In vitro generation of human monocyte-derived dendritic cells within 48 hours: functional characterisation and optimal activation in view of cellular-based immunotherapy

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1. Introduction

Innate and adaptive immunity are the two branches of the immune system. Cells of the innate immune system sense microbial presence by recognizing non-processed antigens, known as pathogen-associated molecular patterns (PAMP) using invariant pattern recognition receptors (PRR) in an antigen-independent manner. Macrophages and granulocytes are subsequently activated, providing „immediate care“ (within hours) while restricting the spreading of an infection.

On the other hand, cells of the adaptive immune system, comprising the T and B lymphocytes, are stimulated in an antigen-specific manner, and rely on additional instructions from the cells of the innate immune system to get activated. The resulting adaptive response is directed against one specific antigen. Furthermore, it is characterised by its immunological memory functions, providing long-lasting protection against invading pathogens, and is responsible for the discrimination between self and non-self.

Antigen-presenting cells (APCs) are critical for the initiation and modulation of adaptive immune responses. Dendritic cells (DCs), known as the most potent APCs, represent a crucial link between the innate and the adaptive immune system, playing a central role in T lymphocyte activation and differentiation into T helper and cytotoxic T cells. In brief, the life cycle DCs can be simplified as follows: immature DCs are ideally positioned as sentinels in the bloodstream, mucosa or residing in tissues, sampling for incoming pathogens. In their immature state, they are highly efficient in antigen capture, processing and presentation with major histocompatibility complex (MHC) molecules - a prerequisite for T-cell activation. Upon activation, they undergo a process of maturation, up-regulating co-stimulatory molecules and migrating to the T cell-rich regions of lymph nodes where priming of corresponding naïve T cells occurs, resulting in the initiation of a T cell-mediated response.

In addition to their pivotal role in bridging the innate to the adaptive immune system, DCs have been found to be involved in the pathology of autoimmune diseases, cancer, organ rejections and graft versus host reactions (Banchereau et al., 2006). In recent years, there has been an increasing interest to exploit DCs to launch a specific T cell-mediated immune response against cancer cells. However, poor results obtained in early clinical trials have dampened the initial enthusiasm about DC-based immunotherapy.

In current studies, factors contributing to the minor success of DC-based immunotherapy against cancer are being identified. Continuous investigation of DC biology will not only be helpful in

understanding the pathophysiology of diseases, but also in improving target-oriented therapeutic approaches. Thus, the generation of highly effective DC eligible for anti-tumour therapy is the main objective of this doctoral thesis.

1.1. Dendritic cells

1.1.1. Dendritic cell subsets

Like all immune cells, DCs originate from hematopoietic stem cells. It is widely accepted that there are two main differentiation pathways of DCs, generating three major DC subsets: (i) myeloid DCs (also known as interstitial DCs), (ii) Langerhans cells (LC)-DCs, and (iii) plasmacytoid DCs (pDCs). Along the myeloid pathway, $CD34^+$ haematopoietic stem cells differentiate in either $CD11c^+CD1a^+$ Langerhans precursor cells or $CD11c^+CD1a^-$ interstitial DC precursor cells (Liu et al., 2001). While epidermal Langerhans cells stimulate cytotoxic T cells, interstitial DCs are localized in the dermis and other tissues, and promote B cell differentiation. Furthermore, the common myeloid progenitor gives rise to monocytes, differentiating into myeloid interstitial DCs under the influence of granulocyte macrophage colony-stimulating factor (GM-CSF) and interleukin-4 (IL-4). $CD34^+$ - pDCs, believed to be of lymphoid origin, are characterized by the secretion of large amounts of type I interferons (interferon-α and interferon-β) in response to viral infections.

Recent murine *in vivo* studies demonstrated that Langerhans cells are derived from monocyte precursors (Banchereau et al., 2006). Though some data support the theory that human monocytes also represent a relevant *in vivo* precursor population for DCs (Banchereau et al., 1998), it remains to be determined whether monocyte-derived DCs have biological significance in humans.

1.1.2 Functional plasticity of dendritic cells

In contrast to the initial belief that the developmental origin exclusively defines the functional specialization of distinct DC subsets, it has become evident that the local microenvironment, antigen dose, nature of microbial stimulus and maturational status of DCs modulate the final outcome of a T-cell mediated response (Pulendran et al., 2004). *In vitro* studies demonstrated that neither a T_H1 (T helper) nor a T_H2 response was an intrinsic feature of specific DC subset (Vieira et al., 2000) : while DCs cultured with IL-10 induced T_H2 responses, interferon-γ-primed (IFN-γ) DCs were found to be potent inducers of T_H1 responses. Similarly, monocyte-derived DCs (moDCs)

matured with interleukin-1β (IL-1β) and tumour necrosis factor-α (TNF-α) were demonstrated to induce a T_H1 response, while the supplementation of prostaglandin E_2 (PGE_2) rapidly induced the secretion of T_H2 cytokines (Kalinski et al., 1998). Likewise, the influence of the microenvironment on DC differentiation was underlined in experiments demonstrating that DCs localized in different tissues elicited different responses. For instance, splenic DCs were found to induce the secretion of T_H1 cytokines, whereas DCs localised in Peyer's patches secreted T_H2 cytokines (Iwasaki et al., 1999).

1.1.3. Antigen uptake, processing and presentation

Immature DCs sense microbial presence via recognition of pathogen-associated molecular patterns (PAMPs) by means of their pattern recognition receptors (PRRs) or through inflammatory cytokines present in the microenvironment. PAMPs represent highly evolutionary conserved patterns found on all microorganisms, that are essential for their survival (and therefore difficult to alter) and distinct from the host's patterns. Following recognition, immature DCs capture antigens *in vivo* by phagocytosis, receptor-mediated endocytosis or macropinocytosis (Guermonprez et al., 2002). Experimentally, DCs have been shown additionally to internalise latex and zymosan beads (Inaba et al. 1993) as well as apoptotic bodies (Parr et al. 1991; Rescigno et al. 2000).

In order to be recognised by T cells, the captured antigens need to be processed and displayed in association with self MHC molecules in a process known as antigen presentation. The presentation of antigenic peptides on either MHC class I or MHC class II molecules determines the type of T cells to be primed – a feature described as the T-cell restriction of the immune system. MHC class I-restricted presentation of intracellular or endogenous antigens leads to activation of $CD8^+$ cytotoxic T lymphocytes (CTL). In contrast, presentation of exogenous antigens on MHC class II molecules activates $CD4^+$ T helper cells, thereby eliciting either a T_H1 or a T_H2 response. While a T_H1 response is characterised by the activation of macrophages, cytotoxic T cells and other effector cells, a T_H2 response results in the activation of B lymphocytes, with the subsequent formation of immunoglobulins.

In addition to these classical pathways of antigen presentation, DCs are capable of presenting exogenous peptides on MHC class I molecules, a process called "cross-presentation" (Trombetta et al., 2005). It represents an important feature of DCs, resulting in the activation of $CD8^+$ T cells against tumour and viral antigens that are otherwise not accessible to the classical MHC class I pathway. Furthermore, cross-presentation in DCs was reported to play an important role in transplantation and autoimmune diseases (Ackerman et al. 2004).

1.1.4. Maturation of dendritic cells

DC maturation is characterised by the development of features optimising the efficacy of DCs in T-cell priming. It is a continuous process, initiated in the periphery following encounter to pathogens, and completed upon T-cell encounter. Microbial-derived stimuli (e.g. Toll-like receptors), pro-inflammatory mediators (PGE_2, TNF-α), T-cell derived signals (CD40 ligand), and immune complexes acting on Fc receptors as well as sensors for cell death are known to induce maturation in DCs.

DC maturation is characterised by the up-regulation of membrane-associated co-stimulatory molecules, such as CD86 (cluster of differentiation) and CD80, as well as increased expression of the specific DC marker CD83 and MHC molecules (Banchereau et al., 1998). CD83 is one of the best-known maturation markers in DCs. Its functional role has not been completely elucidated, but it has been reported to influence T cell differentiation (Lechmann et al., 2002).

Concomitant to the up-regulation of co-stimulatory molecules, DCs undergo co-ordinated changes in the expression of adhesion molecules accompanied by cytoskeletal re-organisations. For instance, mannose-receptors are down-regulated and DCs lose their capacity to capture antigens. Likewise, they become unresponsive to immature DC-associated chemokines such as macrophage inflammatory protein (MIP-) 1α and MIP-3α (Caux et al. 2002).

1.1.5. Migration of dendritic cells

To encounter and activate T lymphocytes, DCs migrate to secondary lymphoid organs. The process of DC maturation is closely related to DC migration: while up-regulating the chemokine receptors CCR7, DCs migrate to the lymph nodes via the afferent lymphatic system or high-endothelial venules (HEV). Upon CCR7 expression, mature DCs respond to MIP-3β (also known as Exodus 3, ELC [EBI ligand chemokine], CCL 19 [chemokine ligand]) and 6Ckine (also known as SLC [secondary lymphoid tissue chemokine], Exodus 2 or CCL21), both highly expressed by stromal cells in the T cell rich zones of lymph nodes. Interestingly, $CCR7^+$ naïve T cells were also reported to enter lymph nodes in response to 6Ckine (Willimann et al., 1998). Likewise, CCR7 expression was found in B cells as well as in non-immune cells in various malignancies (Förster et al., 2008).

The key role of CCR7 in migration was demonstrated in a mouse knock-out model, whereby $CCR7^-$ mice failed to migrate to lymph nodes while exhibiting an impaired immune response (Gunn

et al., 1999). In addition to its central role in chemotaxis, CCR7 was found to exert regulatory effects on DCs. Its expression has been associated with an extended longevity of DCs, caused by an inhibition of apoptosis. Furthermore, an increase in migratory speed was observed in $CCR7^+$ DCs (Sanchez-Sanchez et al., 2004). Concomitant with the up-regulation of CCR7, changes in the cytoarchitecture of DCs were observed in terms of re-organisation of the actin cytoskeleton and the development of dendritic protrusions (Sanchez-Sanchez et al., 2006).

However, in contrast to early assumptions, there is increasing evidence that migration is not limited to mature DCs: immature DCs expressing CCR7 in the absence of inflammatory signals were also found to home to the lymph nodes. These “semi-mature” DCs are believed to contribute to peripheral immunological tolerance against self-antigens (Sanchez-Sanchez et al., 2006).

1.2. Dendritic cells and T-cell mediated immunity

Naïve T lymphocytes rely on interactions with APCs for their differentiation and expansion in effector T cells. DCs deliver three key signals required for the clonal expansion and differentiation of T cells:

- Signal 1: MHC/peptide complex associated with T-cell receptor (TCR)
- Signal 2: co-stimulatory signals required for the survival and expansion of T cells
- Signal 3: co-stimulatory signals involved in directing the differentiation into different subsets of effector T cells

1.2.1. T-cell subsets

While the priming of $CD8^+$ T cells results in a single set of effector cells, namely the cytotoxic T lymphocytes (CTL), the differentiation pathway of the $CD4^+$ subpopulation is more complex giving rise to several types of T helper cells, namely the T_H1, T_H2 or T regulatory cells.

T_H1 cells are characterised by the secretion of IFN-γ, IL-2, TNF-α and TNF-β, enhancing the bactericidal activity of macrophages for the eradication of intracellular pathogens. The predominant inducers of a T_H1 differentiation by DCs are IL-12 and IFN-γ. On the other hand, IL-4, IL-5, IL-6 and IL-13 are the major cytokines secreted by T_H2 cells, optimal for the production of antibodies, and are involved in the elimination of extracellular pathogens, including helminthes and nematodes. The differentiation of T_H2 cells is promoted by the presence of PGE_2, TNF-α, IL-1 and TGF-β

(transforming growth factor) . With regard to T regulatory cells involved in immunological tolerance, the synthesis of IL-10 has been shown to promote their differentiation in the absence of co-stimulatory molecules by DCs.

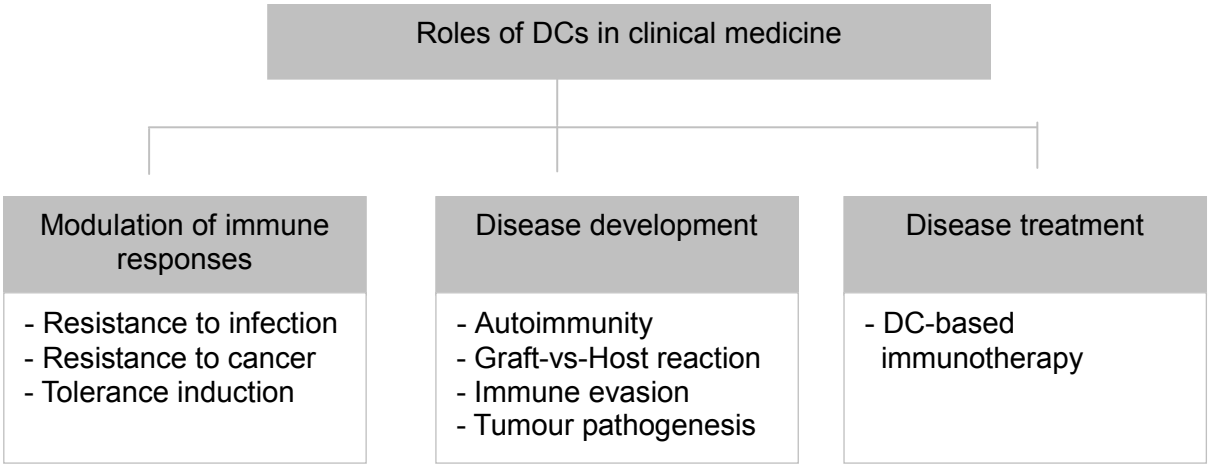
1.2.2. Interleukin-12

Secretion of IL-12 is essential to resist infections against bacteria and parasites. Activation of an antigen-specific T_H1 response represents its major role. As a potent inducer of IFN-γ secretion by natural killer (NK) cells and T cells, it favours the differentiation and proliferation of precursor T helper cells into effector T cells. DCs have been found to be one of the main producers of IL-12. Recent studies have shown that IL-12 also possesses anti-tumour functions. However, the exact mechanisms involved are not fully understood. Unlike its effects on T helper cells, IL-12 exerts no polarising effects on the differentiation of $CD8^+$ T cells, but was found to increase their cytotoxic potential and functional avidity (antigen sensitivity) - an important aspect to be considered in anti-tumour regimes (Xu et al., 2006).

The bioactive form of IL-12 is a covalently linked heterodimer cytokine formed by a p35 subunit (also called IL-12α, a 35 kDa light chain) and a p40 subunit (the heavy 40 kDa chain, also called IL-12β), which is secreted by activated APCs, including macrophages, monocytes and DCs. Although IL-12 is predominantly produced by DCs upon CD40 ligation while interacting with T_H1 cells, IFN-γ or IL-4 have also been found to be required for its secretion (Snidjers et al., 1998). Regarding the IL-12 receptor, it consists of the IL-12R-β1 and the IL-12-β2 polypeptide chains (Presky et al., 1996), mainly expressed by T cells and NK cells. They have been found lately to be additionally expressed by B cells. Interestingly, the IL-12 receptor is absent in most resting T cells, and is up-regulated upon its activation mediated by the T cell receptor.

1.3. Clinical applications of dendritic cells

Steinman's publication about the potential roles of DCs in clinical medicine considered the following three aspects (Steinman et al. 2007):



1.3.1. Dendritic cells and tolerance

One of the fascinating features of the adaptive immune system is its ability to discriminate between self and non-self. DCs play an important role in central as well as peripheral tolerance. By inducing negative clonal selection during T cell development, they ensure the elimination of T lymphocytes recognising self-antigens on thymic cells. In the context of peripheral tolerance, tolerogenic DCs are involved in the induction of anergy (T cells are refractory to activation, due to the absence of co-stimulatory molecules) or deletion of autoreactive T cells (Hawiger et al., 2001).

1.3.2. Dendritic cells and clinical immunology

Considering their pivotal functions in controlling immunity, DCs also play a role in the development of autoimmune diseases, transplantation, immunodeficiency as well as resistance to infection and to tumours. For instance, increased numbers and activations of DCs have been observed in patients with psoriasis and rheumatoid arthritis. Moreover, DCs have been found to be the source of cytokine over-production associated with autoimmunity (Banchereau et al., 2006). Correspondingly, excessive TNF-α was observed in rheumatoid arthritis and psoriasis (Lowes et al., 2005). Likewise, plasmocytoid DCs were found to release large amounts of type I IFN in systemic lupus erythematosus (Benett et al., 2003). In transplantation, DCs have been described

as the key initiators of T-cell induced responses in graft versus host reactions; recipient DCs were found to capture graft antigens and stimulate alloreactive T lymphocytes.

Additionally, DCs are believed to participate in the development of cancer. Tumours suppress immunity by several pathways, and are capable of evading the immune system by exploiting DCs. For instance, tumours have been demonstrated to condition DCs to form suppressive T cells like $FOXP3^+$ (Ghiringhelli et al., 2005) or IL-13-producing $CD4^+$ T cells (Aspord et al., 2007). The secretion of IL-6, vascular endothelial growth factor (VEGF) and IL-10 by tumour cells have also been reported to exert inhibitory effects on DCs (Steinman et al., 2007). On the other hand, DCs infiltrating colon and basal-cell cancers were found to be CD80- and CD83-deficient, emphasizing the necessity of functional DCs to mount an antigen-T cell anti-tumour response.

1.3.3. Dendritic cell-based cancer immunotherapy

In comparison to conventional anti-tumour regimes, the advantages of immunotherapy lie in their non-toxic and specific effects. T-cell mediated immunity is thus a potent therapeutical approach to consider in the treatment of cancer. DCs represent optimal candidates for the treatment of cancer based on the following facts (Steinman et al., 2007):

- DCs have been detected in tumours
- tumour cells express molecularly defined antigens
- DCs are capable of presenting tumour antigens and activating different arms of cell-mediated resistance, namely NK cells and T cells
- *ex vivo* generated DCs retain their immuno-stimulatory capacity in cancer patients
- DC-based vaccines can induce long-term immunity and tumour protection *in vivo*

The induction of a T-cell mediated anti-tumour response can be achieved by two DC-based approaches. In the first approach, *ex vivo* generated DCs are loaded with tumour antigens, and re-injected in the patient. The second strategy is based on the direct targeting of DCs *in vivo*. However, despite the induction of T-cell responses and some cases of tumour regression observed in early clinical trials with DC vaccines, relevant clinical responses and effects on survival are reported only very rarely (Steinman et al., 2007).

