml LPS compared to unstimulated iLC ($p<0.0001$; unpaired Student t test) whereas 1 ng/ml LPS does not have significant impact on IL-12p40 spots. The ”responder frequency” of LPS-stimulated and IL-12p40 SFC at 1 µg/ml LPS is 0.12%.

An estimated responder frequency of LPS-stimulated IL-12p40 SFC of 0.12% demonstrates that the activation process of IL-12p40 secretion needs to surpass a high stimulation threshold. Based on these observations, I stimulated the iLC in the following assays with 1 µg/ml LPS.

3.3.3 PGE_2 effects on the number p40 SFC under LPS stimulation

I quantified PGE_2 influence on the amount of IL-12p40 secreting cells under LPS-stimulation (Fig. 3-12) in ELISPOT and could demonstrate that PGE_2 not only increases the amount of LPS-induced IL-12p40 but also dose-dependently augments the number of IL-12p40 SFC ($p<0.0001$; unpaired Student t test; number of IL-12p40 secreting cells under LPS-stimulation in the presence or absence of 10^{-5} M PGE_2). Significant increase of IL-12p40-secreting cells is reached at a concentration of 10^{-7} M PGE_2.

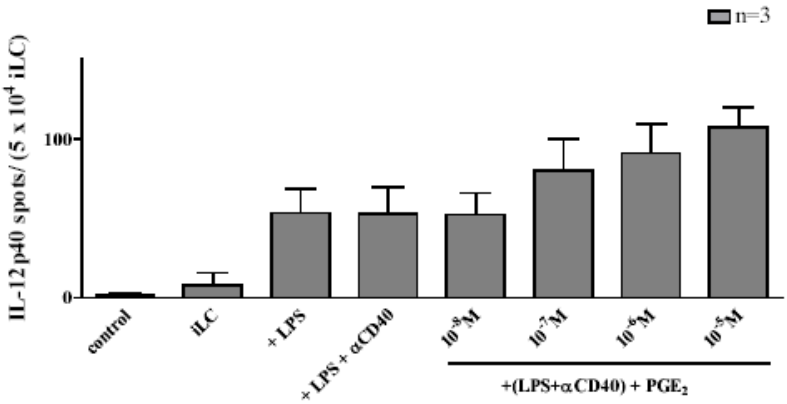

Fig. 3-12: Dose-dependent effects of PGE_2 on the frequency of IL-12p40 secreting iLC.

ELISPOT assay. iLC (50 000 /well in 96-well ELISPOT plates) were incubated with a constant dose of 1 µg/ml LPS in iLC medium. PGE_2 was added in tenfold serial dilutions between 10^{-8} M and 10^{-5} M. Cells were incubated with additives for 24 h. Results represent mean ± SD of three independent experiments with triplicates. Highest effects are reached at 10^{-5} M PGE_2 compared to control ($p<0.0001$; unpaired Student t test; absence/presence 10^{-5} M PGE_2): the mean of IL-12p40-spots is doubled from 53.3 spots to 106.9 spots/50 000 iLC.

3.3.4 Characterization of PGE_2 effects on LPS-stimulated iLC

In the following assays I tried to further characterize these effects of PGE_2. With the help of dbcAMP and Forskolin, I mimicked cAMP which is known to induce signal transduction of the prostaglandin receptors EP_2 and EP_4. Forskolin (Insel and Ostrom, 2003) imitates stimulating G_S-receptor stimulation by activating adenylate cyclase, while dbcAMP is a stable form of cAMP elevating directly intracellular cAMP levels. dbcAMP was diluted in twofold serial dilutions (Fig. 3-13) and added simultaneously with LPS to the iLC. The counted values were related to the iLC stimulated solely with LPS ("+LPS"). The number of spots in the medium control and in the wells of unstimulated iLC is low.

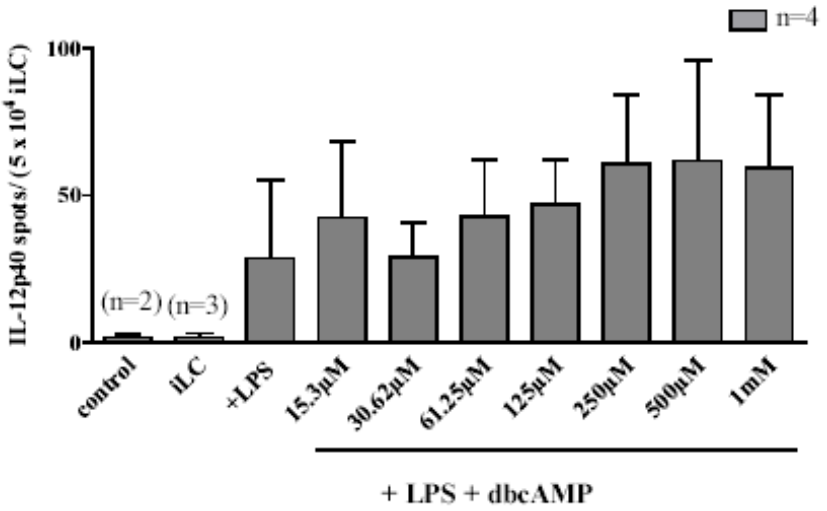

Fig. 3-13: Dose-dependent effects of dbcAMP on the frequency of LPS-induced IL-12p40 SFC.

ELISPOT experiment. iLC (50 000 /well in 96-well ELISPOT plates) were incubated with a constant dose of 1 µg/ml LPS in iLC medium. dbcAMP was directly diluted in the medium added in twofold serial dilutions (15.3 µM, 30.5 µM, 63 µM, 125 µM, 250 µM, 500 µM, 1 mM). Cells were incubated with additives for 24h. Results represent mean ± SD of four independent experiments with triplicates. Optimal effects are obtained at 250 µM dbcAMP compared to control (p=0.005; unpaired Student t-test; absence/presence 250 µM dbcAMP). The number of IL-12p40-spot is doubled under these conditions from 28.75 ± 26.4 (n=4) to 60.7 ± 23.6 (n=4) in presence of 250 µM dbcAMP.

dbcAMP dose-dependently increased the number of IL-12p40 SFC under LPS-stimulation significantly by approximately 100%.

I wanted to reproduce these observed effects with Forskolin. DMSO that was used as a solvent for Forskolin had to be assayed for nonspecific effects and it seemed indeed to increase background effects when added to unstimulated iLC (Fig. 3-14).

Forskolin was diluted in twofold serial dilution (0.3 M to 10 M) (by mistake too high; recommended concentrations at 0.2 mM, compare discussion) and added to the iLC within a period of 10 minutes after their stimulation with LPS. No direct Forskolin-dose-dependent increase of SFC was measured (n=2), although some values were significantly elevated compared to control. I abandoned these tests as the assays that analysed dbcAMP effects demonstrated a dose-dependent relation of the cAMP-stimulation on the number of IL-12p40 secreting iLC.