1.4. Isolation of dendritic cells

DCs can be isolated directly from peripheral blood, or generated *in vitro* from $CD34^+$ hematogenic stem cells or from $CD14^+$ monocytes. Monocytes can be isolated from peripheral blood, buffy coats or leukapheresis by plastic adherence, immunomagnetic selection or elutriation. Alternatively, DCs can be expanded *in vivo* by injections with cytokines termed “DC-poietins” including Flt3-ligand, GM-CSF and G-CSF (Maraskovsky et al., 1996; Pulendran et al., 1999). Their scarcity (accounting for only 0.1 % of the mononuclear cells) and the difficulty encountered in isolating and maintaining them in culture represented the two major obstacles for experimental and clinical use of peripheral blood DCs in early trials.

To date, the gold standard is based on the generation of monocyte-derived DCs *in vitro* from peripheral blood : monocytes are isolated from the peripheral blood mononuclear cells and cultured for 5-7 days with GM-CSF and IL-4 (Sallutsto et al., 1994; Romani et al., 1994), followed by stimulation for another two days with pro-inflammatory mediators (Jonuleit et al., 1997).

1.4.1. Stimuli used for dendritic cell maturation in vitro

1.4.1.1. Pro-inflammatory mediators: Prostaglandin E_2, tumour necrosis factor-α and interleukin-1β

Physiologically, pro-inflammatory mediators are secreted mainly by monocytes and macrophages at inflammatory sites. Previous studies of our group demonstrated that single pro-inflammatory mediators were incapable of inducing phenotypic maturation of monocyte-derived DCs. However, PGE_2 and TNF-α acted synergistically in inducing full DC maturation, and the supplementation with IL1-β enhanced the IL-12 secretory potential of DCs (Dauer et al., 2003). On the other hand, IL-6 was found to be indispensable in the differentiation of *Fast*DC.

PGE_2 is one of the most reliable and powerful mediators known to induce maturation of DCs. More importantly, it has been shown to be indispensable for the development of migratory capacities of DCs by regulating the sensitivity of CCR7; the exact mechanisms involved are not fully understood. Its signalling cascade involves G-proteins, mediated by the subunits EP4 (prostaglandin E receptor) and EP2 receptors in humans (Legler et al., 2006).

IFN-α is a cytokine with antiviral properties secreted predominantly by plasmacytoid DCs. Excessive IFN-α in the serum of systemic lupus erythematosus patients was reported to induce the

differentiation of monocytes into DCs (Blanco et al., 2001). *In vitro* studies with monocyte-derived DCs showed that IFN-α induced maturation when supplemented with other cytokines (Luft et al., 2002). Furthermore, the supplementation of IFN-α was shown to enhance survival and prevent the reversion of DCs to an immature phenotype (Dauer et al., 2006).

1.4.1.2. Pathogen-derived signals: Toll-like receptors

Toll-like receptors (TLRs) are innate receptors that sense microbial products and trigger DC maturation and cytokine production. To date, twelve TLRs have been identified in mammals, and each of them is stimulated by a distinct set of microbial compounds. TLRs are composed of integral membrane glycoproteins with extracellular domains containing varying numbers of leucine-rich repeats and a cytoplasmic signalling domain homologous to the IL-1 receptor, called the Toll/IL-1R domain (TIR domain) (Bowie et al., 2000). Although TLRs are expressed by a large variety of cells including macrophages, B cells, T cells, and non-immune cells such as fibroblasts and epithelial cells, DCs exhibit the broadest repertoire of TLRs. Unlike other receptors recognising microbial ligands, only TLRs are capable of inducing DC maturation directly. Localised in different cellular compartments, they are expressed in a differential manner in distinct DC subsets. Myeloid DCs express all TLRs except TLR7 and TLR9, which are expressed selectively by plasmacytoid DCs (Jarossay et al., 2001). While most of the TLRs are expressed at the cell surface, TLR7, TLR8 and TLR9 are localised in endosomes. However, the exact intracellular localisation of TLR3 remains to be determined (Napolitani et al., 2005). Table 1 gives a summary of the different TLRs as well as their corresponding ligands.

Table 1:

Microbial components	Species	TLR usage
Bacteria		
LPS	Gram negative bacteria	TLR4
Diacyl lipopeptides	*Mycoplasma*	TLR6/TLR2
LTA	Group B *Streptococcus*	TLR6/TLR2
Triacyl lipopeptides	Bacteria and mycobacteria	TLR1/TLR2
Porins	*Neisseria*	TLR2
Flagellin	Flagellated bacteria	TLR5
CpG-DNA	Bacteria and mycobacteria	TLR9
Not determined	Uropathogenic bacteria	TLR11
Viruses		
DNA	Viruses	TLR9
dsRNA	Viruses	TLR3
ssRNA	RNA viruses	TLR7 and TLR8
Hemagglutinin protein	Measles virus	TLR2
Envelope proteins	RSV	TLR4
Fungi		
Mannan	*Candida albicans*	TLR4
Zymosan	*Saccharomyces cerevisiae*	TLR6/TRL2
Glucuronoxylomannan	*Cryptococcus neoformans*	TLR2 and TLR4
Parasites		
Glycoinositolphospholipids	*Trypanosoma*	TLR2
Hemozoin	*Plasmodium*	TLR9
Profilin-like molecule	*Toxoplasma gondii*	TLR11

(modified from Akira et al., 2006)

The engagement of TLRs by microbial components triggers the activation of signalling cascades, which initiates the induction of the gene expression of pro-inflammatory mediators, involved in antimicrobial host defence. The two signalling pathways identified in TLRs are the inflammatory MyD88-dependent pathway and the interferon-dependent pathway. The inflammatory signalling

pathway acts via the adapter molecule, MyD88 (myeloid differentiation factor 88), binding to the cytoplasmic portion of all TLRs except TLR3, or TIRAP (Toll-like receptor adaptor protein), which binds only TLR2 and TLR4. Both lead eventually to the activation of NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) (Fitzgerald et al., 2001). The activation of NF-κB via TLRs results in the secretion of mediators such as IL1, IL6, IL12 and TNF-α. On the other hand, via the MyD88-independent pathway, IFN-α or IFN-β are secreted in response to stimulation with TLR3, 4, 7, and 9 (Akira et al., 2006). The release of these cytokines is a key process channelling signals of the innate system to the induction of a cellular response. The set of TLRs stimulated determines the cytokine profile induced, resulting in diverse adaptive immune responses. Interestingly, it has been demonstrated that the maximum cytokine production was induced by a combination of TLR agonists as compared with single TLR agonists. TLR synergy was equally observed in the T_H1 polarising capacity of DCs (Napolitani et al., 2005).

In the experiments described below, the TLR agonists R848 and LPS were used as microbial-derived signals for DC maturation. The physiological ligands for human TLR8 have been identified as GU-rich ssRNA (Heil et al., 2004; Diebold et al., 2004), whereas the synthetic nucleoside analogs binding to TLR7/8 comprise imiquimod (R837) and resiquimod (R848), as well as the guanine nucleotide analogs like loxoribine. Showing high homology, TLR7 and TLR8, recognise viral ssRNA with different but overlapping specificities. TLR8-activation results in the induction of type I IFN secretion, acting antivirally. Additionally, their intracellular localization reflects their optimisation in detecting micro-organisms reaching the cytosolic compartments, as it is in the case in viral invasion. Clinically, TLR8 agonists are used for their antiviral properties. Lipopolysaccharides (LPS), recognised by the extracellular TLR4, were originally known as endotoxins, are compounds consisting of a lipid and polysaccharide part, found in the structural cell wall of Gram-negative bacteria. During infections, LPS is liberated from bacterial cell walls.

1.4.1.3. T-cell derived signals: CD40 ligand and interferon-γ

CD40 ligand (CD40L) is a 50 kDa glycoprotein of the TNF receptor superfamily, which is primarily expressed by T cells. It activates CD40-expressing DCs leading to the expression of high levels of co-stimulatory molecules. CD40-CD40 ligand interaction is additionally associated with an enhanced survival in DCs (Caux et al., 1994) and is indispensable in the priming and expansion of antigen-specific $CD4^+$ T cells.

IFN-γ is a potent antiviral cytokine, blocking viral replication and eliminating viruses from infected cells. Early studies have demonstrated that mice deficient in IFN-γ had impaired immune

responses *in vivo*, with an increased susceptibility to microbial pathogens and viruses (Szabo et al., 2003). While NK cells and T cells are the most potent sources of IFN-γ, macrophages, B cells and DCs have also been identified as IFN-γ-secreting cells. IFN-γ has been demonstrated to enhance the production of IL-12 by DCs, matured with either LPS or pro-inflammatory mediators (Vieira et al., 2000). IFN-γ polarises immature DCs to stimulate T_H1 cells. T_H1 cells secreting IFN-γ activate macrophages, thereby potentiating the resulting inflammatory response (Grewal et al., 1998).

1.5. Short-term culture protocols

During the past few years, it has been shown repeatedly that monocyte-derived DCs can be generated in a shorter time span than above. Short-term protocols using calcium-mobilising agents for maturation (Czerniecki et al., 1997) or maturation with IL-4 or IFN-α (Dauer et al., 2003; Xu et al., 2003) have been described. The interest in short-term protocol is based on the assumption that a more rapid differentiation process represents more closely physiological conditions *in vivo*. On the other hand, in terms of methodology, short-term protocols confer the advantages of reducing costs and labour as well as the risks of contamination.

Previously, in our working group, a novel protocol was established, whereby monocyte-derived DCs undergoing all the processes of DC-differentiation were generated within only 48 hours, referred to as *Fast*DCs (Dauer et al., 2003). In this work, higher levels of co-stimulatory molecules were measured when moncocytes were incubated for 24 hours with GM-CSF and IL-4, and the conventional stimulatory combination, PGE_2, TNF-α and IL1-β, was added for further 24 hours of incubation, as compared with a single-step differentiation (GM-CSF and IL-4 plus conventional stimulatory combination added at the beginning of the culture for 48 hours). Additionally, it was found that monocytes cultured with GM-CSF or GM-CSF and pro-inflammatory mediators in the absence of IL-4 failed to develop DC features.

Comparative analysis between the different pro-inflammatory mediators revealed that complete immunophenotypic DC maturation (CD83> 50%) was elicited only in combinations including PGE_2 and TNF-α, or when IL-1β was substituted for TNF-α in the presence of PGE_2. Moreover, IL-1β was found to synergise with PGE_2 and TNF-α to induce the secretion of IL-12. In contrast, IL-6 was found to be superfluous in the maturation of *Fast*DCs.

IL-12 secretion is a key event in the activation of T cells and a critical requirement in DC-based immunotherapy. Low levels ofIL-12 could be detected in mature *Fast*DC, which was enhanced

upon addition of CD40L and IFN-γ. On the other hand, no IL-12p70 secretion could be induced unless supplemented by CD40 ligand and IFN-γ. With respect to their ability in activating $CD8^+$ T cells in an antigen-specific manner, *Fast*DCs and moDCs were found to be equally capable of inducing T cell proliferation using tetanus toxoid (TT) as a model antigen. While comparable levels of IFN-γ were measured in the supernatants of both *Fast*DC and moDC, no IL-4 was detected in all supernatants.

The functional characteristics o*f Fast*DCS and monocyte-derived DCs were further analysed in a comparative study (Dauer et al., 2005). Interestingly, in comparison to moDCs, a higher yield with more than 95% purity and viability was obtained with *Fast*DCs. Regarding their migratory capacities, both populations were readily capable of migrating in response to 6ckine. Likewise, *Fast*DCs were found to be capable of equally priming tumour-antigen specific cytotoxic T cell responses as shown in a melanoma antigen model: While a higher lytic activity was observed in the *Fast*DC population in the chromium release assay, comparable levels of IFN-γ-producing $CD8^+$ T cells were measured in monocyte-derived DCs and *Fast*DC. Though superior in *Fast*DCs, only low levels of IL-12p70 could be detected.

Considering that the maintenance of a mature DC phenotype while homing to the lymph node is a pre-requisite for T cell priming, the following set of experiments were performed : DC viability and immunophenotype were assessed in moDCs cultured for additional 6 days without further supplementation of growth factors or cytokines (wash-out cultures) following maturation. Moreover, comparative studies were conducted to analyse the influence of CD40L, IFN-γ and IFN-α. To this end, monocytes were cultured for 24 hours with GM-CSF and IL-4 on day 1, while maturation was induced with PGE_2, TNF-α, IL-6 and IL1-β for 36 hours. CD40 ligand and IFN-γ were added during the last 24 hours of maturation whereas IFN-α was added on day 1: after 24 hours of maturation, 90% of DCs were alive and exhibited the typical mature DC immunophenotype $CD83^+CD80^+CD86^{high}MHC^{high}$. An increased viability and enhanced maturational degree were observed in mature DCs supplemented with CD40 ligand and IFN-γ. Furthermore, their supplementation was associated with a lower re-expression of CD14 (monocytic phenotype). Supplementation with IFN-α did not affect DC yield and survival, neither its ability to prime autologous naïve T cells or migratory potential. In contrast, a higher expression of co-stimulatory molecules was observed while CD14 re-expression was delayed. Both effects were more pronounced when CD40L and IFN-γ were added in the last 24 hours of incubation. IL-12p70 deficiency was observed in all wash-out cultures, independent of IFN- α supplementation (Dauer et al., 2006).

In summary, the previous works in our working group established a new short-term protocol for the generation of DCs within 48 hours. *Fast*DCs were capable of exhibiting a fully mature immunophenotype and migrating in response to 6ckine. Moreover, the ability of antigen-pulsed *Fast*DCs to prime a tumour-antigen-specific cytotoxic T cell response was demonstrated in the aforementioned experiments. Upon maturation with the set of pro-inflammatory mediators including PGE_2/TNF-α/IL-1β, IL-6 was found to be dispensable. Under the above conditions, *Fast*DCs were found to be poor inducers of IL-12p70. The maturity cocktail consisting of PGE_2, TNF-α and IL1-β for the maturation of DCs was used in the experiments described below and referred to as the "conventional" stimulatory combination.

1.6. Questions addressed

Based on the experimental data available and our own results on the generation of *Fast*DCs in previous experiments, the following questions were addressed in the work described here:

- The influence of IFN-α on the differentiation of *Fast*DCs
- Maintenance of a mature immunophenotype of *Fast*DCs in wash-out cultures
- Comparison between proinflammatory-based and pathogen-derived activation of *Fast*DCs
- Optimisation of the *Fast*DC protocol for DC-based cancer immunotherapy

2. Methods

2.1. Reagents and materials

2.1.1. Apparatus

Incubator	Heraeus (Hanau, D)
FACSCalibur	Becton Dickinson (Heidelberg, D)
Centrifuge Sepatech Omnifuge	Heraeus (Munich, D)
Centrifuge 5417 R	Eppendorf (Hamburg, D)
Vortexer	Janke & Kunkel (Staufen, D)
Weighing scale	Sartorius (Göttingen, D)
Ice machine	Ziegra (Isernhagen, D)
Phase contrast microscope	Zeiss (Jena, D)
MidiMACS	Miltenyi Biotec (Bergisch Gladbach, D)
1480 Wizard 3_ γ Counter	Wallac Oy (Turku, FI)
Multistep pipette (Multipette plus)	Eppendorf (Hamburg, D)
Pipettes (Eppendorf reference)	Eppendorf (Hamburg, D)
Pipettus (Pipetus akku)	Hirschmann (Eberstadt, D)
LaminAir HB 2472S	Heraeus (Munich, D)
96 well MicroCellHarvester	Skatron (Ismaning, D)
ELISA-Reader MRX	Dynatech Laboratories (Burlington, USA)

2.1.2. Plastic materials

175 cm^2/175 cm^2 culture flasks	Greiner (Frickenhausen, D)
6- / 12- / 96 well round bottom tissue culture plates	Greiner (Frickenhausen, D)
96-well flat bottom tissue culture plates	Greiner (Frickenhausen, D)
Cell scraper	Eppendorf (Hamburg, D)
Pipette tips	Sarstedt (Nümbrecht, D)
Transwells 5.0 µm pore size, 6.5 mm diameter	Corning Incorporated (NY, USA)
MACS Separation columns LS	Miltenyi Biotech (B-Gladbach, D)
5 ml polystyrene round bottom tubes	BD Labware Europe (Meylan, F)

2.1.3. Reagents

Heparin-sodium	Ratiopharm (Ulm, D)
Sodium chloride (NaCl) 0.9%	Baxter (Lessines, B)
Brefeldin A	SIGMA (Missouri, USA)
FIX & PERM Reagents	Invitrogen Corporation (Carlsbad CA, USA)
Strep-Tactin PE	IBA GmbH (Göttingen; D)
Recombinant MHCI-Strep	IBA GmbH (Göttingen; D)
FITC-Dextran	Sigma-Aldrich (Steinheim, D)
Tween 20	Sigma-Aldrich (Steinheim, D)
Trypan blue	Sigma-Aldrich (Steinheim, D)
Triton X	Sigma-Aldrich (Taufkirchen, D)
Ortho-mune-Lysis Reagent	Ortho-Clinical Diagnostics (Neckarsgmünd, D)
Phorbol-Myristat Acetate (PMA)	Sigma-Aldrich (Steinheim, D)
Calcium-Ionomycin	Sigma-Aldrich (Steinheim, D)
$Na_2{}^{51}CrO_4$	Hartmann Analytic (Braunschweig, D)
FACS Flow	Becton Dickinson (Heidelberg, D)
FACSSafe	Becton Dickinson (Heidelberg, D)
Ethylen-Diamin-Tetra-Acetic acid (EDTA)	Sigma-Aldrich (Steinheim, D)
5,6-Carboxyflurescein Diacetat Succinimidyl Ester (CFSE)	Sigma (St. Louis, USA)

2.1.4. Frequently used buffers

RPMI-complete media (=DC-media)

RPMI-media
2% volume human AB serum
100 IU/ml penicillin
100 µg/ml streptomycin
2 mM L-glutamine

MACS-buffer:

PBS)
0.5% volume HSA
2 .68 mM EDTA
pH = 7.2

Erythrocyte-lysis buffer :

Ortho-mune lysis reagent in PBS

2.1.5. Reagents for cell culture

RPMI 1640 media	Biochrom (Berlin, D)
Human AB-Serum	BioWhittaker (Walkersville, USA)
Phosphate buffered saline (PBS)	PAA (Linz, A)
2% volume human AB serum	BioWhittaker (Walkersville MD, USA)
Ficoll Hypaque	Biochrom (Berlin, D)
Penicillin	PAA (Linz, A)
Streptomycin	PAA (Linz, A)
L-glutamine	PAA (Linz, A)
Phosphate buffered saline (PBS)	PAA (Linz, A)
Human Serum Albumin (HSA)	Baxter (Lessines, B)
Bovine serum albumin (BSA)	Sigma (St. Louis, USA)
Fetal calf serum (FCS)	Gibco BRL (Paisley, UK)

2.1.6. Monoclonal antibodies

Name	**Specificity**	**Clone**	**Fluorescence**	**Company**
Anti-human CCR7	CCR7	150503	APC	R&D Systems
Anti-human CD83	CD83	HB15e	FITC	BD Biosciences
Anti-human CD14	CD14	M5E2	APC	BD Biosciences
Anti-human CD80	CD80	L307.4	PE	BD Biosciences
Anti-CD4	CD4	RPA-T4	APC	BD Biosciences
Anti-human IFN-γ	IFN-γ	B27	ALEXA 488	CALTAG Laboratories
Anti-human CD86	CD86	2331(FUN-1)	PE	BD Biosciences
Anti-CD8	CD8	RPA-T8	APC	BD Biosciences
Anti-HLA-DR	HLA-DR	L243	PerCP	BD Biosciences
Anti-CD3	CD3	HIT3A	FITC	BD Biosciences

2.1.7. Cytokines and chemokines

GM-CSF	Novartis (Basel, CH)
Interleukin-1β	Strathmann Biotech GmbH (Hannover, D)
Interleukin-2	Strathmann Biotech GmbH (Hannover, D)
Interleukin-4	Promega (Madison, WI, USA)
Interleukin-7	Strathmann Biotech GmbH (Hannover, D)
Interferon-α	Strathmann Biotech GmbH (Hannover, D)
PGE_2	Sigma-Aldrich (Steinheim, D)
TNF-α	R&D Systems (Wiesbaden, D)
CD40 ligand	Amgen (Thousand Oaks, CA USA)
Recombinant human Interferon-γ	Strathmann Biotech GmbH (Hannover, D)
Recombinant Human Exodus-2/CCL21	PeproTech Inc. (USA)
Ultra Pure *E.Coli* LPS	InvivoGen (San Diego CA, USA)
R848	Kindly provided from Dr. V. Hornung (Division of Infectious Diseases and Immunology, University of Massachusetts Medical School, MA, USA)

2.1.8. Enzyme-linked immunosorbent assays (ELISA)

Human IL-12 (p70) ELISA set	BD Biosciences (San Diego CA, USA)
Human IL-4 ELISA set	BD Biosciences (San Diego CA, USA)
Human IFN-γ ELISA set	BD Biosciences (San Diego CA, USA)

2.1.9. Magnetic activated cell sorting (MACS)

Pan T Cell isolation kit II human	Miltenyi Biotec (Bergisch Gladbach, D)
CD14 MicroBeads human	Miltenyi Biotec (Bergisch Gladbach, D)

2.1.10 Peptides

The MART-1 peptides (ELAGIGILTV, position 26-35) were produced at Jerini Peptide Technologies (Berlin, D). The HIV-POL peptides (ILKEPVHGV, position 476-484) were synthesised on a multiple peptide synthesizer (peptide synthesizer 433A, Applied Biosystems, Forster City, CA) at the GSF Research Institute Munich.

2.1.11. T2 cell line

The T2 cell line was obtained from the American Type Culture Collection (ATCC CRL-1992). A transporter associated with antigen (TAP)-deficient cell line composed of the fusion of a lymphoblastic B-cell line with a lymphoblastic T-cell line. The T2 cell line is HLA-$A2^+$ and was maintained in FCS-media for use as target cells in the chromium release assay.

2.2. Cell isolation and cell culture

2.2.1. Cell culture

All cell cultures were maintained in a cell culture incubator at 37°C in 5% CO_2 and 5% air humidity. Cell manipulations were performed in a sterile environment under a laminar air flow hood. Unless mentioned otherwise, cell cultures were maintained in DC-media (RPMI-media, 2% volume human AB serum, 100 IU/ml penicillin, 100 µg/ml streptomycin and 2 mM glutamine).

2.2.2. Counting of cells and cell viability

Cell viability and cell counts were assessed by the trypan blue exclusion test. Trypan blue, a dye taken up by dead cells, and excluded by living cells, was used to distinguish between viable and non-viable cells. To this end, 90 µl of trypan blue was added to 10 µl of the cell suspension. 10 µl of the mixture was then transferred in a hemocytometer, where the non-stained viable cells were counted under the light microscope.

2.2.3. Enrichment of monocytes by cell adhesion

PBMC was prepared using the Ficoll Hypaque gradient centrifugation. 200 ml of full blood was obtained from volunteer healthy donors by venous punction, heparinised (50 IU heparin/ml blood), and diluted 1:1 with sodium chloride (NaCl). 30 ml of this dilution was stratified cautiously on 15 ml Biocoll (Ficoll) and centrifuged 1000g at 20°C for 20 minutes. The buffy coat, containing predominantly monocytes (found between the upper layer [plasma] and the Ficoll-Paque layer) was collected and washed three times with NaCl. Cells were re-suspended in fresh DC-media (20 ml DC-media/200 ml blood) and incubated at 37°C for 1 hour in tissue culture flasks. After two washings with PBS, non-adherent cells (mainly lymphocytes) were either preserved for further cell

isolations or discarded when not needed. The adherent fraction was incubated overnight at 37°C with DC-media (20 ml DC-media/200 ml blood), and harvested the next day with the cell scraper. Cell viability and cell counts were assessed by the trypan exclusion method.

2.2.3.1. Generation of FastDC in vitro

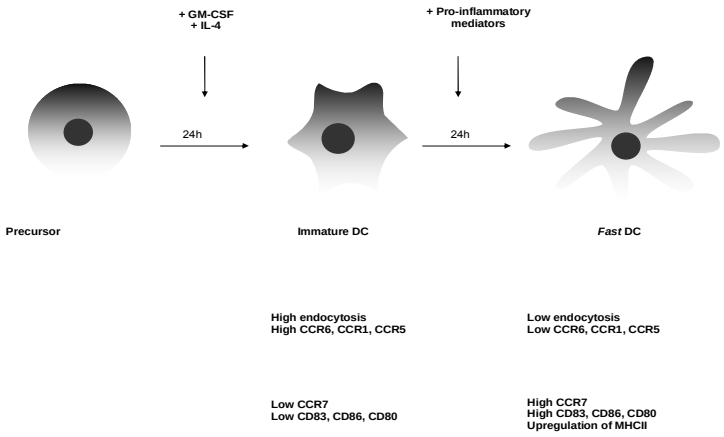


Diagram 1 - Generation of conventional FastDCs

The following protocol, established in our laboratory, illustrates the generation of mature DCs from monocytes within 48 hours (Dauer et al., 2003): PBMC was prepared by Ficoll Hypaque gradient density, and monocytes were isolated by cell adhesion. Cells were adjusted to a concentration of 10^6 cells/ml DC-media in a 6 well-plate. Monocytes were cultured for 24 hours with either GM-CSF (1000 U/ml) and IL-4 (500 U/ml), or supplemented additionally with IFN-α (500 U/ml), and followed by maturation with the following stimuli, either alone or in combinations for another 24 hours: PGE_2 (1 µM), TNF-α (1000 U/ml), IL-1β (10 ng/ml) , LPS (1 µg/ml), R848 (2.5 µg/ml), CD40L (500 ng/ml), IFN-γ (1000 U/ml). DCs were harvested with a cell scraper, washed twice with PBS and re-suspended in fresh DC-media for further investigation. Subsequently, DCs were counted manually with the hemocytometer. DC-immunophenotype was analysed by flow cytometry. The cell culture supernatant was collected and frozen to -20°C for further cytokine analysis. DCs generated with this protocol are referred to as *Fast*DCs.

2.2.3.2. Standard protocol for the generation of monocytes-derived dendritic cells

Analogous to the protocol described above, monocytes were cultured for 6 days with GM-CSF (1000 U/ml) and IL-4 (500 U/ml), or supplemented additionally with IFN-α (500 U/ml), followed by maturation with the following stimuli, either alone or in combinations for another 24 hours: PGE_2 (1 µM), TNF-α (1000 U/ml), IL-1β (10 ng/ml) , CD40L (500 ng/ml), IFN-γ (1000 U/ml). Immunophenotype was analysed by flow cytometry. The cell culture supernatant was collected and frozen to -20°C for further cytokine analysis. DCs generated with this protocol are referred to as monocytes-derived DCs (moDCs).

2.2.3.3. Immature DC

Monocytes cultured with GM-CSF and IL-4 without additional stimuli are referred to as immature DCs in this work.

2.2.4. Isolation of T-cells by magnetic cell sorting (MACS)

T-cells were isolated from the non-adherent fraction of the PBMC using the Pan T Cell isolation kit (Miltenyi Biotech, B-Gladbach) by negative selection of MACS following the manufacturer's instructions. In this case, the non-T-cell fraction (B cells, natural killer cells, DC, monocytes, granulocytes and erythroid cells) was magnetically labeled, while the unlabeled T-cell fraction eluted through the column : the cell suspension was first incubated with a cocktail of biotin-conjugated antibodies against CD14, CD16, CD19, CD36, CD56, CD123, and CD235a (Glycophorin A), followed by magnetic labeling with Antibiotin-Microbeads. Next, the cell suspension was loaded onto the MACS column placed in the magnetic field of the MACS Separator. Non-T cells were retained on the column, and the effluent containing the unlabeled enriched T cell fraction, consisting of all the T-cell subpopulations (so-called panT-cells) was collected for further use.

2.3. Analytical methods

2.3.1. Phase-contrast microscopy

Cell morphology was analysed by phase-contrast microscopy, by 10-100x magnification.

2.3.2. Enzyme-linked immunosorbent assay (ELISA)

The enzyme-linked immunosorbent assay (ELISA) was used to determine the concentration of proteins released in the supernatants of cell cultures. The protocol was performed according to the manufacturer's recommendations: in a first step, monoclonal antibodies against the cognate protein were coated to a 96 well-plate overnight. Standards were prepared by serial dilutions from the stock standard provided. Following to coating, the standards and supernatant were added to the well-plates for 2 hours (cognate protein binds to the coated immobilised antibody). Next, unbound proteins were washed away, and the „detection antibody"(specific against the coated antibody), a biotinylated-antibody, was added, followed by incubation with Streptavidin-horseradish peroxidase, producing an antibody-antigen-antibody „sandwich". The wells were washed and the substrate (enzyme catalysing a reaction, forming a coloured substance) was added. The resulting change in colour, proportional to the concentration of the protein examined, was analysed by a photometer. Together with the standard serial dilutions, the concentration of the examined protein was quantified.

2.3.3. Fluorescent-activated cell-sorting (FACS)

Fluorescent-activated cell-sorting is a specialised type of flow cytometry, used for counting, examining and sorting heterogeneous mixture of biological cells simultaneously, based upon the specific light scattering and fluorescent characteristics of each cell. In short, the mixture to be analysed was passed as a stream of fluid through a beam of light. Depending on the cell volume, or on the granularity, i.e. shape of nucleus, amount and type of cytoplasmic granules or membrane roughness, scattered light was detected by either the forward scatter (cell volume) or the side scatter SSC (granularity). In another step, cells were incubated with fluorescence-conjugated antibodies. These fluorescent molecules were excited to emit light by the laser, which was then detected.

2.3.3.1. Staining of cell surface CD (cluster of differentiation) molecules

Cell surface CD molecules were detected by staining with FITC-, PE-, APC- or PerCP-labeled antibodies specific for these CD molecules. Here, 3.5 µl of the labeled antibodies were incubated in the dark for 30 minutes with 100.000 DCs/100 µl. Next, DCs were washed twice with 3 ml PBS, and re-suspended in 100 µl PBS, analysed by flow cytometry and evaluated by CellQuest software (Becton, Dickinson).

2.3.3.2 Staining of intracellular interferon-γ

A modified version of immunofluorescent staining was used to detect IFN-γ intracellularly and characterise the immunophenotype of T cells simultaneously in one experiment. First, T cells were activated in a co-culture with antigen-loaded DCs, and cell surface molecules, CD8, CD4 and CD3, were identified in the conventional staining. Next, cells were fixed to preserve their morphological scatter characteristics, and permeabilised, thereby allowing fluorescent-labeled antibodies to access intracellular structures. To maximise IFN-γ synthesis, ionomycin (calcium ionophore) and Phorbol-Myristat Acetate (PMA) [phorbol ester/PKC activator] were supplemented (activating the signalling pathway of IFN-γ secretion). To block the secretion of IFN-γ, Brefeldin A (inhibitor of the Golgi apparatus and thus the secretory potential of cells) was added at a later point.

T cells were co-cultured with Melan-A-loaded DCs, and intracellular IFN-γ staining was performed in a second re-stimulation from co-cultured T cells (concentration: 4x 10^6 cells/ml with loaded DCs at a concentration of 0.4 x 10^6 cells/ml): Cell suspension was incubated in a 4ml-tube for two hours at 37°C in the incubator. For positive controls, ionomycin (500 ng/ml) and PMA (50 ng/ml) were added to the cell suspension, as well as the isotype control ALEXA-FITC. At the end of incubation, 100 µl of Brefeldin A (1 µg/ml), were added to all samples for four hours at 37°C. To phenotype cells, conventional cell surface staining for CD3, CD8 and CD4 was performed: samples were incubated for 15 minutes on ice in the dark, with 4 µl of each of the corresponding fluorescent-labeled antibodies. Next, all samples were incubated for 15 minutes at room temperature with 100 µl of Reagent A (Fixation Media). Cells were washed with PBS, and 100 µl of Reagent B (Permeabilization Media) was added, followed by the addition of 3.5 µl of ALEXA-FITC to all samples and isotype control, and incubated for 20 minutes at room temperature. Next, all samples were washed twice with PBS, and the pellets were re-suspended in 100 µl PBS, and analysed by flow cytometry.

2.3.3.3 Streptamer Fluorescent T-labeling and isolation via FACS

One of the pre-requisite for T-cell activation is the presentation of its specific antigen in the context of MHC molecules (major histocompatibility complexes). The *Strep*tamer technology enables the reversible staining of antigen-specific functional T cells. *Strep*-tags are short peptides binding to *Strep*-Tactin. Recombinant proteins attached to *Strep*-tags can be isolated from immobilised *Strep*-tactin by elution. In a modified approach, MHC I streptamers (in our case, MHCI-*Strep* HLA-A*201 MART 1 peptide) were multimerised with *Strep*-Tactin labeled with R-Phycoerythrin. Upon binding of T lymphocytes expressing the corresponding T-cell receptor, antigen-specific T cells can be detected by FACS analysis. After the selective isolation of these T cells, the MHCI *Strep*-tag fusion proteins dissociate spontaneously from the TCR, and the staining can be removed by the addition of biotin. Thus, the isolated T cells can be re-used for further experiments. Under our experimental settings, the use of MHC I streptamers was restricted to the staining of $CD8^+$ T cells specific for MART 1 peptide in stimulated T cells cultured with Melan-A-loaded DCs.

A master mix was prepared, including 5 µl *Strep*-Tactin-PE, 4 µl MHC I-*Strep* HLA-A*0201 MART-1 peptide, and 41 µl FACS-buffer (PBS + 0.5% BSA) per sample, and incubated on ice in the dark for 45 minutes. Co-cultured T cells were prepared to a concentration of 3 x 10^6/ml and were incubated with 50 µl of the pre-incubated *Strep*-Tactin-PE/MHCI master mix for 45 minutes in the dark. For isotype staining, one sample was treated similarly without the master mix. During the last 20 minutes of incubation, 3 µl of CD8-APC were added to the samples for the additional identification of the $CD8^+$-subpopulation. Finally, samples were washed twice with the FACS-buffer, and resuspended in 100 µl FACS-buffer for flow cytometrical analysis.

2.3.3.4 Endocytosis assay (FITC-dextran uptake)

Monocytes were isolated from PBMC and cultured with GM-CSF/IL-4, or GM-CSF/IL-4 plus IFN-α (500 U/ml) for 24 hours. Next, immature DCs (10^6 cells/ml) were harvested and incubated with FITC-dextran (0.5 mg/ml) for 37°C for two hours. Cells were counterstained with CD14-APC for 30 minutes in the dark. Finally, cells were washed extensively with PBS. FITC-dextran uptake was analysed by flow cytometry and quantified as mean fluorescence intensity. Unspecific FITC-dextran uptake was assessed by incubating cells for two hours on ice.

2.3.3.5. Pinocytosis assay (Panc-tumor-cells uptake)

Pancreas tumour cells (Panc-cells) were detached from tissue culture flasks with 0.2% EDTA (ethylene diamine tetraacetic acid) for 5 minutes at 37°C and washed extensively with PBS. Panc-cells (5 x 10^6/ml) were incubated with CFSE (10 µM) for 20 minutes in the dark. Cells were washed twice and re-suspended in culture media. Next, panc-cells were incubated in a water bath at 43°C for two hours. Cells were transferred to tissue culture flasks and incubated overnight at 37°C.

Monocytes were prepared from PBMC and incubated with either GM-CSF/IL-4, or GM-CSF/IL-4 plus IFN-α (500 U/ml) for 24 hours. Panc-cells (10^6/ml) were incubated with immature DCs (10^6/ml) at 37°C for two hours. Next, cells were counterstained with MHCII-PerCP. Uptake of panc-cells was analysed by flow cytometry and quantified by mean fluorescence intensity. Unspecific panc-cells uptake was assessed by incubating cells on ice for two hours.