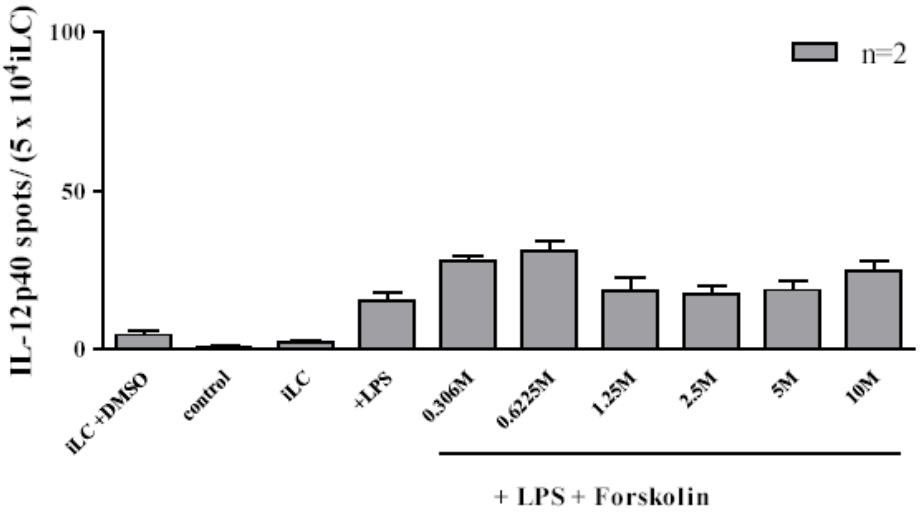

Fig. 3-14: Dose-dependent effects of Forskolin on the number of LPS-induced IL-12p40 SFC.

ELISPOT experiment. iLC (50 000/well in 96-well ELISPOT plates) were incubated with a constant dose of 1 µg/ml LPS in dendritic cell medium. Forskolin, solved in DMSO, was diluted in the medium added in twofold serial dilutions (0.3 M to 10 M). The solvent DMSO was assayed at a dilution of 1:1000. Cells were incubated with additives for 24 h. Results represent mean ± SEM of two independent experiments with triplicates. The test was only performed twice.

The ionophor Ionomycin is capable of extracting Ca^{2+} and other divalent cations from an aqueous into an organic phase and elevates intracellular Ca^{2+} levels. In this manner it bypasses Ca^{2+} as second messenger as implicated in inhibitory G-protein coupled receptor signaling known for prostaglandin receptor EP_1 and EP_3. Fig. 3-15 presents the results of 2 independent experiments. While the controls measuring unstimulated iLC, medium background, DMSO-effects and cell stimulated with LPS were in accordance with previous assays, the addition of Ionomycin added in tenfold serial dilutions (10^{-10} M to 10^{-6} M) to LPS-stimulated (1 µg/ml) iLC did not lead to significant changes in the number of SFC.

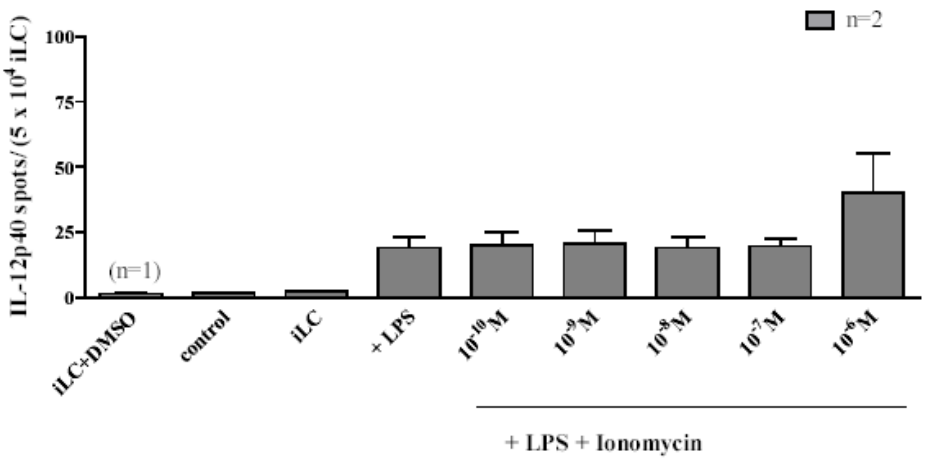

Fig. 3-15: Effects of Ionomycin on the frequency of LPS-induced IL-12p40 secreting iLC.

iLC (50 000/well in 96-well ELISPOT plates) were incubated with a constant dose of 1 µg/ml LPS in dendritic cell medium. Ionomycin, solved in DMSO, was diluted in dendritic cell medium added in tenfold serial dilutions (10^{-10} M to 10^{-6} M). The solvent DMSO was assayed at a dilution of 1:1000. Cells were incubated with additives for 24h. Results represent mean ± SEM of two independent experiments with triplicates.

3.3.5 Modulation of the amount of LPS-induced IL-12p40 SFC by PGD_2

LPS- (1 µg/ml) and αCD40 Ab-treated (1µg/ml) iLC were additionally stimulated with different concentrations of PGD_2 (10^{-5} M to 10^{-8} M, tenfold serial dilutions) (Fig. 3-16).

Neither medium nor unstimulated cells or cells stimulated with PGD_2 alone led to significant amounts of spots while the number of SFC in the controls corresponds with previous assays. The number of spots obtained under stimulation with LPS (1 µg/ml) in combination with PGD_2 (10^{-5} M) is reduced significantly ($p=0.0023$; unpaired Student t test) compared to LPS-stimulated cells in the absence of PGD_2.

The inhibitory tendency of PGD_2 on LPS-induced IL-12p40 secretion in my results is supported in recent publications.

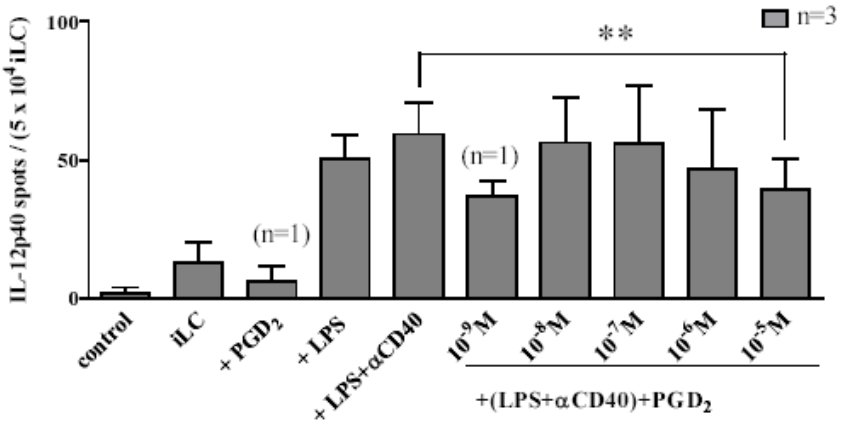

Fig. 3-16: Dose-dependent effects of PGD_2 on the number of LPS-induced IL-12p40-secreting cells.

IL-12p40 ELISPOT experiment iLC (50 000 /well in 96-well ELISPOT plates) were incubated with a constant dose of 1 µg/ml LPS and 1 µg/ml αCD40 solved in iLC medium in the absence of growth factors. PGD_2 was diluted in tenfold serial dilutions (10^{-9} M to 10^{-5} M). Cells were incubated with additives for 24h. Results represent mean ± SD of three independent experiments with triplicates. The amount of IL-12p40 secreting cells in the presence of PGD_2 is significantly reduced (p=0.0025 as determined with Whitney-Mann test) compared to incubation of the cells in the absence of PGD_2. 1 value was excluded.

3.3.6 Influence of αCD40 on the number of IL-12p40 SFC

The results in Western blot assay indicate an increase of IL-12p40 in cell culture supernatant (Fig. 3-10) when cells were stimulated with αCD40 Ab additionally to LPS. I tested the stimulating αCD40 antibody 3/23 for effects in the IL12p40-ELISPOT and could not detect any differences in the amount of SFC compared to iLC stimulated with LPS alone. By mistake, assays in ELISPOT were performed with 1 µg/ml αCD40 instead of 5 µg/ml as recommended (Hasbold et al., 1994) while in the iLC that were prepared for Western blot and RT-PCR the recommended 5 µg/ml were used.

3.4 IL-12p40 expression in supernatants of CpG-stimulated iLC

3.4.1.1 CpG induction of IL-12p40 secretion in iLC

The supernatants obtained from CpG-stimulated iLC were assayed for total IL-12p40 secretion in Western blot with the αmoIL-12p40 antibody BAF499 (Fig. 3-17).

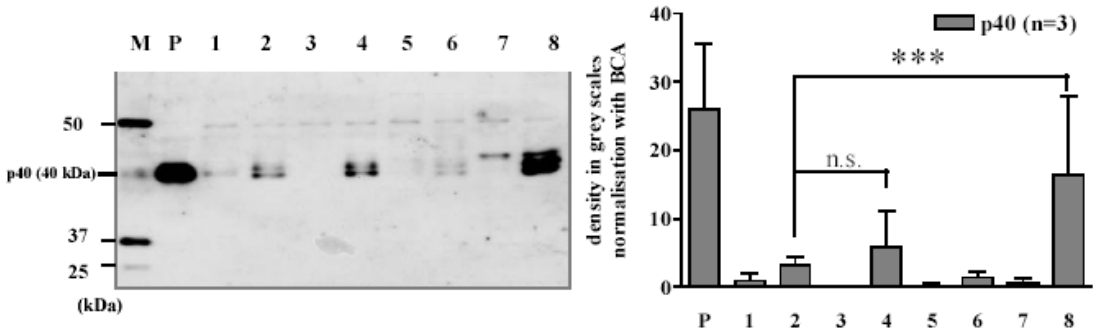

Fig. 3-17: CpG-induced IL-12p40 secretion in iLC.

Out of 3 experiments performed under the same conditions, one representative Western blot is shown. The Western blot was performed with biotinilated antibody BAF499 at a concentration of 0.2 µg/ml. Supernatants were concentrated with 10 kDa Centriprep®-filters to one-fourth of their volume. Protein-amount was normalised to a constant 25 µg/lane, protein mass was determined with the BCA assay Supernatants were isolated from 2.5 x 10^6 iLC incubated for 20 h with the following stimulants solved in iLC medium in the absence of growth factors: *lane 1:* unstimulated iLC, *lane 2:* iLC + 2,5 µM CpG, *lane 3:* iLC + 10^{-6} M PGE_2, *lane 4:* iLCC + 10^{-6} M PGE_2+ 2,5 µM CpG, *lane 5:* iLC +10^{-5} M PGD_2, *lane 6:* iLC +10^{-5} M PGD_2 +2,5 µM CpG, *lane 7:* iLC + 5 µg/ml αCD40 Ab, *lane 8:* iLC + 5 µg/ml αCD40 Ab + 2,5 µM CpG solved in 10 ml of total volume. The recombinant control contains 50 ng of recombinant protein (P). Precision plus® marker was used with auto-luminescent protein bands at 37 kDa and 50 kDa (M). 50 ng of rec. $(p40)_2$ was applied in the first lane (P). Results represent mean ± SD of three independent experiments. PGE_2 increases the IL-12p40 levels and PGD_2 reduces the IL-12p40 levels comparing a stimulation with CpG alone. αCD40 strongly induces IL-12p40 levels ($p<0.001$; unpaired Student t test compared to stimulation with CpG alone).

I demonstrate that CpG induced IL-12p40 secretion in iLC supernatants, alone or in combination with PGE_2, PGD_2 or stimulating αCD40 Ab. In combination with stimulating αCD40 Ab CpG leads to massively increased IL-12p40 protein levels in iLC supernatants compared to iLC stimulated with CpG alone. Apart from this, the results correspond to the data obtained under LPS-stimulation: PGD_2 in combination with CpG rather seems to decrease the IL-12p40 level. CpG in combination with PGE_2 seems to lead to higher IL-12p40 supernatant levels compared to isolated CpG stimulation.

Additionally to these observations, a third band of 50 kDa could be observed in lane 7 and 8 were stimulatory αCD40 Ab was added. This band represents IgG antibody fragments from the added antibody according to papain cleavage.

3.4.1.2 CpG dose-dependent affection of the number of IL-12p40 SFC

Next, I performed assays, analysing dose-dependent effects of CpG on the number of IL-12p40 secreting iLC, using concentrations of CpG between 100 nM to 12800 nM in

twofold serial dilutions (Fig. 3-18). Optimal concentrations for TLR-stimulation (personal opinion of A. Krieg) are 100 nM to 1 µM CpG.

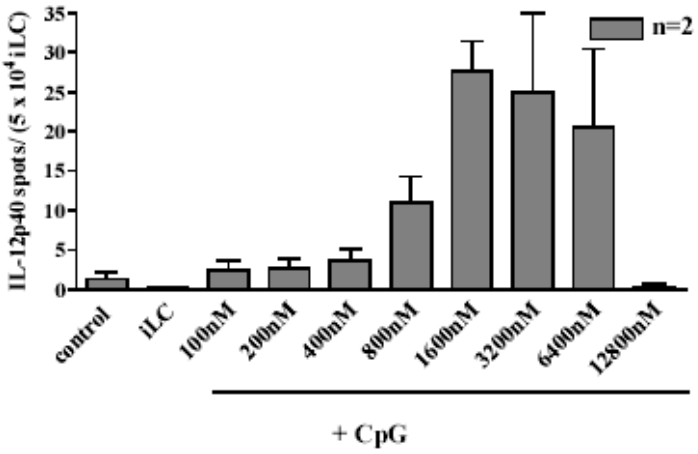

Fig. 3-18: Dose-dependent effects of CpG in iLC.

iLC (50 000/ well in 96-well ELISPOT plates) were incubated with varying doses of CpG (100 nM to 12800 nM; twofold serial dilutions) in iLC medium and assayed in IL-12p40 ELISPOT assay. Cells were incubated with additives for 24 h. Results represent mean ± SEM of two independent experiments with triplicates. Optimal stimulation is observed at 2.5 µM CpG.