2.3.4. Cell migration assay

DCs migrate upon maturation, expressing the chemokine receptor CCR7, to the secondary lymphoid organs in response to 6Ckine, secreted by the T-lymphocytes. To analyse the *in vitro* chemotactical response of DCs, a cell migration assay was performed. To this end, transwell inserts (diameter: 6.5 mm; pore size: 5.0 µm; tissue culture treated) were used, across which $CCR7^+$ -DCs migrate through the pores of a membrane in response to 6Ckine found in the lower compartments of the well plates (diagram 1).

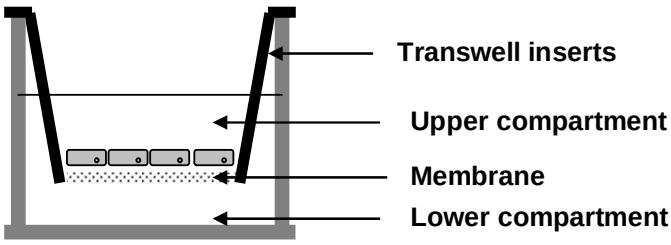


Diagram 2 – Cell migration assay

To this end, transwell inserts were placed in 24-well plates. 600 µl of DC-media alone or supplemented with 6CKine (100 ng/ml) were added in the lower chambers of 24-well plates. Next, freshly prepared mature *Fast*DCs (2 x 10^4 cells/ml) were loaded in the transwell inserts and incubated for 2 hours at 37°C. Following incubation, DCs were harvested and concentrated to a volume of 50 µl. Cells were counted with a hemocytometer. All assay conditions were triplicated.

2.3.5. Induction of antigen-specific T-cell responses

2.3.5.1. Co-culture of FastDC with autologous T-cells

Autologous T-cells were isolated from the non-adherent fraction of the PBMC using the Pan T Cell isolation kit (Miltenyi Biotech, B-Gladbach) by negative selection of MACS following the manufacturer's instructions, and were incubated with culture media containing IL-2 (10 U/ml) and IL-7 (10 ng/ml), which was replaced every 2 days. *Fast*DCs were prepared from healthy HLA-A*0201 donors according to the protocol described above and pulsed with Melan-A/MART-1 (10 µM) or left unloaded during the last four hours of maturation. Next, DCs were harvested and washed extensively, and co-cultured with autologous T cells at a ratio of 1:10. Culture media (including IL-2 and IL-7) was replaced every two days. Re-stimulations were performed weekly for two weeks with freshly prepared unloaded or peptide-pulsed *Fast*DCs. CTL were harvested five days after the second re-stimulation and used in the standard ^{51}Cr release assay.

2.3.5.2. Radiolabeling of target cells

HLA-A2 positive T2 cells, obtained from the American Type Culture Collection (ATCC CRL-1992), were used as target cells in the ^{51}Cr release assay. T2 cells were incubated with either the cognate or control peptide (MelanA-MART1 or HIV-pol, both at a concentration of 10 µM) for four hours at 37°C. Next target cells were radiolabeled with 100 µCi $Na_2{}^{51}Cr\ O_4$ for 1 hour at 37°C. Target cells were washed four times and adjusted to a concentration of $3x10^3$ cells/ml.

2.3.5.3. Standard ^{51}Cr release assay *****

The chromium release assay was used to determine the lytic potential of cytotoxic T cells (effector cells) activated by mature *Fast*DCs loaded with MelanA-MART-1 (melanoma-associated antigen most frequently recognised by tumour-reactive CTL derived from HLA A*0201 melanoma patients). This assay is based on the antigen-specific recognition and lysis of target cells, previously radiolabeled with ^{51}Cr and loaded with MelanA-MART1. The Cr released in the supernatant, proportional to the lysis of target cells, is measured in a gamma-counter.

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* (in cooperation with Prof. Dr. R. Wank, Department of Immunonlogy, Ludwigs-Maximilian-Universität, Munich).

Next, 100 µl of CTL were added at various effector: target cells (E: T ratios ranging from 80:1 to 20:1) to 100 µl of target cells in 96-well round bottom plates. After four hours of incubation at 37°C, 50 µ of the culture supernatant was harvested and counted in a gamma-counter. The maximum Cr release was determined by the addition of 10% Triton X and spontaneous release was assessed by adding complete media to target cells without effector cells. The percentage specific lysis was calculated with the formula: [(experimental counts – spontaneous counts)/(maximal counts – spontaneous counts)] x 100 %. All assay conditions were triplicated.

2.3.6. Statistical analysis

Data were presented as arithmetical mean + SEM (standard error of the mean). The statistical significance was determined by means of the paired student t-test. Differences were considered statistically significant for $p < 0.05$ (represented by asterisks in bar charts).

3. Results

3.1. Influence of interferon-alpha on *Fast*DC differentiation, terminal maturation and antigen uptake

3.1.1 Differentiation and terminal maturation

Survival and maintenance of a mature phenotype after withdrawal of cytokines and growth factors are essential for the use of DC preparations in vaccination protocols. Standard monocyte-derived DCs (Feuerstein et al, 2000) as well as *Fast*DCs (Dauer et al, 2006) survive for up to five to six days in wash-out cultures without further replacement of cytokines or growth factors. ere, we analysed the influence of IFNα on the expression of maturation markers in *Fast*DCs in wash-out cultures. To this end, monocytes were incubated either with GM-CSF and IL-4, or with GM-CSF and IL-4 plus IFN-α. After 24 hours, maturation was induced by incubation with PGE_2, TNF-α, and IL-1β for further 24 hours. The maturational stage was determined by assessing the phenotypical expression of the cell surface molecules CD83, CD80, CD14 and MHC II at 24, 48, 76 and 96 hours after stimulation, without further replacement of cytokines or growth factors.

As shown in figure 1, a fully mature phenotype ($CD83^{+}CD80^{+}MHC^{High}CD14^{-}$) was exhibited 24 hours after maturation in unprimed or IFN-α–primed-*Fast*DCs. Without further cytokine supplementation, CD80 and MHCII expression persisted up to 76h after maturation whereas the expression of CD83 prevailed up to 48h, and was downregulated thereafter. No significant difference was observed in the pattern of expression of CD83, CD80, and MHC II in *Fast*DCs treated with IFN-α. Likewise, independent of IFN-α-treatment, a marginal up-regulation of CD14 in *Fast*DC was observed 96 hours following maturation.

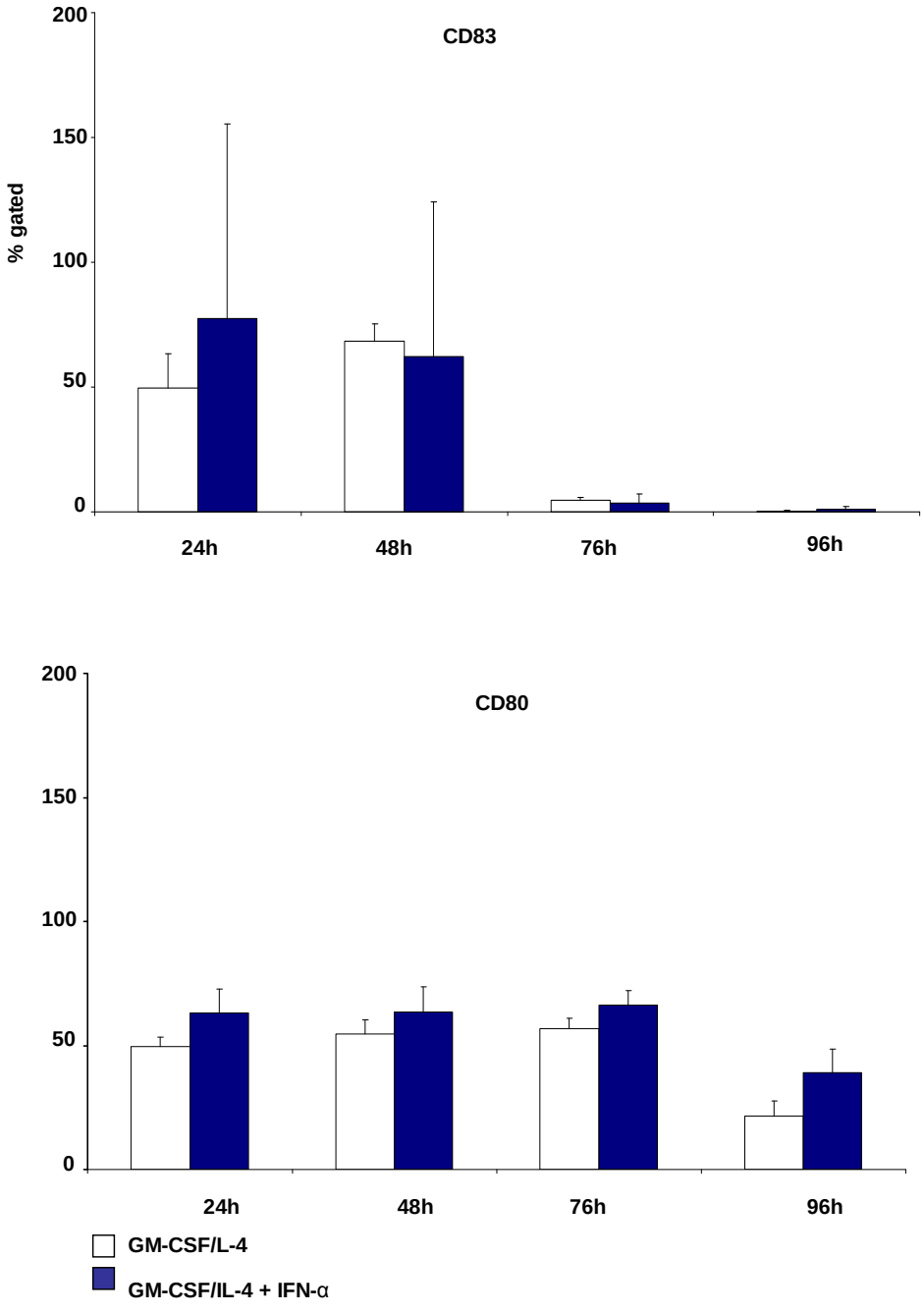


Fig. 1: *Fast*DCs maintain mature immunophenotype and are CD14$^-$ in wash-out cultures. Monocytes were cultured 24 hours with either GM-CSF/IL-4 or GM-CSF/IL-4+IFN-α, and further matured for 24 hours with pro-inflammatory mediators PGE_2, TNF-α, and IL-1β. 24h, 48h, 76h and 96h after addition of pro-inflammatory mediators, *Fast*DCs were analysed by flow cytometry.

3.1.2 Antigen uptake

3.1.2.1 Uptake of FITC-labeled dextran

Antigen uptake, process and presentation in the context of MHC molecules are hallmarks of immature DCs. The uptake of FITC-labeled dextran (dextran is a polymer of anhydroglucose) occurs via receptor-mediated endocytosis, similar to the uptake of exogenous antigens. To this end, we examined the uptake of FITC-labeled dextran after culture with GM-CSF/IL-4 or GM/IL-4 plus IFN-α for 24 and 48 hours. Next, unprimed immature *Fast*DCs and IFN- α-primed immature *Fast*DCs were incubated for 30 minutes at 37°C or on ice (representing the negative control) and quantified as mean fluorescence intensity by flow cytometry.

Fig. 2 shows that supplementation with IFN-α left the endocytic capacity of immature *Fast*DCs unaffected. A 3-fold increase in FITC-dextran uptake was measured in immature DCs cultured for 48 hours as compared with 24 hours.

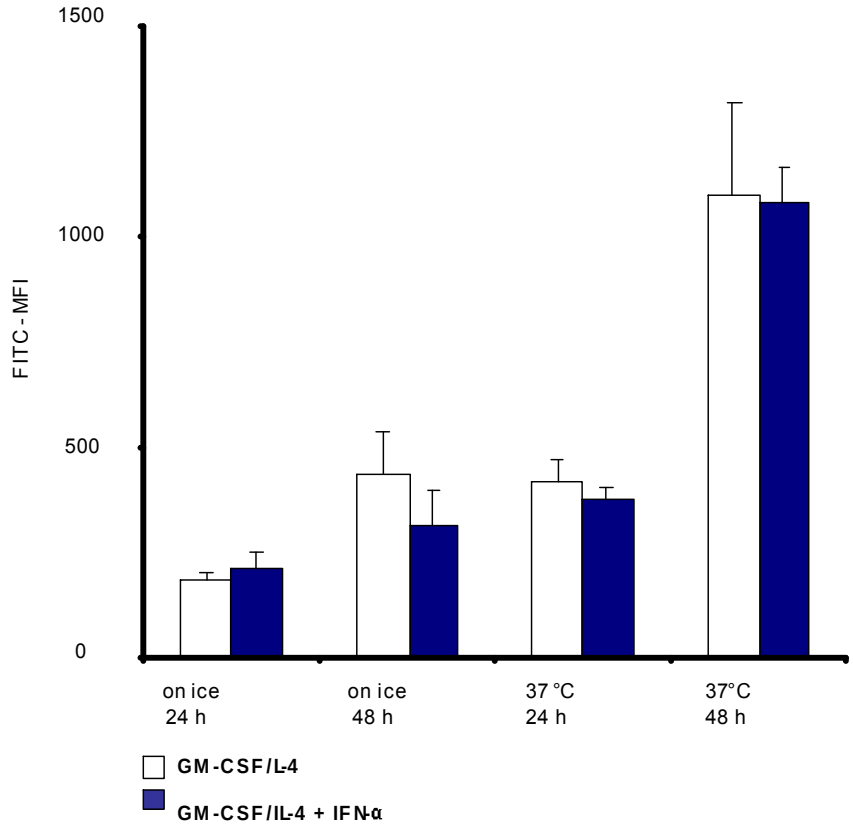


Fig. 2: Endocytosis of FITC-dextran by immature *FastDC*. Monocytes isolated from peripheral blood were differentiated for 24 hours or 48 hours with GM-CSF/IL-4 or GM/IL-4 plus IFN-α. Unprimed or IFN-α-primed immature DCs were incubated for 30 minutes with FITC-dextran, at 37°C or on ice (negative controls). The uptake of FITC-dextran was quantified as mean fluorescence intensity (MFI) by FACS-analysis.

3.1.2.2 Uptake of CFSE-labeled apoptotic pancreatic carcinoma cells by FastDC

DCs have been reported to take up apoptotic tumour cells or cell fragments, so called apoptotic bodies, and subsequently present tumour antigens to T cells. To this end, the ability of *Fast*DCs to take up and process antigens from apoptotic pancreatic tumour (PANC1-) cells was investigated. As shown in figure 3, unprimed as well as IFN-α-primed immature *Fast*DCs were equally able to take up CFSE-labeled apoptotic pancreatic tumour cells.

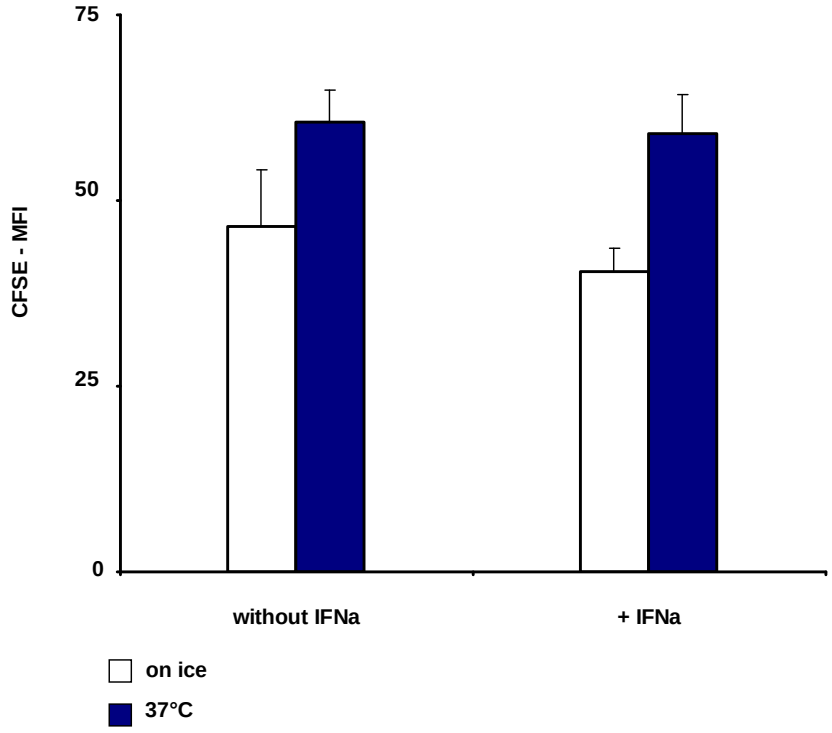


Fig. 3: Endocytosis of CFSE-labeled apoptotic pancreatic tumour cells in immature *FastDC*. Monocytes isolated from peripheral blood were cultured with GM-CSF/IL-4 or GM-CSF/IL-4 plus IFN-α for 24 hours. Immature *Fast*DC were then incubated for two hours with CFSE-labeled apoptotic pancreatic tumour cells, at 37°C or on ice (negative control). The uptake was analysed by flow cytometry, and measured as MFI.

Taken together, in this first set of experiments we were able to show that *Fast*DCs are terminally differentiated DCs maintaining a mature phenotype even after withdrawal of cytokines and growth factors, and are capable of efficiently taking up soluble and whole cell-derived antigens. Supplementation of *Fast*DC cultures with IFN-α influenced none of these functions significantly and can thus be omitted from the *Fast*DC generation protocol.