First effects are observed at a CpG concentration of 100 nM. An intense stimulation of the iLC in ELISPOT assay is obtained at concentrations between 1600 nM to 3200 nM. Concentrations higher than 3200 nM lead to high background effects. Contrary to these observations the relatively highest effective dose in RT-PCR is reached at a concentration of 500 nM. In order to standardize the assays I used a constant concentration of 2500 nM in all assays. Remarkably, the spot quality detected under stimulation with CpG differs from the spot quality in LPS-stimulated cells (Fig. 3-19).

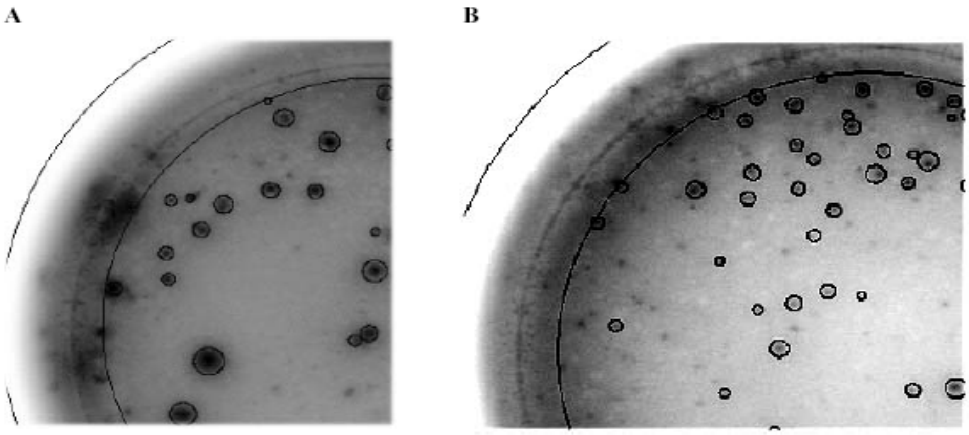

Fig. 3-19: Comparison of spot quality under stimulation with CpG and LPS as measured with AELVIS ELISPOT plate scanner.

Figures **3-19 A** and **B** are details of a αIL-12p40 (BAF499)-BCIP/NBT-coloured ELISPOT plate image of 50.000 iLC incubated with **A** 2.5 µM CpG or **B** 1 µg/ml LPS. Spot colour intensity, spot diameter, spot circularity and the number of spots varied. The images are taken from a single plate, assaying both CpG and LPS-stimulation effects.

Microscopically, aggregation of cells under CpG stimulation was observed, an observation that might have impaired the results.

Prostaglandin effects on CpG-stimulated iLC were tested in the following three assays under the same conditions but due to high background effects, results have only limited value.

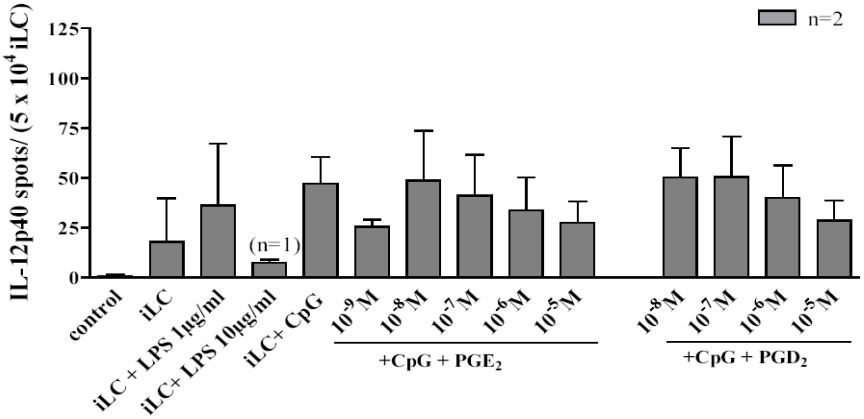

Fig. 3-20: Prostaglandin D_2 and E_2 effects on the amount of CpG-stimulated IL-12p40 SFC.

For the IL-12p40 ELISPOT experiment iLC (50 000 /well in 96-well ELISPOT plates) were incubated with a constant dose of 2.5 µM CpG. Both reagents PGD_2 and PGE_2 were diluted in tenfold serial dilutions and incubated with CpG-stimulated iLC. The assay was repeated 4 times. Due to high background effects, only two assays could be statistically evaluated. In these assays, both PGE_2 and PGD_2 had depressing effects on the number of IL-12p40 secreting cells.

3.5 IL-12p40 expression in iMDC supernatants

While transcription of all needed subunits is described in RT-PCR no IL-12p40 protein could be detected in cell culture supernatants in four out of five IL-12p40 Western blot assays according to the established protocol. Also under non-reducing conditions in ELISPOT no IL-12p40-linked protein could be detected with the established αmoIL-12p40 ELISPOT.

4 Discussion

These assays were consistent with a model of early Th1 mediated inflammation in which I analysed activating or inhibiting effects of PGE_2 and PGD_2 and potentially 15d-PGJ_2 and cytokines (especially IL-12 related cytokines) on memory Th1 cells. The effects of these substances produced by bystanding stromal cells or immune cells, such as iLC, are yet poorly characterized.

The data were assessed by RT-PCR (analysing the mRNA transcription levels of IL-12 cytokine subunits), Western blot assays (analysing isolatedly the IL-12p40 cytokine subunit in the cell culture supernatant under prostaglandin influence) and ELISPOT assays that provided information about the actual immunological effects of prostaglandins on single cell level. As priming signals I tested the influence of differential TLR-stimulation in both iLC and iMDC which I further compared in their alloproliferative capacities.

4.1 Modulation of Th1 cell activity by PGE_2 and PGD_2

It has long been accepted that prostaglandins have an effect on T lymphocytes during the effector phase. In regard to IL-2 and IFN-γ, PGE_2, even at nanomolar concentrations, clearly inhibits the expression of Th1 cells. When measuring the number of memory Th1 cells in IFN-γ ELISPOT in a model of MHC-dependent adaptive immune response mediated by either Con A-stimulation or stimulation with syngeneic DC in the presence of OVA I could observe a moderate decrease of IFN-γ SFC under stimulation with PGE_2. In previous assays of the "Foundation Immune Pharmacology" the extend of PGE_2-mediated suppression on the number of IFN-γ secreting memory Th1 was more significant. The extent of inhibition of antigen-specific activated Th1 clones allows us to estimate with a more sophisticated and qualitative method, the extent of prostaglandin-mediated effects as compared to previous ELISA-based assays. The extent of PGE_2-induced suppression was quantitatively lower in ELISPOT single cell assay as compared to previous ELISA studies (Hilkens et al., 1996) – a result that is due to different experimental settings and might reflect the actual immunological reaction more precisely: the activation of single cells and not the plasma-levels of cytokines leads to the clonal expansion of immune response.

Despite that Th1 are not supposed to express DP_1 and the second PGD_2 receptor CRTH2 seems to be exclusively expressed on Th2, probable premature degradation of prostaglandins was considered, especially under the premise that PGD_2 products are active. The concentrations of active prostaglandins can only be estimated under assay conditions. While PGE_2 in supernatants of intact cells seems to be more stable, PGD_2 effects rapidly reduce and overlap with 15d-PGJ_2 effects reaching at least 10% of originally applied PGD_2 after 12 h. Beside CRTH2, the other 15d-PGJ_2 ligand PPAR-γ is supposedly expressed in Th1 and might be regulated by added PGD_2, effects, that I could not detect although the necessary stimulation time for the optimal stimulation of this receptor might be longer.

The relatively high concentrations of prostaglandins in my model designed to imitate bystander cell products - in this case especially immune cells, keratinocytes and fibroblasts – should therefore be understood in first line in regard to the rapid catabolism of prostaglandins and in a time-dependent context. Gilroy et al. established a model in which a sequential liberation of PGE_2 by PMN in the first hours of inflammation and the liberation of PGD_2, 15d-PGJ_2 by monocytes in late inflammation serves as an inhibitory feedback mechanism to prevent excessive inflammation (Gilroy et al., 1999).

These described prostaglandin effects on the effector cells have to be separated from the induction phase of the immune response mediated by the activation of local DC.

4.2 Modulation of IL-12-related cytokines by prostaglandins in iLC and iMDC

4.2.1 mRNA expression of IL-12-related cytokines

The immune cells that are influenced most by modulating skin bystander products are iLC in the epidermis and iMDC in the dermis. Both cell types have tissue-specific allostimulatory properties dependent on their state of maturation. I focused especially on the LC that, although also bone marrow-derived, constitute a distinct unique DC subset.

In semi-quantitative mRNA of IL-12 related cytokine subunits in the iLC cell line XS52 I detected high levels of p40 – induced by LPS as described by Torii et al. (Torii et al.,

1997) but also by CpG – and a constitutive expression of the p19 subunit. I could not detect p35 and - only in low levels under additional CD40-stimulation - p28. Prostaglandins and in most assays stimulating αCD40 Ab did not seem to influence the transcription of these subunits in as far as this could be interpreted in results of semi-quantitative RT-PCR. The parallel expression of both p19 and p40 allow the assembly of the IL-23 that in fact could be detected in later assays performed by the "Foundation Immune Pharmacology" in cell culture supernatants. According to my data, it seems that XS52 do not produce IL-12 but are inducible for IL-23 and eventually under additional CD40-stimulation for IL-27 expression. Some authors claimed to have detected IL-12 in XS52 (Bouis et al. detected only IL-12p40; Thatcher et al. used C15.6 and the antibody kit OpTEIA consisting of 9A5 and C17.8) in immunoassays but no direct RT-PCR or Western blot assay evidence of the two subunits had been brought forward for XS52. It is currently unclear if Langerhans cells produce IL-12 at all and great part of the pertinent publications ignoring the existence of IL-23, were actually based on IL-12p40 assays and therefore have to be questioned in the post-IL-23 era. Morelli et al. (Morelli et al., 2005) characterized a human migratory $CD1a^+$ $CD14^-$ skin DC fraction that did not express IL-12 but IL-23, IL-10 and TGF-β that (in combination with IL-6) has been reported to be responsible for the generation of Th17 out of naïve and memory T lymphocytes (Bettelli et al., 2006). By contrast solitary stimulation with TGF-β seems to engender tolerogenic regulatory T lymphocytes. While XS52 have not been assayed for TGF-β, although they are known to express TGF-β_1 receptors, the role of this factor is known to be crucial for Langerhans cell differentiation. XS52 might resemble the epidermal cell fraction described by Morelli in different aspects.

The significance of the expression of IL-23 in the skin inflammation and not only in Langerhans cells has been meanwhile underlined.

Ghilardi et al. (Ghilardi et al., 2004) studied IL-23p19 and IL-23p40 knockout mice and outlined a severe impairment of delayed-type hypersensitivity skin reaction (DTH) and of humoral immune response indicating a significant role for IL-23 in the activation of memory T lymphocytes in the epidermis. Furthermore Kopp et al. (Kopp et al., 2003) established a transgenic expression model of IL-23 in the basal epidermis inducing a atopic dermatitis-like inflammation with increased numbers of LC and allostimulatory activity inducing accelerated rejection in a graft rejection model. While Kopp et al. only detected the p19 subunit in keratinocytes (murine model), Piskin et al. described a high

expression of IL-23 in psoariatic lesions by human keratinocytes. Beside IL-23, I could further detect the IL-27p28 subunit that was only expressed under costimulation with αCD40 Ab as previously described by Pflanz et al.. When dimerized with EBI3, that I did not analyse, the assembly of IL-27 would be possible. IL-27 has been characterized as a IL-12 response supporting cytokine but also as as antagonist of IL-2 expression (as a limiting factor for clonal Th expansion) and might, without overrating this observation, be a factor of autoregulation of IL-23 response in my assays.

Contrary to XS52, the myeloid JAWS II cell line expressed mRNA of both IL-12 and IL-23 subunits and also low amounts of p28 upon αCD40 stimulation. The regulation of the IL-12 subunits in this cell line was more complex, as p40 but also p35 and p19 were upregulated upon confrontation with LPS. This cell line has been characterized as a common precursor for MΦ and DC according to Jorgensen et al. and might be in fact representative for an undifferentiated precursor with the ability to differentiate into a majorly IL-12- as well as IL-23-expressing DC. Suprisingly when I tested this cell line in ELISPOT and Western blot assay I could not detect the p40 protein in cell culture supernatant upon stimulation with LPS although it was obviously expressed on mRNA level, suggesting that although already transcribed, the protein delivery process is blocked or inefficient. While it has been suggested that p40 is regulated on the level of transcription, the expression of p35 (that is known to undergo complex posttranscriptional modifications) and probably p19 is considered to be regulated posttranslationally (Abdi, 2002), so at least, the detection of p40 should have been expected in cell culture supernatants. This observation displays the complex translation of mRNA to actual protein that is tightly regulated and requires the activity of diverse processing enzymes.

4.2.2 Protein expression of IL-12-related cytokines

In the past decade the wrongly attributed contribution of actual IL-23 effects to the IL-12 protein (without the detection of the existence of IL-23 in reliable immunological assays) diminished the meaning of these previous works. Until now no specific IL-12 antibody detecting both subunits simultaneously or detecting one of the subunits exclusively exists. Due to the very restricted stability of the heterodimer proteins and

the vast excess of p40 monomer and homodimer, a valid immunologic detection requires therefore the detection of both subunits.

To address this issue, I verified the antibody specificity of commercial p40 antibodies.