3.2. Optimisation of the *Fast*DC protocol: use of toll-like receptor agonists for stimulation

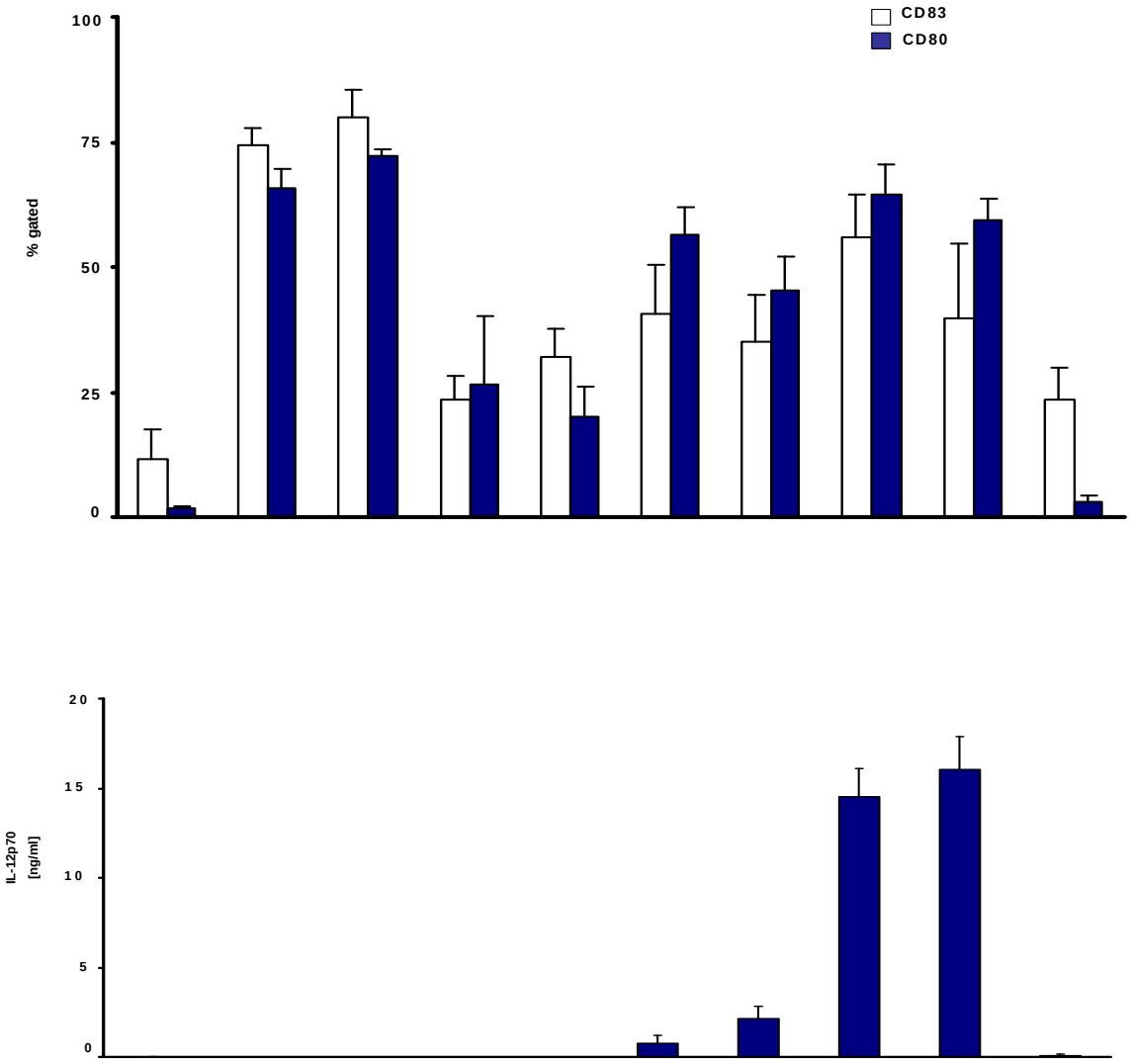
*Fast*DCs matured with pro-inflammatory mediators expressed a fully mature phenotype and migrated in response to 6Ckine, but showed inefficient secretion of IL-12p70, which is essential for the promotion of a T_H1 response.

The aim of the following experiments was thus to investigate the potential role of TLR agonists in the stimulation of *Fast*DCs and their effects on maturation, cytokine production, migration and T cell activation. To this end, a combination of TLR agonists, namely LPS and R848, as well as the additional influence of the T-cell derived signals CD40L and IFN-γ, was investigated in *Fast*DCs.

*3.2.1. Fast*DC *immunophenotype and IL-12p70 synthesis upon TLR-based activation*

DC maturation is a pre-requisite to induce efficiently antitumour cytotoxic T cell responses, while IL-12p70 has been demonstrated repeatedly to be necessary for promoting a T_H1 response. In the following experiment, we analysed the effects of the different stimuli mentioned below, alone or in combination, on the immunophenotype of *Fast*DCs. Isolated monocytes were cultured with GM-CSF/IL-4 for 24 hours according to the *Fast*DC protocol, followed by maturation for another 24 hours. Maturational status, as characterised by the up-regulation of CD83 and CD80, was assessed by FACS analysis. The supernatants were collected for the measurement of IL-12p70 by ELISA.

Fig. 4 shows that stimulation with the pro-inflammatory mediators PGE_2/TNF-α/IL-1β led to a greater up-regulation of maturation markers as compared to TLR agonists used alone or in combination. R848 was found to be a moderate inducer of maturation, whereby additional stimulus with either IFN-γ, CD40L or LPS did not raise its potential to up-regulate CD80 or CD83. As already known, *Fast*DCs treated with PGE_2/TNF-α/IL-1β failed to induce the synthesis of IL-12p70. Similarly, LPS-treated *Fast*DCs were deficient in IL-12p70. Although stimulation with R848 alone, or in combination with IFN-γ, could trigger IL-12p70 secretion, cytokine secretion was enhanced when R848 was used in combination with CD40L and IFN-γ; IL-12p70 secretion could be even maximised upon additional stimulation with LPS.



GM-CSF/IL-4	+	+	+	+	+	+	+	+	+	+
PGE_2/TNF-α/IL-1β	-	+	+	-	-	-	-	-	-	-
LPS	-	-	-	+	+	-	-	-	+	-
R848	-	-	-	-	-	+	+	+	+	-
IFN-γ	-	-	+	+	+	-	+	+	+	+
CD40L	-	-	+	-	+	-	-	+	+	+

Fig. 4: Immunophenotype after maturation with either pro-inflammatory mediators or TLR-derived signals in *Fast*DC. Monocytes were isolated from PBMC, and incubated 24 hours with GM-CSF/IL-4, and matured subsequently for another 24 hours with different combinations of stimuli as shown above. The control represents monocytes incubated with GM-CSF/IL-4 for 48 hours only. Expression of surface molecules was determined by FACS analysis. The secretion of IL-12p70 was measured by ELISA.

3.2.2. Migration and CCR7 expression by FastDC upon TLR-based activation

In addition to the expression of maturation markers and secretion of functional IL-12, we investigated the migratory capacity and CCR7 expression by TLR-activated *Fast*DCs.

As shown in figure 5, the highest migratory potential in response to the CCR7 ligand, 6Ckine, was observed in *Fast*DCs stimulated in the presence of PGE_2 (so-called "conventional *Fast*DC") and could not be enhanced by additional T-cell derived signalling. In contrast, only minimal migration could be observed in TLR-activated *Fast*DC (<15% vs. > 50% in conventional *Fast*DCs). Less than 5% of the *Fast*DCs subsets migrated spontaneously in the absence of 6Ckine. Monocytes incubated with only GM-CSF and IL-4 did not show migration in response to 6Ckine.

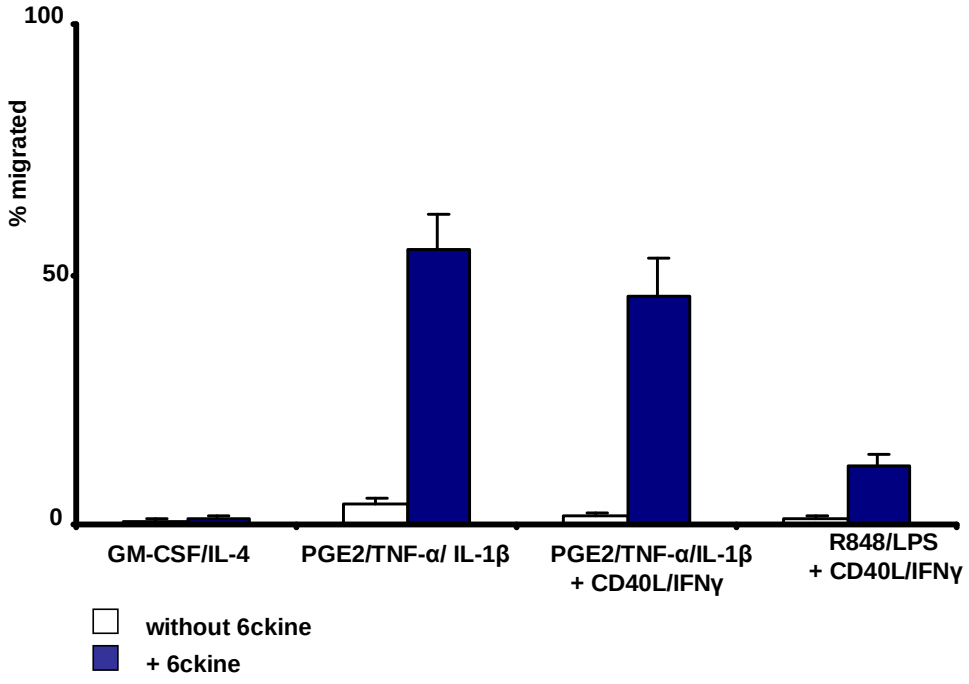


Fig.5: Migration in response to 6ckine in differently matured *Fast*DC. Monocytes were isolated from PBMC, stimulated 24 hours with GM-CSF/IL-4, and subsequently matured as indicated above After maturation, the *Fast*DCs were analysed in a transwell system for migration in response to a 6CKine gradient.

3.2.3. PGE_2 and TLR-based FastDC

Next, we examined the correlation between the different modes of activation on CCR7 expression and migratory response to 6Ckine. Furthermore, we analysed the influence of PGE_2 on the migration and secretion of IL-12p70 in TLR-stimulated *Fast*DCs.

As shown in figure 6, phenotypical maturation and CCR7 expression were achieved under all conditions, although the highest level of CCR7 expression could be observed in *Fast*DCs stimulated with TLR agonists, T cell-derived signals and PGE_2. Irrespective of CCR7 expression, no migration was observed in TLR-treated *Fast*DCs. However, upon supplementation of TLR-derived *Fast*DCs with PGE_2, a higher percentage of migration could be induced in comparison to conventional *Fast*DCs matured with PGE_2/TNF-α/IL1β. With respect to IL-12p70 secretion, consistent with previous results, IL-12p70 deficiency was observed in conventional *Fast*DC whereas high levels of IL-12p70 were measured in TLR-activated *Fast*DCs. Surprisingly, the addition of PGE_2 to TLR ligands restored the migratory capacity of *Fast*DC without affecting the high secretion of IL-12p70 (figure 7).

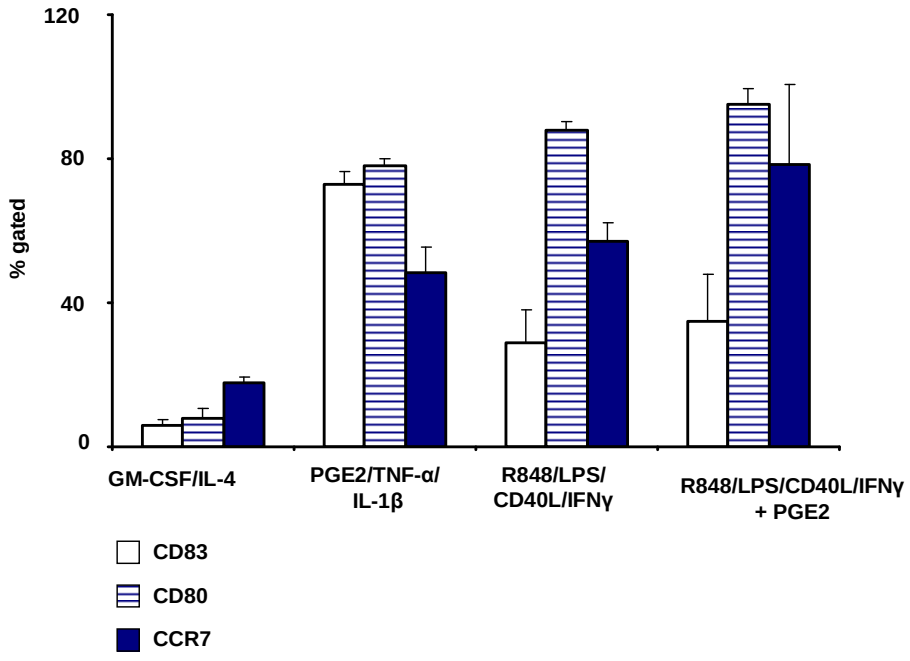


Fig.6: *Fast*DC after maturation with either pro-inflammatory mediators, TLR-derived signals or TLR-derived signals plus PGE_2. Monocytes were isolated from PBMC, stimulated 24 hours with GM-CSF/IL-4, and subsequently matured for 24 hours as indicated above. Immunophenotype was analysed by flow cytometry.

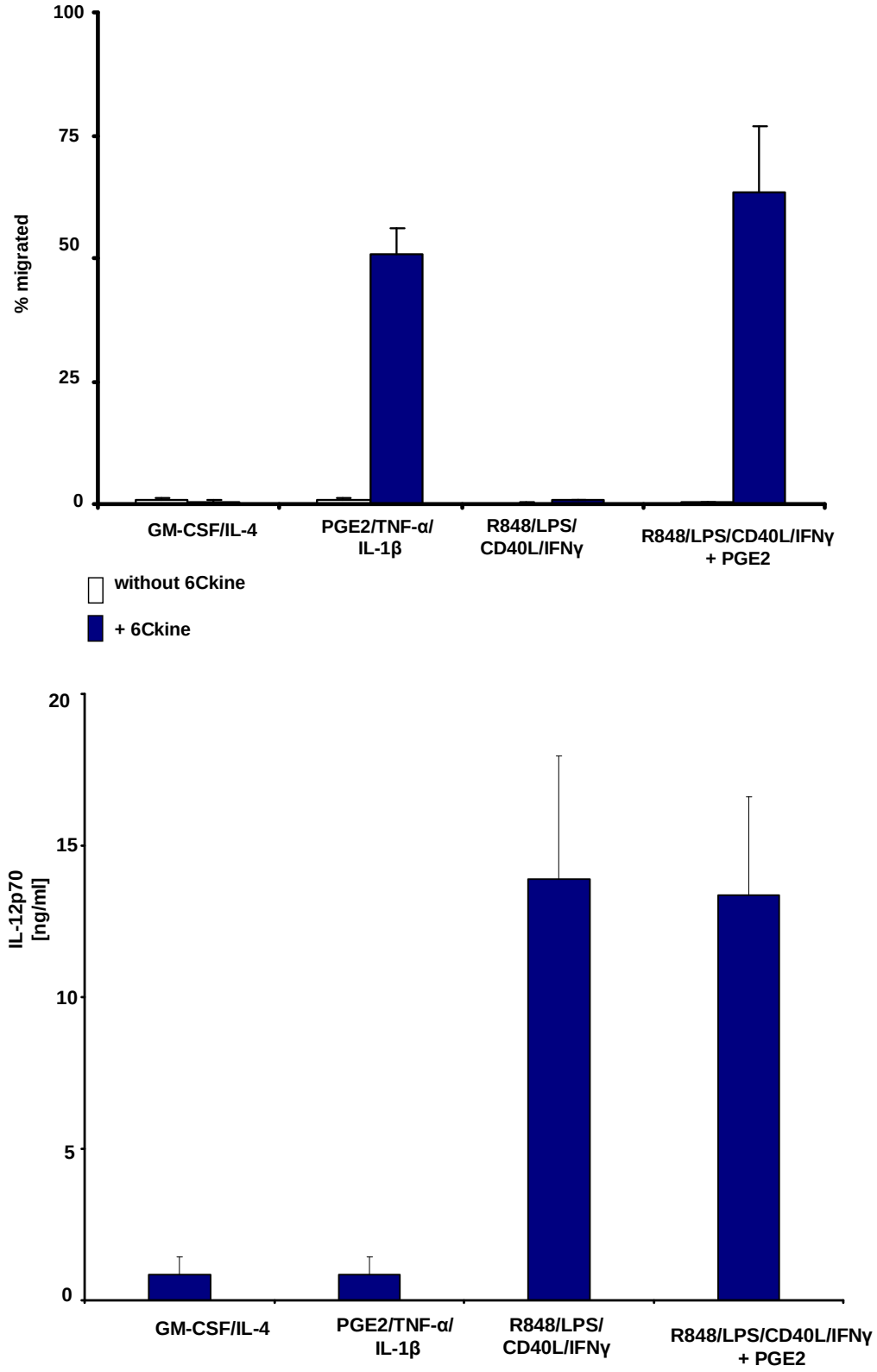


Fig.7: *Fast*DC after maturation with either pro-inflammatory mediators, TLR-derived signals or TLR-derived signals plus PGE_2 . Monocytes were isolated from PBMC, stimulated 24 hours with GM-CSF/IL-4, and subsequently matured for 24 hours as indicated above. After maturation, *Fast*DCs were incubated for 2 hours, either with 6Ckine, or with DC-media. Migration was analysed by the cell migration assay. The supernatant was collected for measurement of IL-12p70 by ELISA.

Taken together, although conventional *Fast*DCs matured with PGE_2/TNF-α/IL-1β showed the highest degree of phenotypical maturation and the highest migratory potential, they completely failed to secrete IL-12p70. On the other hand, the combined maturation with TLR agonists and T-cell derived signals resulted in *Fast*DCs exhibiting an inferior degree of phenotypical maturation, yet inducing very high levels of IL-12p70 secretion. In the absence of PGE_2, TLR-activated *Fast*DCs did not migrate in response to 6Ckine despite high levels of CCR7 expression. This specific defect was overcome by the addition of PGE_2 without impeding the capacity for secretion of functional IL-12p70.

3.2.4. Generation of multifunctional FastDCs using pro-inflammatory mediators in combination with TLR agonists

The next experiments were performed to investigate on the influence of T-cell derived signals as well as PGE_2 on TLR-based *Fast*DCs with respect to immunophenotype, CCR7 expression, and migration as well as IL-12p70 secretion.

As shown in figure 8, in the absence of T-cell derived signals, CD40L and IFN-γ, CCR7 expression was up-regulated in *Fast*DCs stimulated with R848 and LPS. CCR7 expression could be further enhanced in the presence of PGE_2. Likewise, the migratory capacity of *Fast*DCs matured with R848 and LPS was restored upon supplementation with PGE_2, independent of T-cell signalling (figure 9). However, though PGE_2 did not abort IL-12p70 secretion in TLR-stimulated *Fast*DCs, IL-12p70 secretion was maximised in TLR-stimulated *Fast*DCs in the presence of CD40L and IFN-γ.