Aside from the producer-declared cross-reactivity (Fig. 2-3), I tried to verify the antibody specificity in serial Western blots under reducing SDS-PAGE conditions using either rec. $(p40)_2$ protein or concentrated supernatants of LPS-stimulated iLC as target protein. I could detect under reducing conditions with the declared αIL12p40 Ab BAF499 and C17.8 but also with the sc-1283 Ab a 40 kDA protein as double-band – a phenomenon that Westermeier contributed to the sulphate-linked protein subunits that partial refold after separation and thus have different running fronts. It is suggested to use alcylating agents such as iodacetamide to prevent refolding. The band intensity was optimal with the Ab BAF499 which was therefore chosen for subsequent Western blot assays. I tried in a single assay to detect the rec. $(p40)_2$ and possibly assembling p40 products in supernatants of LPS-stimulated iLC in a non-reducing Western blot assay (data not shown). While I could detect the intact rec. $(p40)_2$ and the p40 monomer in supernatant of LPS-stimulated iLC, I did not detect either $(p40)_2$, IL-23, IL-12 or a possible p40-p28 dimer. A reason for this might be the poor stability of p40-linked proteins that might be responsible for the dissociation of the supernatant protein that underwent a technical necessary freeze-thaw cycle in my assays.

Antagonizing effects of p40 momomer and more important of the p40 dimer have been described [$(p40)_2$ has been only detected in the mouse model] (Gillessen et al., 1995; Holscher et al., 2001) but might have been overestimated due to lacking knowledge of IL-23. The total amount of in vivo detected p40 monomer and dimer is small. Hölscher et al. estimated its amount to 5-30% of totally detected p40. But Kopp et al. described that only 8% of intact IL-23 compensated the antagonising effects of p40 both monomer and dimer.

While p35 could not be detected in RT-PCR, I focused on the p19 subunit. No commercial antibodies were available at this time for this subunit. Based on the protein structure information for p19 of the NIH protein bank, we selected a p19 epitope that was recombinantly synthesized and vaccined to rabbits (www.eurogentec.com). Because of a delay in the antibody production, I could not analyse these effects.

Despite Abdi et al. argued that p35 and p19 is rate-limiting and best-correlating to actual heterodimeric cytokine amounts, it constituted for me under these conditions the only possible approach to test the regulated p40 subunit alone. In case of the XS52 cell line at least, p40 might correlate well with total IL-23: the p19 subunit was constantly transcribed on mRNA level while the p40 subunit was regulated upon LPS and CpG stimulation. Further it is assumed that p19 is only secreted when assembled with p40. In a single Western blot experiment I could describe that IL-12p40 in LPS-stimulated iLC was overadditively upregulated by PGE_2 and αCD40 Ab while it was downregulated by PGD_2. These data were later confirmed by ELISPOT experiments. Under ELISPOT cell culture conditions, it might be assumed that at least a part of the detected IL-12p40 spots reflects intact IL-23. The responder frequency of 0.2% thereby was congruent with the data in a human IL-12p40 system analysing the amount of IL-12p40 secreting PBMC (Ozenci et al., 2000).

I also focused on the influence of PGE_2 in IL-12p40 ELISPOT experiments.

Using the reagents Forskolin and dbcAMP the stimulatory effects observed under PGE_2 were found to reflect intracellular cAMP levels. Whereas Forskolin was probably used at too high concentrations (Betz et al. used 10^{-5} M Forskolin) the effects of dbcAMP correlated with the observed PGE_2 effects indicating a mediation of IL-12p40 promotion by EP_2 and EP_4 signaling. Ionomycin was ineffective, underlining this thesis.

Surprisingly until now, PGE_2 pathways are one of the only known differences between IL-12 and IL-23 secreting cells. Sheibanie et al. described an PGE_2-induced increase of total IL-23 in supernatants of murine MDC while p35 levels were not altered. Schnur et al. using human monocyte-derived DC suggests, that G protein-coupled receptors acting through cAMP EP_2 and EP_4, histamine receptor H_2, adenosine receptor $A_{2\alpha}$ and adenosinetrisphosphate receptor P2Y downregulate (synergistically with IL-10 and TGF-β) the expression of IL-12 and IL-12p40 but upregulate the p19 subunit leading to higher levels of IL-23. PGE_2-stimulated DC had in that way a lower capacity to induce IFN-γ secreting naïve T lymphocytes. In my assays I could not directly analyse a differential regulation of IL-12 and IL-23, as the used iLC were more differentiated and did not express IL-12. But I did observe an upregulation of IL-12p40 and presumably of total IL-23 in ELISPOT. Although the iMDC did express both, the results in semi-quantitative RT-PCR did not allow qualitative interpretation. Hence I focused on

possible differential effects of TLR4 and TLR9. The diversity of TLR indicate different pathways leading to the expression of IL-12 related cytokines.

4.3 Differential effects of TLR4 and TLR9 stimulation

TLR have become defining receptors for DC subtypes: with regard to human DC, it has been shown that myeloid DC express TLR2 and TLR4, whereas plasmacytoid DC express preferentially TLR7 and TLR9 (Kadowaki et al., 2001; Krug et al., 2001) and it has been suggested that - based on different pattern of Toll-like receptor (TLR) expression - these two cell types detect different classes of microbial molecules. In murine LC Mitsui et al. detected TLR2, TLR4, TLR9 but not TLR7 that is contrarily expressed in XS52 cells. Differential effects of TLR stimulation on IL-12 subunit expression have been demonstrated: It was demonstrated that IL-23 expression of myeloid DC upon confrontation with Klebsiella pneumoniae is TLR4-dependent (Happel et al., 2005; Re and Strominger, 2001). Though I did not analyse the receptors directly, I detected a specific response of the cells towards receptor-specific antigens. Focusing on the mRNA expression of cytokines upon TLR4 and TLR9 stimulation, I could not detect differences in cytokine expression as analysed by semi-quantitative RT-PCR, whereas I observed differences at the protein level. While LPS alone or in combination with prostaglandins or αCD40 Ab led reproducibly to detectable p40 expression in Western blot and ELISPOT, only CpG stimulation in combination with aCD40 led to significant bands in Western blot. In IL-12p40 ELISPOT I focussed on the effects of prostaglandins under CpG in the absence of αCD40, which led to inconsistent results. In a similar manner Will et al. (Waibler et al., 2006) could not detect intact IL-23 in cellular supernatant of CpG-stimulated iLC as measured with ELISA in the absence of T cell costimulation. SFC in ELISPOT when detected had a remarkable large halo while the number of total spots was smaller compared to IL-12p40 ELISPOT under LPS-stimulation indicating a different cell biology such as upregulation of adhesion molecules under the influence of TLR9 stimulation. On the other hand, there have been signs that CpG has lymphotoxic side effects under certain conditions (Heikenwalder et al., 2004). The observed effects of CpG on the expression of DNA polymerase-γ under CpG might be a indicator for this.

4.4 Combined effects of prostaglandins and IL-12-related cytokines

I described in 4.1 and 4.2 the isolated effects of prostaglandins on either Th1 alone or on iLC alone. The combined effects of prostaglandins on the two cell lines together seem however to be more complicated and have to respect the time and the place of stimulation. Gilroy et al. outline the time-dependence of PGE_2 and PGD_2 release. Further the microenvironment in the tissue and the differentiation state of the immune cells are outstanding factors that influence the prostaglandin pattern and the cellular reactivity of the immune cells. This overview (Fig. 4-1) reflects some of the properties contributed to prostaglandins in different cell systems and is consistent with my results.