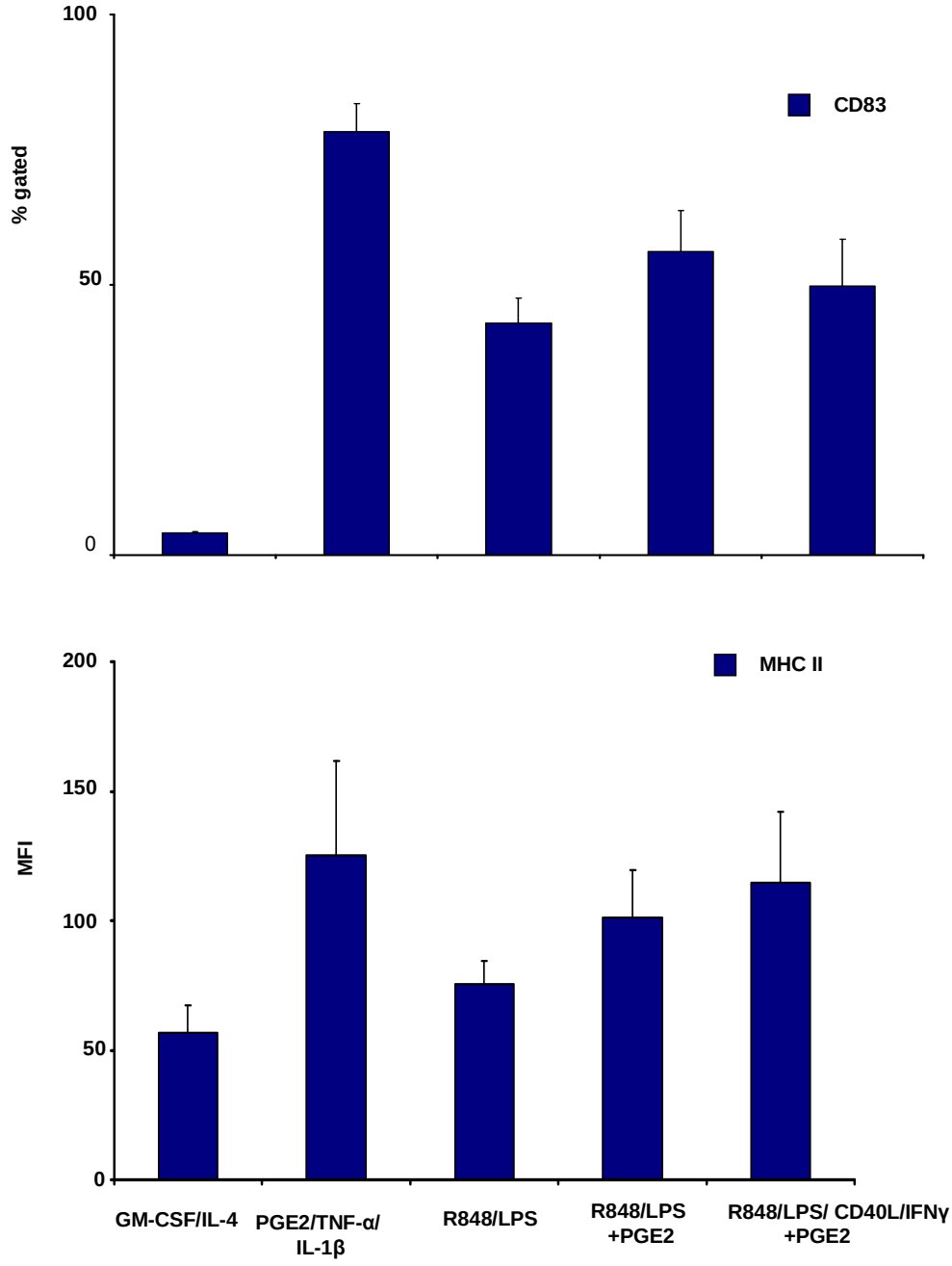


Fig. 8a: Up-regulation of co-stimulatory molecules and chemokine receptor CCR7 after maturation with different combinations of pro-inflammatory mediators, TLR-agonists and T cell-derived signals. Monocytes were isolated from PBMC, and incubated 24 hours with GM-CSF/IL-4, and matured for another 24 hours with the different combinations of stimuli as indicated. Immunophenotye was determined by FACs analysis. The control represents monocytes incubated with GM-CSF/IL-4 for 48 hours.

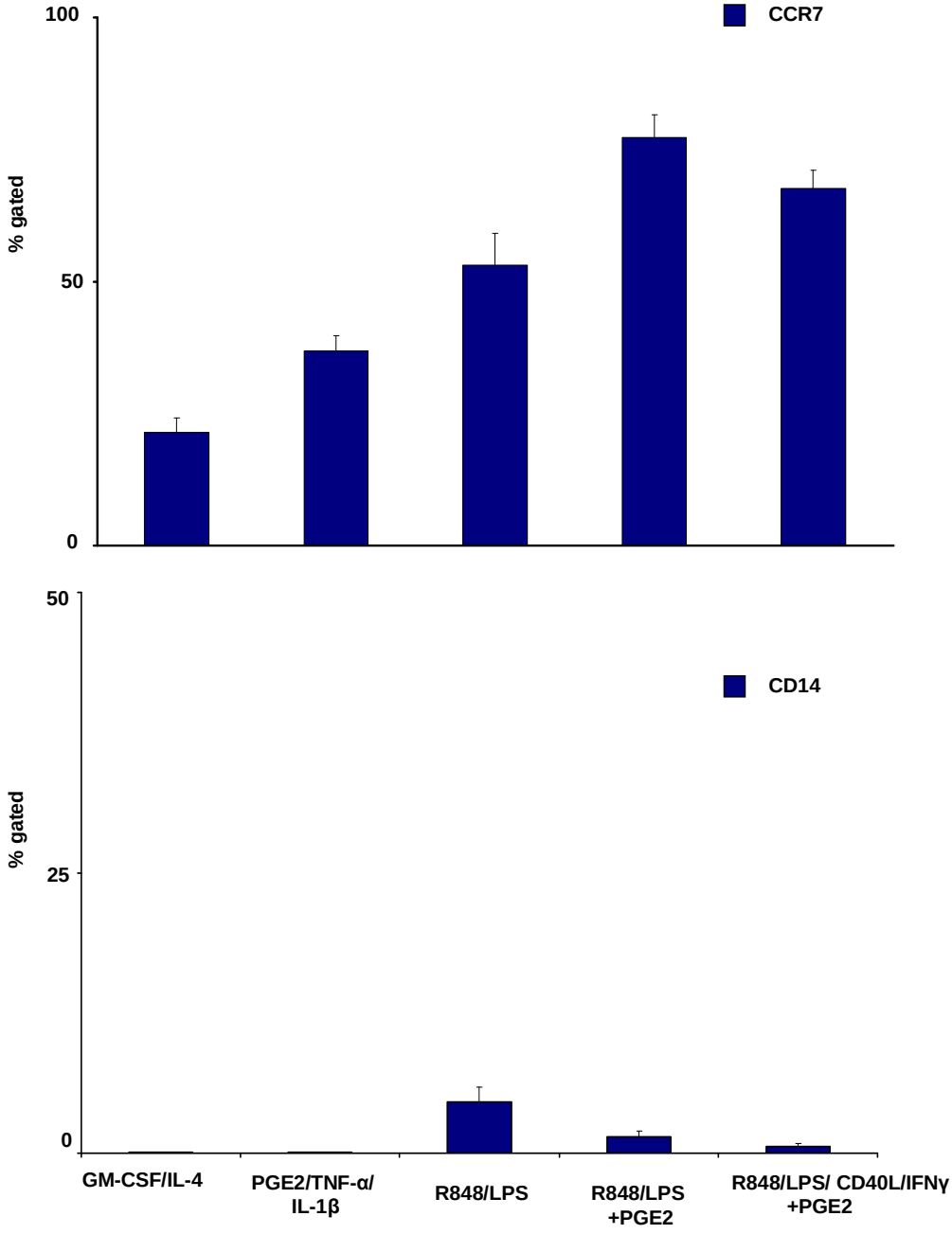


Fig. 8b: Up-regulation of co-stimulatory molecules and chemokine receptor CCR7 after maturation with different combinations of pro-inflammatory mediators, TLR-agonists and T cell-derived signals. Monocytes were isolated from PBMC, and incubated 24 hours with GM-CSF/IL-4, and matured for another 24 hours with the different combinations of stimuli as indicated. Immunophenotye was determined by FACs analysis. The control represents monocytes incubated with GM-CSF/IL-4 for 48 hours.

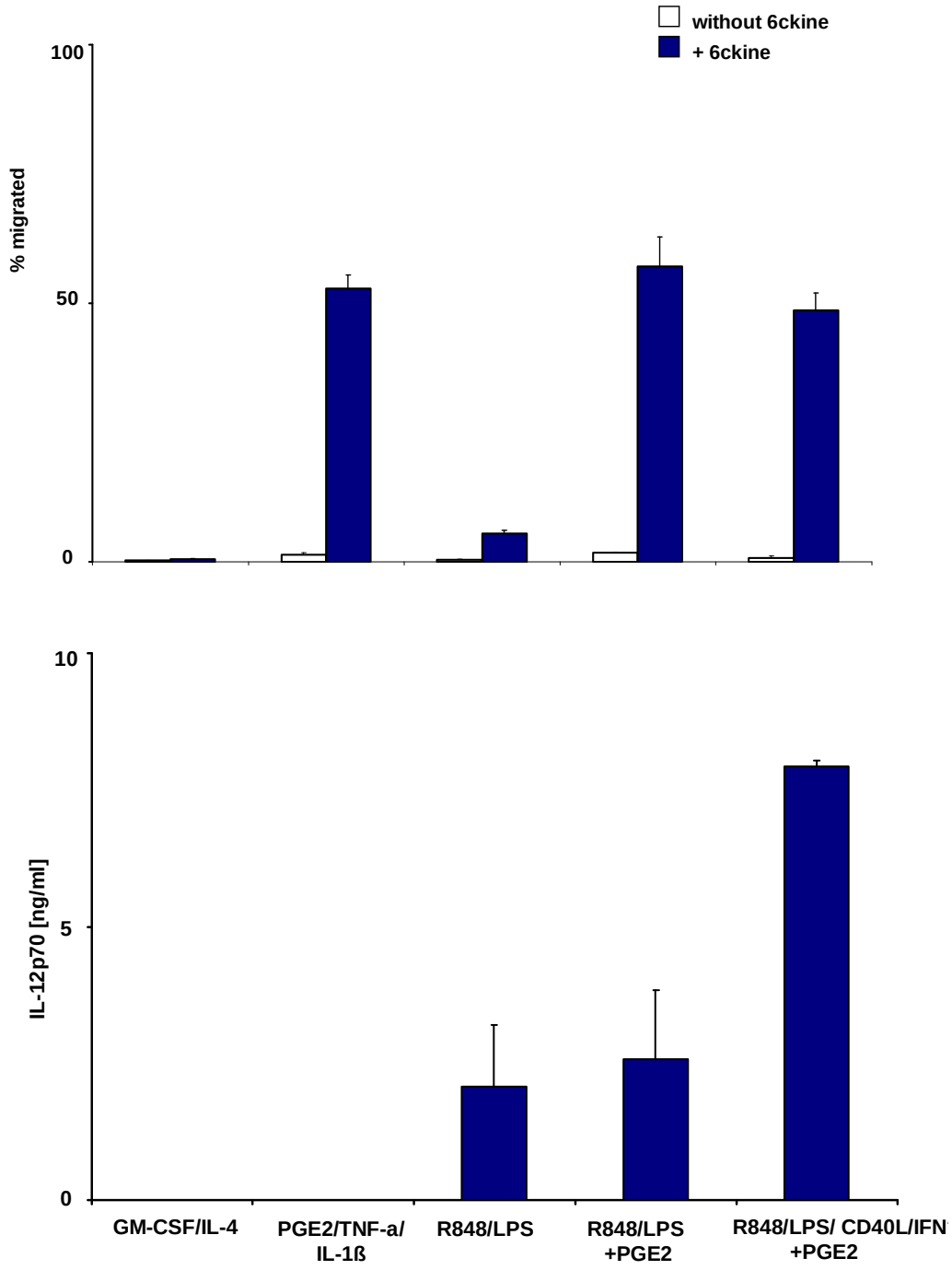


Fig. 9: PGE_2 restores migratory capacities of TLR-activated *Fast*DC without affecting IL-12p70 synthesis. Monocytes were isolated from PBMC, stimulated 24 hours with GM-CSF/IL-4, and matured as indicated above. After maturation, *Fast*DCs were incubated for 2 hours, either with 6Ckine, or with DC-media, and chemotaxis was analysed in a cell migration assay. The supernatant was collected for measurement of IL-12p70 by ELISA.

Taken together, the combination of LPS and R848 with PGE_2 resulted in the generation of multifunctional *Fast*DC capable of migration and IL-12p70 secretion. Migration was restored in the presence of PGE_2 in TLR-activated *Fast*DCs. IL-12p70 secretion was not aborted in the presence of PGE_2. Furthermore, the secretion of IL-12p70 could be maximised upon supplementation with T-cell derived signals, CD40L and IFN-γ.

3.3. Induction of antigen-specific T-cell responses by *Fast*DC

The induction of a T-cell mediated response is a critical requirement for the use DCs in anti-tumoural vaccination. The following experiments were performed to investigate and compare the efficiency of *Fast*DCs to induce a tumour antigen-specific T cell response upon activation with TLR agonists or pro-inflammatory mediators (as described in the "materials and methods" section) : lytic activity of cytotoxic T cells in a chromium release assay, measurement of the IFN-γ released in the co-cultures by ELISA, and the identification of $CD8^+$ binding specifically to fluorescently labeled MHC-I/peptide complexes (streptamers).

As shown in figure 10, TLR-activated *Fast*DC were readily capable of, but not superior to their conventional counterparts, in priming Melan-A-specific cytotoxic T cell responses *in vitro,* as detected by the chromium release assay using Melan-A-loaded T2 cells as target cells. The differently matured *Fast*DCs displayed equal target specificity: lytic activity was neither enhanced in co-cultures of unpulsed *Fast*DCs nor in cultures of T2 cells loaded with the irrelevant peptide HIV-pol.

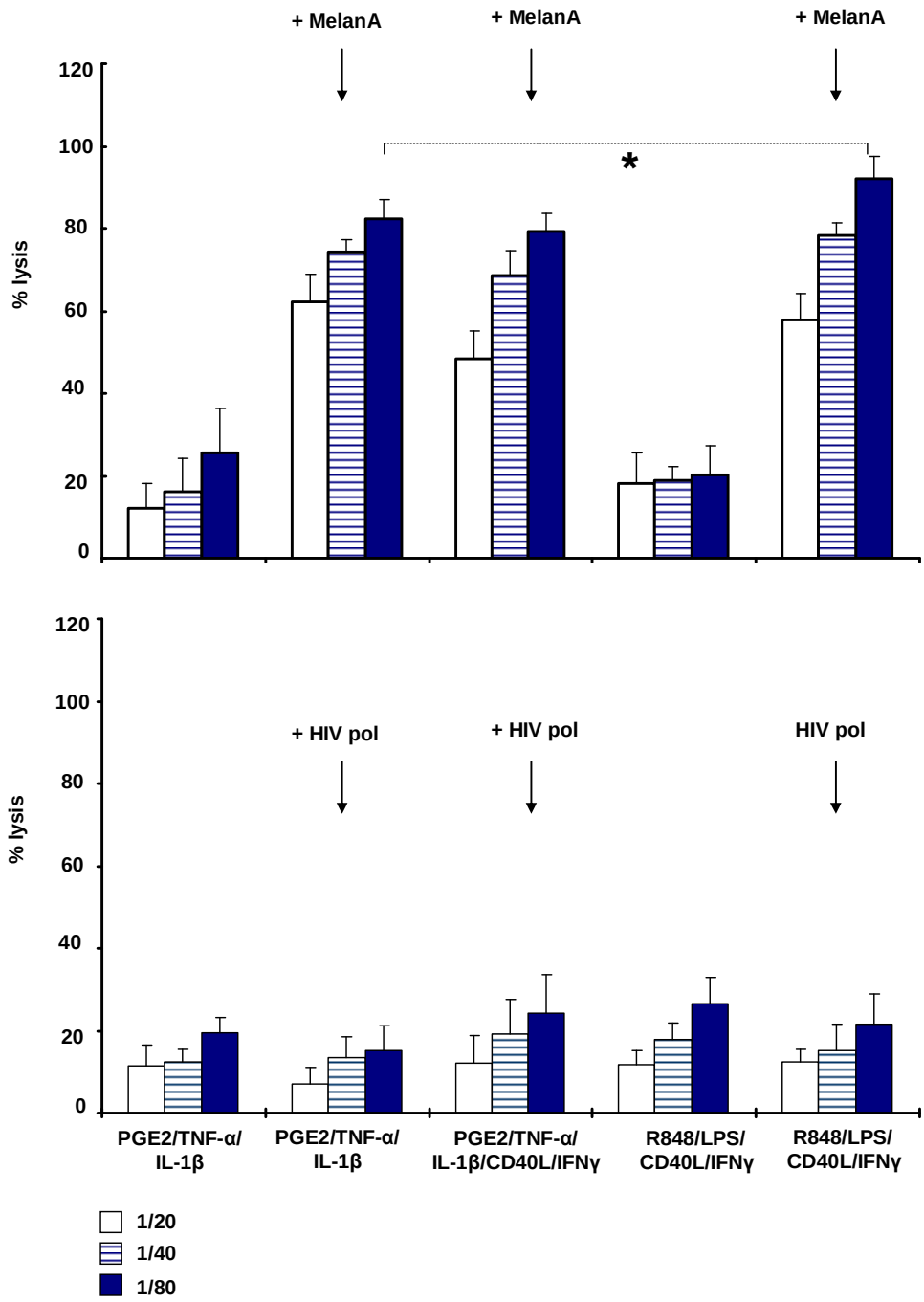


Fig. 10: Priming of Melan-A-specific cytotoxic T cells by differently matured *Fast*DC- A co-culture of autologous T cells with Melan-A-pulsed or unpulsed *Fast*DC stimulated with the above stimuli was performed. Five days after the second re-stimulation, T cells were harvested and incubated with ^{51}Cr-labeled T2 cells (at different ratios) loaded previously with Melan-A or the irrelevant peptide, HIV pol. Supernatants were collected four hours later for measurement of the Cr released specific lysis was calculated.