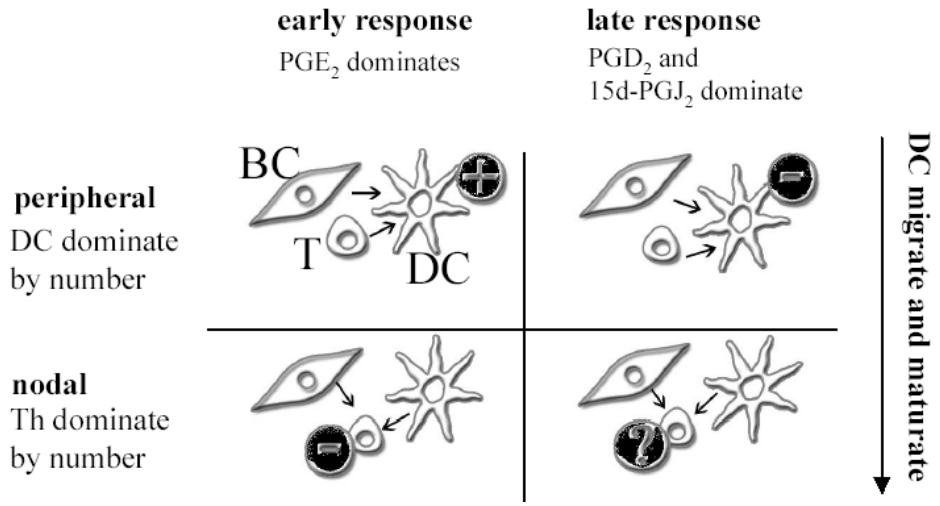

Fig. 4-1: Model of possible effects of modulatory bystander products in different tissues, at different times and in different states of maturation of the effector cell.

Either immature or mature DC communicate with T lymphocytes (T) and stromal bystander cells (BC) in peripheral and lymphatic tissue. Dependent on the maturation state and the ratio of the number of DC and T lymphocytes predominantly either the DC or the T lymphocyte is directed to a specific maturation by bystander cell products. While the prostaglandins are sequentially secreted by different bystander cells and the prostaglandin profile differs at inflammation sites and in lymph nodes, the predominant cell might act very differently dependent on these factors. Indices that underline the theoretical model above are: a) the principle of autoregulation of immune processes; b) the cell ratio: T lymphocytes are concentrated in lymph nodes while LC are widely distributed in the skin; c) PGE_2 was shown to be a very potent promoter of DC migration. As it acts early at the site of inflammation, this helps the activated LC to home to the lymph node early after antigen-confrontation; d) in lymph nodes a rich activity of PGD synthase has been recently observed (Trivedi et al., 2006) that might potentially modulate the activation of specific T lymphocytes by DC.

In the IFN-γ ELISPOT assays Con A stimulated the memory Th1 lymphocyte clone (Fig. 3-3) to a relatively high responder frequency of 11 % of IFN-γ positive cells indicating a competent activation. In contrast to responder frequencies of 0.2 % in iLC under stimulation with LPS or CpG, this constitutes a specific adaptive stimulation of a

specific memory Th clone with the priming protein OVA (the decrease of the responder frequency from 42 % in 2002 to 11% might be consistent with the loss of ability of the T cell clone to cope with complex proteins under cell culture conditions, often observed in cell culture). This adaptive stimulation requires physical contact - contrary to the nonspecific innate IL-12/IL-23 mediated T cell activation – and proceeds by activating the TCR to a T-bet-mediated release of IFN-γ. Because it is specific and potent, this is a very strong stimulus for Th differentiation and led in the described Th1 ELISPOT model to IFN-γ secretion. Physiologically, this is a very rare event and Th1 stimulation is preferentially promoted by a) IL-12 (not IL-23) considered to be indispensable for STAT4-induced IFN-γ secretion (Magram et al., 1996) and alternatively by b) the cytokines IL-15, IL-21, IL-18 (Strengell et al., 2003).

Overlapping of these two axes of T cell activation a) adaptive TCR-mediated (auto)antigen-presentation and b) innate immunity activation via TLR-signaling via TGF-β and IL-6, later upheld by IL-23 are suggested to be implicated in the formation of autoreactive Th17.

4.5 Conclusion and Outlook

The balance of prostaglandins and other tissue hormones play a decisive role as modulators of early steps in the development of Th differentiation. When this balance collapses, potential harmful Th subtypes like the proposed Th17 may lead to auto-aggressive diseases. I established a coculture model and examined the influence of modulatory prostaglandins with special attention on their effects on DC and its IL-12 related cytokine subunits. Immunological therapies targeting IL-12 related cytokine subunits are now in development and have, at least in the mouse model, shown positive effects. While therapeutical targeting the p35 subunit is obsolete, the p40 and p19 subunit constitute major targets in recent studies. Advantages of the use of αIL-12p40 treatment in a phase I study of psorias vulgaris patients (Kauffman et al., 2004) were outlined and it was later demonstrated that the blockage of IL-12p40 is equally effective as the blockage of IL-23p19 (Chen et al., 2006). The specificity of the used antibodies in human will however constitute a severe problem as cross-reactivity to potentially protective subunits has been observed so far. New insights in the biology of Th cells may furthermore provide new and more specific targets.

Summary

IL-12-related cytokines produced by dendritic cells are considered to be major inducers of adaptive immune system activation upon innate antigen-sensing. IL-23 specifically is currently being discussed to support the differentiation of potentially auto-aggressive Th17 cells. Prostaglandins as bystander cell products are known to modulate the translation of this process. While previous studies focused therefore on IL-12, ignoring the existence of new IL-12-related cytokines IL-23 and IL-27, this study analysed effects of prostaglandin E_2, D_2 and 15d-PGJ_2 on the secretion pattern of these subunits in the murine immature Langerhans cell line XS52 and the murine immature myeloid dendritic cell line JawsII under TLR4 (LPS) and TLR9 (CpG) stimulation as well as effects of prostaglandins on the murine Th1 cell line IF12 in coculture and upon Con A treatment.

In serial semi-quantitative RT-PCR of the IL-12 related cytokines of the XS52 cell line and the JawsII cell line, the p40 subunit was upregulated in both DC cell lines upon TLR-stimulation, the IL-23p19 subunit constantly expressed in XS52 and upregulated in JawsII upon TLR-stimulation, while the IL-27p28 subunit was only weekly expressed under additional stimulating αCD40 Ab treatment. IL-12p35 could only be detected in the immature myeloid cell line. The protein expression of the p40 subunit was measured in Western blot assays following SDS-PAGE under reducing conditions in XS52. The Western blot-based antibody specification allowed the establishment of a p40-specific ELISPOT assays, where overadditive upregulation of the number of LPS-stimulated spot forming XS52 cells was observed under stimulation with PGE_2 while PGD_2 depressed the number of LPS-stimulated cytokine secreting cells. Contrary IL-12p40 could not be detected in supernatants of the JawsII cell line.