While abundant IFN-γ was detected in the supernatants of all cultures after the second re-stimulation with the different Melan-A-pulsed *Fast*DCs, very low levels or no IL-4 (T_H2 polarising cytokine) could be detected (Fig. 11 and 12). However, levels of IFN-γ in co-cultures with Melan-A-pulsed TLR-activated *Fast*DC were more than 2 times higher than those from co-cultures with conventional Melan-A-pulsed *Fast*DCs.

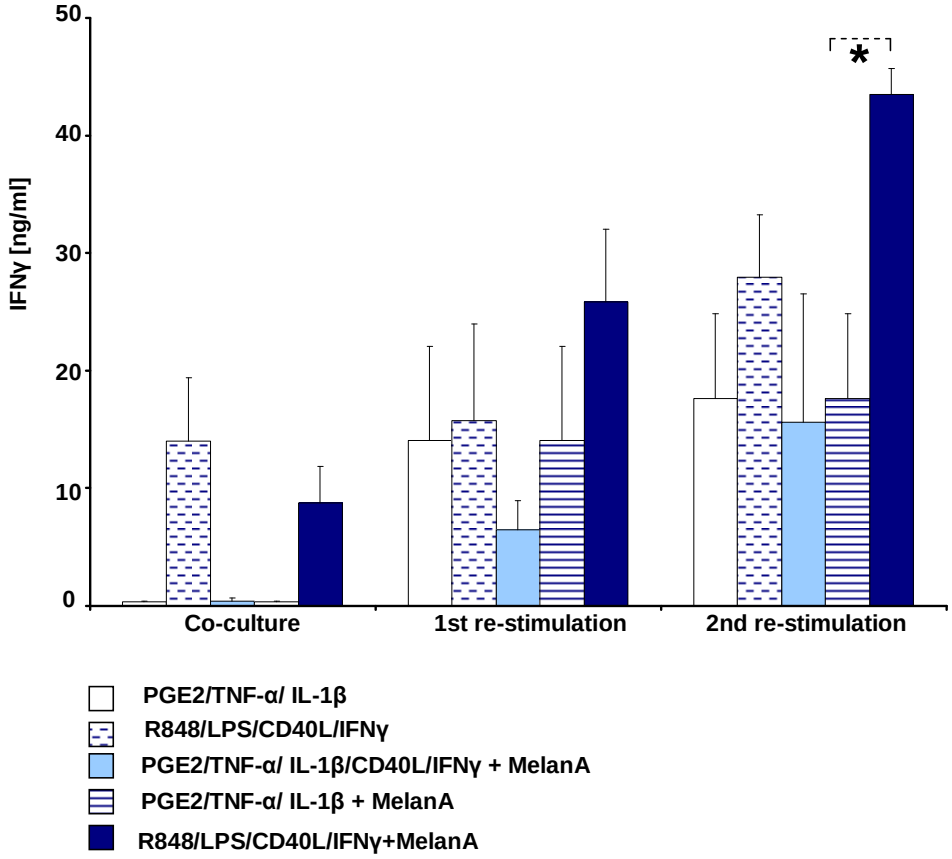


Fig. 11: IFN-γ secretion in co-cultures of autologous T cells with differently activated *Fast*DC. *Fast*DC generated as indicated were pulsed with Melan-A for 4 hours or left unpulsed, followed by the co-culture with autologous T cells. 2 days after each re-stimulation with freshly prepared *Fast*DCs, the supernatants were collected and the secretion of IFN-γ was quantified by ELISA.

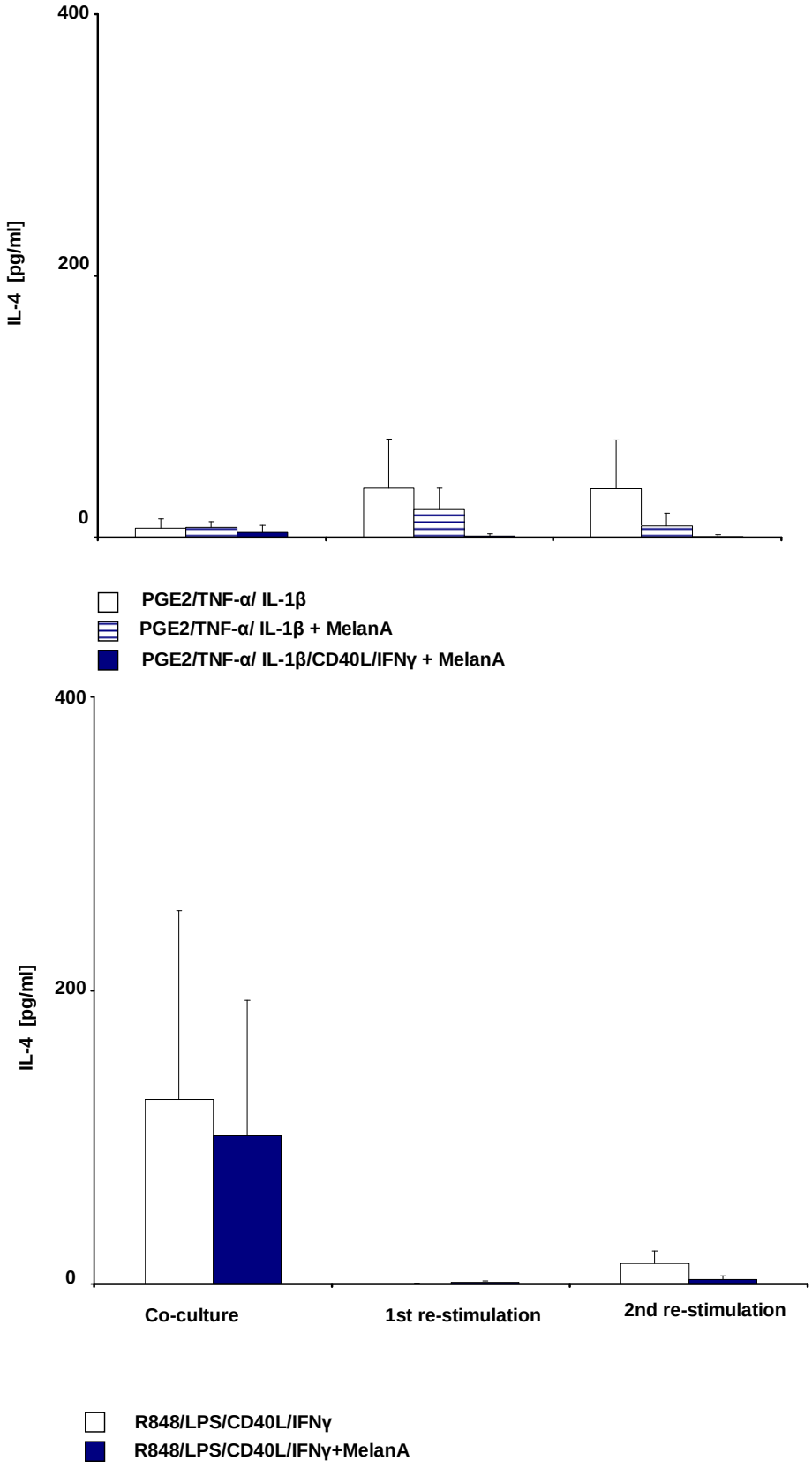


Fig. 12: IL-4 secretion in co-cultures of autologous T cells with differently activated *Fast*DC. *Fast*DC generated as indicated were pulsed with Melan-A for 4 hours or left unpulsed, followed by the co-culture with autologous T cells. 2 days after each re-stimulation with freshly prepared *Fast*DCs, the supernatants were collected and the secretion of IL-4 was quantified by ELISA.

Intracellular cytokine staining and analysis of MHC-I/Melan-A-peptide complexes binding of CD8+ T cells confirmed the activation of tumour-antigen specific CTL in co-cultures with either conventional or TLR-activated *Fast*DCS : Intracellular staining of IFN-γ in $CD8^+$ T cells was detected in both TLR-stimulated and conventional Melan-A-loaded *Fast*DCs. The frequency of MHC-I/Melan-A peptide complexes binding to $CD8^+$ in the flow cytometric analysis was higher, though not significantly, in the co-cultures of TLR-stimulated Melan-A-pulsed *Fast*DCs as compared with the conventional counterparts (Fig. 13 and 14).

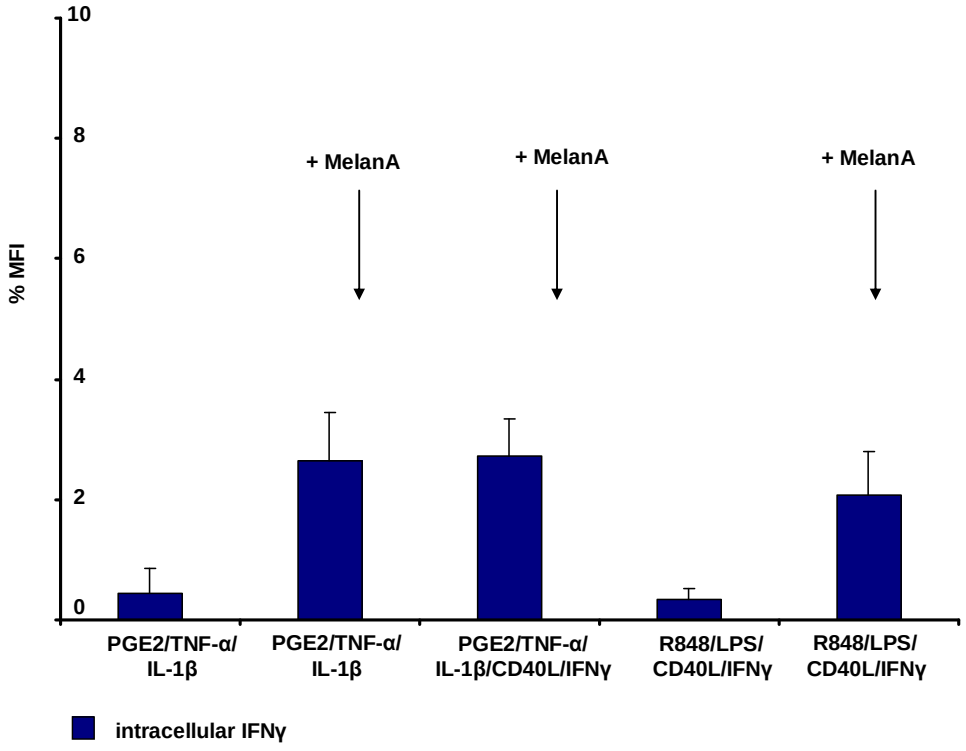


Fig. 13: Intracellular IFN-γ-staining in $CD8^+$ T cells of co-cultures with the different *Fast*DC preparations. Differently matured *Fast*DCs were co-cultured with autologous T cells, followed by two successive re-stimulations with newly generated unpulsed *Fast*DC or *Fast*DC pulsed with Melan-A. Two days after the second re-stimulation, intracellular IFN-γ-staining of $CD8^+$ T cells from the different co-cultures as described in the "materials and methods" section was performed and analysed by flow cytometry.

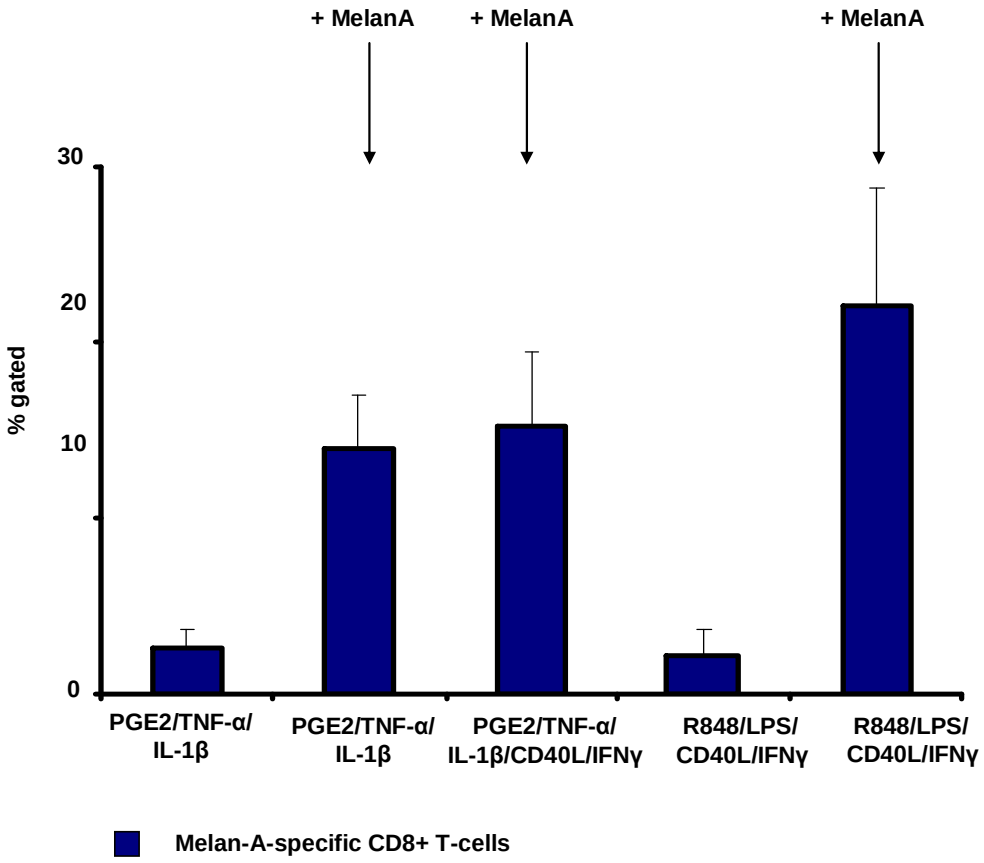


Fig. 14: MHC-I/Melan-A-peptide complexes binding of CD8$^+$ T cells after co-cuture with the differently activated *Fast*DC. After co-culture and two re-stimualtions of autologous T cells with the differently activated *Fast*DC, binding of CD8$^+$ T cells to MHC-I/Melan-A-petide complexes was determined by FACS analysis. T cells from the co-culture with unloaded *Fast*DCs either stimulated with pro-infalmmatory mediators or TLR- ligands served as controls.

As expected, relevant levels of IL-12p70 secretion could only be detected in co-cultures with TLR-activated *Fast*DCs, despite comparable activation of antigen-specific T cell responses in conventionally activated *Fast*DCs (figure 15).

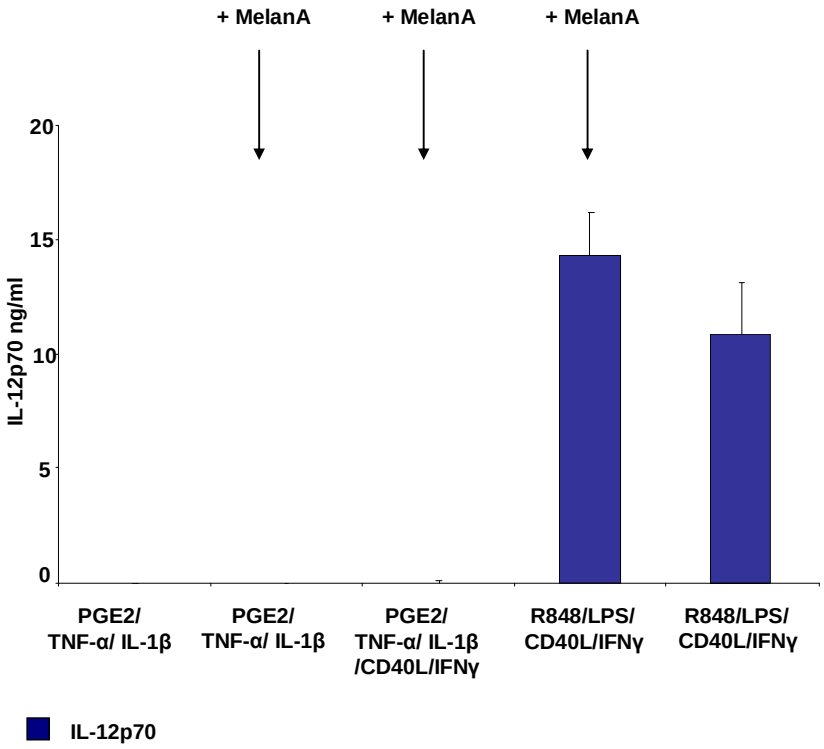


Fig. 15: IL-12p70 secretion in *Fast*DC in the differently activated *Fast*DC. Monocytes were isolated from PBMC, and incubated 24 hours with GM-CSF and IL-4, and matured subsequently for another 24 hours with the different combinatory stimuli.

Taken together, in the set of experiments described above, TLR-activated *Fast*DCs showed equal capacity to prime tumour antigen-specific CTL responses while having a higher potential to elicit a T_H1 immune response as compared to their conventional counterparts. While no significant differences in the level of unspecific T cell-activation was observed, higher levels of IFN-γ secretion could be detected in co-cultures of autologous T cells with TLR-activated *Fast*DCs, in accordance with their unique capacity to secrete high levels of IL-12p70.

4. Discussion

4.1. Summary of the results

With regard to the positive effects of IFN-γ on DC differentiation and function reported in the literature, we first investigated whether the addition of IFN-α to GM-CSF/IL-4 during the differentiation of *Fast*DCs from monocytes influenced terminal differentiation, survival or the capacity for antigen uptake and could detect no significant effects. *Fast*DCs maintained mature immunophenotype in wash-out cultures and readily took up soluble as well as cell-derived antigens independent of additional supplementation with IFN-α.

The main objective of the second part of this doctoral thesis was to modify the *Fast*DC protocol in order to generate DCs fulfilling all the criteria required to improve the efficiency of DC vaccines:

- mature immunophenotype
- migration in response to 6Ckine
- secretion of high levels of IL12-p70
- ability to prime effectively antigen-specific cytotoxic T cells

To this end, we tried to overcome the specific defect of the so-called conventional *Fast*DC activated with the pro-inflammatory mediators PGE_2/TNF-α/IL-1β to secrete functional IL-12p70 by activation with the TLR agonists R848 and LPS, either used alone or in combination with the T cell-derived stimuli CD40L and IFN-γ.