Both DC cell lines were further tested for differential response towards different TLR stimulation described as a defining feature of DC subsets. While subunit expression on transcription level did not differ, only LPS-treatment led to constant IL-12p40 expression in supernatants of XS52. CpG-treatment of XS52 cells led to constantly high IL-12p40 levels under additional αCD40 Ab treatment.

In IFN-γ ELISPOT assays, prostaglandin effects were further analysed in IF12 Th1 cells upon Con A treatment or alternatively upon treatment in a coculture model with the

syngeneic cell line XS52 and the T lymphocyte-specific protein ovalbumin. While PGE_2 depressed the amount of activated Th1, PGD_2 showed no effect.

In conclusion, a coculture model has been generated that allows the analysis of DC and TC interactions. The importance of prostaglandins as differential regulators in time- and tissue-dependence in inflammatory processes has been demonstrated. These results accord with recent observations of an upregulation of IL-23 secretion upon PGE_2 treatment.

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This work is dedicated to my parents who supported me during the last 28 years.

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Zusammenfassung

Die von dendritischen Zellen freigesetzten Zytokine der IL-12 Gruppe sind maßgeblich an der Übersetzung der unspezifischer Aktivierung des angeborenen Immunsystems zur adaptiven Immunantwort beteiligt. Besonders Interleukin-23 wird gegenwärtig als unterstützendes Zytokin einer potentiell autoaggressiven Th17-Antwort diskutiert. Es wurde gezeigt, daß die von Stromazellen freigesetzten Prostaglandine diese Aktivierungsmechanismen beeinflussen. Einflüsse auf die kürzlich charakterisierten IL-12-verwandten Zytokine IL-23 und IL-27, von denen ersteres eine gemeinsame Untereinheit mit IL-12 teilt, wurden in früheren Studien nicht berücksichtig.

Die vorliegenden Arbeit stellt eine Untersuchung der Effekte der Prostaglandine PGE_2, PGD_2 und dessen Abbauprodukts 15d-PGJ_2 auf die Expression der Untereinheiten der IL-12 Gruppe in der murinen immaturen Langerhans Zellinie XS52 und der murinen immaturen myeloiden dendritischen Zellinie JawsII nach TLR4- (LPS) und TLR9-(CpG) Stimulierung dar. In weiteren Versuchen wurde der Prostaglandineinfluß auf Th1 Zellen (IF12) in Cokultur als auch nach Con A-Stimulierung untersucht.

Ergebnisse von seriellen semi-quantitativen RT-PCR der Zytokinuntereinheiten der IL-12 Gruppe in XS52 und JawsII Zellen zeigten eine Hochregulierung der p40-Expression auf mRNA-Ebene nach TLR-Stimulation. Die IL-23p19 Untereinheit wurde in XS52 Zellen konstant transkribiert, in JawsII Zellen nach TLR-Stimulation hochreguliert. Die IL-27p28 Untereinheit wurde nur schwach und nur nach Stimulierung mit zusätzlichen αCD40-spezifischen stimulierenden Antikörper transkribiert. IL-12p35 konnte nicht in XS52 Zellen nachgewiesen werden, wohl aber in JawsII Zellen. Mittels Western blot assays nach reduzierenden SDS-PAGE wurde p40 in Zellkulturüberständen detektiert. Die Antikörperspezifierung durch Western blot assay erlaubte die Etablierung eines p40-spezifischen ELISPOT assays. Überadditiv steigerte PGE_2 unter LPS-Stimulierung die Anzahl der Spot forming cells, während PGD_2 die Anzahl der aktivierten Zellen verringerte. In JawsII-Zellkulturüberständen konnte p40 nicht nachgewiesen werden.

Beiden Zellinien wurde auf eine differentielle Expression der IL-12 Untereinheiten nach verschiedenartiger TLR-Stimulierung, als definierendes Merkmal von unterschiedlichen DC Linien untersucht. Während die Transkription unbeeinflußt blieb, wurde nur in Zellkulturüberständen von XS52 nach LPS-Stimulierung eine konstante

Proteinexpression nachgewiesen. CpG-Behandlung führte zu konstant hoher Expression nach zusätzlicher stimulierender αCD40-Stimulierung.

In IFN-γ ELISPOT assays wurden Prostaglandineffekte auf die Th1 Zellinie IF12 nach Con A-Behandlung und alternativ im Cokultur Modell mit syngenen XS52 und dem T Lymphozyten-spezifischen Protein Ovalbumin untersucht. Während PGE_2 die Anzahl der aktivierten Th1 senkte, hatte PGD_2 keine Wirkung.

Zusammenfassend wurde in dieser Arbeit ein Cokultur Modell entwickelt, das die Untersuchung von DC and TC Interaktionen erlaubt. Die Wichtigkeit von Prostaglandinen als differentielle Regulatoren in Zeit- und Ortsabhängigkeit bei inflammatorische Prozessen wurde dargelegt. Diese Ergebnisse stimmen mit kürzlich veröffentlichten Publikationen, die eine isolierte Hochregulierung der IL-23 Sekretion bei unveraänderter IL-12 Expression nach PGE_2-Behandlung beschreiben, überein.

Curriculum vitae

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- Ärztliche Vorprüfung bestanden im September 2001
- Erster Abschnitt der ärztlichen Prüfung bestanden im September 2002
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06/1998- 07/1999: Zivildienst in der Pflegeabteilung des „Neuroorthopädischen Rehabilitationszentrums" in Bad Orb

09-1995-06/1998: Besuch des „Grimmelshausen Gymnasium Gelnhausen"; Abitur

09/1989- 07/1995: Besuch des Gymnsiums der Gesamtschule Wächtersbach

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Frankfurt, September 2006

Ehrenwörtliche Erklärung

Ich erkläre hiermit ehrenwörtlich, daß ich die dem Fachbereich Medizin zur Promotionsprüfung eingereichte Arbeit mit dem Titel

„Influence of prostaglandin E_2, D_2 and J_2 on IL-12-related cytokine subunits in murine dendritic cells" - "Einfluß von Prostaglandin E_2, D_2, J_2 auf die Expression von Untereinheiten der IL-12-verwandten Zytokine in murinen dendritischen Zelllinien"

im "Institut für Allgemeine Pharmakologie" – "Dr.-Hans-Schleussner-Stiftung Immunpharmakologie"

unter Leitung von Prof. Dr. H.H. Radeke

ohne sonstige Hilfe selbst durchgeführt und bei der Abfassung der Arbeit keine anderen als die in der Dissertation angeführten Hilfsmittel benutzt habe.

Ich habe bisher an keiner in- und ausländischen Medizinischen Fakultät ein Gesuch um Zulassung zur Promotion eingereicht noch die vorliegende Arbeit als Dissertation vorgelegt.

Frankfurt, den _____

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(Unterschrift)