In comparison with TLR-activated *Fast*DCs, the conventional maturation cocktail was found to trigger the highest expression of co-stimulatory molecules, despite the inefficacy in IL-12p70 secretion. In contrast, TLR agonists, alone or in combination, could not induce complete mature immunophenotype, unless used in combination with T-cell derived signals, CD40L and IFN-γ. High levels of IL-12p70 were produced by *Fast*DCs stimulated with TLR-agonists used alone or in combination, which could be maximised in *Fast*DCs after the combined use of TLR7/8 and TLR4-agonists, together with CD40L and IFN-γ. TLR-activated *Fast*DCs expressed equal levels of CCR7 as compared to conventional *Fast*DCs. However, irrespective of CCR7 expression and semi-mature phenotype, they failed to migrate towards

6Ckine, unless supplemented with PGE_2. Importantly, elevated levels of IL-12p70 could still be induced in the presence of PGE_2 in TLR-derived *Fast*DCs.

Irrespective of the mode of activation, *Fast*DCs were capable of inducing the antigen-specific activation and expansion of $CD8^+$ T cells. However, CTLs derived from co-cultures with TLR-stimulated *Fast*DCs appeared to be moderately superior in priming $CD8^+$ T-cells. CTLs primed by conventional or TLR-stimulated *Fast*DC displayed equal target specificity. Importantly, multifunctional *Fast*DCs, capable of both migration and cytokine secretion, could be generated using TLR agonists and PGE_2 in combination.

4.2. Comparison with the literature

*4.2.1. Fast*DC *and short term protocols*

The traditional 7-day protocol has long been considered as the gold standard to generate DCs for experimental and clinical purposes: monocytes are incubated with GM-CSF and IL-4 for 5 days, followed by maturation for additional 2 days with cytokines, T-cell derived signals or microbial-derived stimuli. However, increasing evidence from animal studies implies that only 24 to 48 hours are required for the differentiation of dendritic cells from monocyte precursors *in vivo* (Palucka and Banchereau, 2002; Randolph et al., 1998), emphasizing that rapid culture techniques reflect more closely the process of DC differentiation *in vivo*.

The development of short-term protocols was pioneered by the team of Czerniecki using calcium-mobilizing agents for maturation. Alternatively, the use of IL-4 or IFN-α has also been reported to induce maturation within 2-3 days. Recently, our working group showed that functional mature dendritic cells, so-called *Fast*DC, could be generated within 48 hours, with equal capacity for antigen uptake, T-cell priming and migration. In comparison to the conventional protocol, a higher yield with more than 95% purity and viability was achieved (Dauer et al., 2003). Technically, the *Fast*DC protocol is not only more convenient in terms of practicability, but additionally lowers costs, labour, time and risks of microbial contamination.

According to a recent comparative study, the *Fast*DC protocol was demonstrated to be adaptable to full-scale GMP-production of DC required for clinical trials. In this respect, it may represent a promising strategy enabling doctors to treat more patients in a shorter period of time (Jarnjak-Jankovic et al., 2007). Taking these advantages into consideration, the short-

term protocol illustrates a superior model for the study of human DC biology and represents a highly standardised method to generate dendritic cells in a reproducible manner, eligible for cellular-based immunotherapy.

4.2.2. Determination of antigen-specific T-cell responses

Cellular immune responses are currently assessed by measuring cytotoxicity, proliferation or release of cytokines by T-cells. The chromium release assay is a semi-quantitative assay, measuring the lytic potential of cytotoxic T lymphocytes. However, in addition to being time-consuming, labour-intensive and not very sensitive, it does not provide information about the type of cells involved. Similarly, while total cytokine secretion is essentially analysed by enzyme-linked immunoassays, it is not possible to determine the type and number of cells actually secreting those cytokines.

On account of this, T-cell responses can additionally be assessed with new single-cell assays. Using the streptamer approach, the frequency of $CD8^+$ T-cells binding specifically to Melan A-MHC I-StrepTag-PE can be measured by FACS analysis. In order to detect simultaneously the cytokine produced, and also to define the subtype and frequency of IFN-γ-secreting cells, an intracellular staining as well as a conventional staining of cell surface molecules were performed, and analysed by flow cytometry. Both assays showed a similar trend, with an enhancement in the frequency of $CD8^+$ T cells detected in Melan-A-pulsed populations as compared with unloaded populations. Consistent with previous published data, Melan-A-specific $CD8^+$ T cells were detected to a higher level in the streptamer assay than intracellular $IFN\gamma^+$ T cells (Rothenfusser et al., 2004; Whiteside et al., 2003). However, this disparity observed might be due to different measuring time points. Nevertheless, these improved techniques are not entirely satisfactory. For instance, the necessity to identify the patient's haplotype, and the limited availability of MHC class I/peptide multimers restrict their use on a larger scale. Therefore, the above assays used exclusively to monitor T cell responses would be suboptimal, and a combination of several assays will show more validity in experimental settings as well as in clinical immunomonitoring.

4.2.3. FastDC and interferon-α

It is currently believed that DCs represent terminally differentiated cells, having a low survival rate after the withdrawal of cytokines and growth factors (Feuerstein et al., 2000). Our working group showed previously that *Fast*DCs in wash-out cultures survived up to six days and reverted to an immature phenotype unless stimulated with T-cell signals (Dauer et al., 2006). IFN-α shows *in vivo* controversial effects on the differentiation of dendritic cells. IFN-α was reported to be a poor inducer of DC maturation in the absence of other stimuli and was demonstrated to impair maturation in moDCs (Dauer et al., 2003). However, in combination with IL-1β and IL-4, it was found to enhance CD40L-mediated IL-12p70 secretion (Luft et al., 2002). Furthermore, analogous to moDCs, maturation was superior in *Fast*DCs treated with PGE_2, TNF-α, and IL-1β in the presence of IFN-α (Luft et al., 2001; Dauer et al., 2006). Interestingly, in contrast to moDC, no reversion to an immature phenotype was observed in IFN-α-primed *Fast*DC. With respect to endocytosis, IFNα-primed *Fast*DCs showed an inferior tendency, though not significantly, to internalise FITC-dextran or CFSE-labeled pancreas tumour cells than unprimed *Fast*DCs.

4.2.4. IL-12p70 deficiency and migration in conventional **Fast*DC***

PGE_2, required at early stages of maturation, has been demonstrated to be indispensable to deploy the migratory capacities of DCs (Legler et al., 2006). Consistent with previous published data, conventional *Fast*DCs migrated readily in response to 6Ckine (Scandella et al. 2002, Luft et al. 2002). In that regard, chemotaxis in $CCR7^+$ DC deployed by PGE_2 has been attributed to podosome dissolution, induction of high-speed migration (Van Helden et al. 2006) and the formation of less adherent cells, linked with a lower expression of activated β_1-integrin, thereby favouring migration.

It is generally accepted that IL-12p70 deficiency limits significantly the use in DC cancer vaccines. In accordance with several studies, we could reaffirm the inhibitory effects of PGE_2 on IL-12p70 secretion in conventional *Fast*DCs. Previous studies with conventional *Fast*DCs showed that total IL-12 (IL-12p70 + IL-12p40) could be induced upon additional stimulation with CD40L, whereas moderate levels of IL-12p70 secretion could not be enhanced, unless supplemented with IFN-γ (Dauer et al., 2003). Under our experimental settings, no IL-12p70 secretion was triggered upon additional IFN-γ-stimulus. The underlying mechanisms of the antagonistic effects of PGE_2 on IL-12p70 secretion have not been completely clarified.

Bioactive IL-12p70 is not secreted constitutively, but regulated by the synchronous expression of subunits, p35 and p40, in the same cell (Trinchieri et al. 2003). The selective induction of IL-12p40 homodimers has been reported to account for the suppression of IL-12p70 by PGE_2 (Kalinski et al. 2001). On the other hand, an increase in cAMP, caused by PGE_2 has also been associated with a deficiency in IL-12p70 secretion (Van der Pouw Kraan et al. 1998).

4.2.5. Maximum IL-12p70 secretion in TLR-derived Fast*DC*

DCs exhibit the broadest repertoire of TLRs, expressed constitutively or inducible in different cell types (Kadowaki et al. 2001; Jarossay et al. 1998). In accordance with several studies of moDCs, despite comparable maturation with single TLR agonists, high levels of IL-12p70 secretion could be elicited unless stimulated with a combination of TLR agonists, confirming the synergistic effects reported by Napolitani et al. Likewise, IL-12p70 secretion could be maximised upon CD40 ligation in the presence of IFN-γ (Snidjers et al. 1998). These findings illustrate the potential of dendritic cells to secrete large amounts of IL-12p70, developing only in response to multiple microbial signals combined with T-cell derived signals. Furthermore, IL-12p70 secretion was suggested to be regulated by a feedback loop, in which DC:T-cell contact via CD40-CD40L induces IL-12p70 secretion, followed by the secretion of IFN-γ, which sustains DCs to secrete IL-12p70 (Boullart et al., 2008). The concept of TLR-based activation with T-cell derived signals is also supported by a very recent study showing that co-electroporation of moDCs with mRNA encoding CD40L and TLR4 resulted in high levels of IL-12p70 secretion (Dauer et al., 2008). Moreover, an induction in the expression of the p35 subunit (the limiting factor in the synthesis of bioactive IL-12p70) was associated with CD40 ligation (Schulz et al. 2000). Recent experimental data suggests that the synergetic effects of TLR4 and TLR8 in moDCs result from the activation of both TLR transducing signalling pathways, the MyD88-dependent and interferon-dependent pathways (Bohnenkamp et al. 2007). Moreover, Napolitani et al. demonstrated that IL-12p70 secretion could be elicited only when different TLR ligands were added in a time window of four hours. This finding implies that the engagement of several TLRs and the subsequent synergistic effects induced are tightly controlled. This may represent *in vivo*, a “combinatorial security code”, ensuring that an immune response will be initiated only in the presence of invading pathogens (expressing several TLR ligands simultaneously).

4.2.6. Poor migration in TLR-stimulated Fast*DC*

Irrespective of the up-regulation of CCR7 expression, PGE_2 is imperative to license migration in DCs (Scandella et al. 2002, Luft et al. 2002). This finding could be reaffirmed in our experiments. Migration was observed in conventional *Fast*DCs, whereas $CCR7^+$ TLR-stimulated *Fast*DCs failed to undergo chemotaxis to 6Ckine under our experimental settings. The physiological significance or the exact mechanisms involved in rendering CCR7 in a responsive state by PGE_2, or other leukotrienes found at inflammatory sites (Randolph et al., 2005) are still under debate. It is assumed that PGE_2 interferes in the signalling pathways of CCR7, resulting in a cytoarchitectural reorganization during migration. A stronger adherence was observed in DCs treated with R848 or IFN-γ in comparison to PGE_2 (Lehner et al., 2008). This finding could explain the immobility of TLR-activated *Fast*DCs. At the molecular level, stimulation of PGE_2 via EP2 and/or EP4 receptors has been shown to be a requisite to license the functionality of CCR7 (Scandella et al. 2002). On the other hand, in a murine study, CD38 has also been identified to be involved in CCR7 licencing (Partida-Sanchez et al. 2004).

4.2.7. Migration is restored and IL-12p70 secretion is not aborted in TLR-matured Fast*DC supplemented with* PGE_2

Poor DC migration and low IL-12p70 secretion are drawbacks of the currently used DC vaccines. The use of PGE_2 in maturity cocktails have been reported repeatedly to account for the low secretory potential of moDCs. Under our experimental settings, supplemented PGE_2 could restore the migratory capacities of *Fast*DC matured with agonists of TLR7/8 and TLR4, in the presence of CD40L and IFN-γ, without inhibiting their IL-12p70 secretions. Recent findings demonstrated that the secretion of IL-12p70 could not be further intensified upon prolonged stimulus with LPS (Kalinski et al. 1997) or CD40L (Langenkamp et al. 2000), implying that IL-12p70 is secreted in a primary and a secondary manner. Under the assumption that PGE_2 is acting on the secondary cytokine secretion, it is believed that the TLR-derived stimuli launch the primary cytokine secretion, which cannot be neutralised by PGE_2. Moreover, PGE_2 could counteract the enhanced adherence observed in DCs treated with TLR agonists.

Interestingly, migration and secretion of IL-12p70 could be triggered, though to a lesser extent, in *Fast*DCs treated with R848, LPS and PGE_2 in the absence of CD40L and IFN-γ.

On the other hand, PGE_2 supplemented with CD40L and IFN-γ failed to induce IL-12p70 secretion. These findings underline the necessity of T-cell derived signals upon maturation with PGE_2 in the absence of TLR signals, and inversely imply that TLR signals are sufficient to trigger the secretion of IL-12p70 in a T-cell independent manner.

4.2.8. Migratory vs. pro-inflammatory dendritic cells

Its inevitable requirement to initiate migration in DCs, and its antagonistic effects on the secretion of IL-12p70, make PGE_2 one of the most controversial mediators used in DC protocols. The induction of a T_H1 response is dependent on the production of IL-12p70 by DCs. Maturation in the presence of PGE_2 resulted in the formation of migratory *Fast*DCs incapable of inducing the secretion of IL-12p70. On the other hand, DCs matured under the influence of TLR-derived stimuli and T-cell derived signals, without PGE_2, developed into "sessile" *Fast*DCs with high levels of IL-12p70. This central dogma observed already in early studies with moDCs led to the concept of a "pro-inflammatory phenotype" or "cytokine-secreting" phenotype under the influence of PGE_2 (Luft et al., 2002).

What could be the functions of such DCs migrating to the lymph nodes, but unable to "converse with T cells"? Recent works have suggested that this subset of DCs might be involved in self-tolerance, presenting self-antigens in a "steady-state". Moreover, inflammatory mediators are not only secreted in response to infection, but also to trauma or tissue injury, during which DCs mature without TLR-derived stimuli. In this way, IL-12p70 deficient DCs "involuntarily activated"can prevent an excessive or inadequate activation of a cell-mediated response.

Migration to the lymph nodes offers the greatest opportunity for DC to encounter T cells. What could be the physiological relevance of "sessile" CCR7-positive DCs"conditioning their environments" with the secretion of large amounts of IL-12p70, as generated in TLR-derived *Fast*DCs? It has been suggested that $CCR7^+$ immobile *Fast*DCs with maximum IL-12p70 secretion could correspond *in vivo* to sessile DCs localised originally in lymph nodes (where CD40 ligation would also occur), sampling soluble antigens which have drained there directly (Randolph et al. 2005). Alternatively, they might be immobilised purposely at the sites of challenge, recruiting there either naïve "passerby" T cells or other immune cells with killing properties to confine the inflammatory processes, or sustain DC:T-cell dialog facilitating maturation while recruiting T effector cells.

4.2.9. Activation of an antigen-specific immunity

In contrast to $CD4^+$ T cells, IL12-p70 has been reported to be involved in enhancing functional avidity in $CD8^+$ T cells (i.e. the activation of cytotoxic T cells with potent capacity to recognise and kill tumour cells), but showed no influence on cytokine secretion (Xu et al. 2006). Although we observed a tendency towards enhanced activation of Melan-A-specific CTL in TLR-derived *Fast*DCs, conventional *Fast*DCs deficient in IL-12p70 secretion were found to sensitise equally $CD8^+$ T cells to lyse target T2 cells. This implies that in our *in vitro* model, T-cell activation occurred predominantly in an IL-12p70-independent manner. Likewise, the secretory profile of IFN-γ was comparable in both sets of *Fast*DCs, independent of the secretion of IL-12p70. Importantly, in another study of our working group, the supplementation of PGE_2 did not influence the capacity of *Fast*DCs to activate Melan-A-specific CTLs. Accordingly, equal levels of IFN-γ were measured in co-cultures of TLR-derived *Fast*DCs and TLR-derived *Fast*DCs in the presence of PGE_2. Analogous to our previous experiments, compared to conventional *Fast*DCs, more than a two-fold increase was induced in co-cultures of TLR-derived *Fast*DCs (Dauer et al., 2008).

4.2.10. IL-12p70 secretion and antitumour immunity

The ability of IL-12p70 to induce antigen-specific immunity is reflected not only by its ability to induce T_H1 and CTL responses, but also to increase the production of opsonizing and complement-fixing IgG antibodies, shown to have antitumour activity *in vivo*. These findings indicated that the direct administration of IL-12p70 could be efficient in antitumour therapies. However, excessive toxicity in the form of inflammatory responses and the moderate responses in clinical trials have dampened this enthusiasm greatly (Trinchieri et al., 2003). Thus, the induction of IL-12p70 secretion *in vivo* in DC vaccines represents a more reliable and efficient pathway with less adverse effects to mount an antitumour response. On account of this, the identification of factors inhibiting IL-12p70 secretion is another important aspect to be considered to improve DC-based cancer vaccines. For example, tumour-induced suppression has been associated with the inhibition of IL-12p70, caused by IL-10 and TGF-β. The latter were shown to be potent cAMP-inducers and inhibitors of the transcription of IL-12 genes (Trinchieri et al., 2003; Tarbell et al. 2006).

4.3. Clinical relevance

4.3.1. Minor success of first-generation DC vaccines

Although tumour regression has been observed occasionally, no studies have yet shown to improve survival in DC-based vaccines (Steinman et al. 2007). The route of administration, the loading of the antigens, or the selected antigens pulsed with DCs were also reported to be suboptimal (Steinman et al. 2007; Aarntzen et al. 2008). The migratory potential of PGE_2 in maturity cocktails was utilised at the expense of IL-12p70 deficiency. Only 1% of DCs injected was found to migrate to the secondary lymphoid organs (De Vries et al. 2003). Moreover, DC vaccines in early clinical trials were applied exclusively to late-stage cancer patients. Recent findings have shown that CTL responses following DC-based vaccination were found to be stronger and long-lasting in stage II melanoma patients with minimal residual disease than stage IV melanoma patients with large tumours. Antitumour immunosuppression by tumour cells has been another factor recently identified to counteract the effects of DC vaccines. Furthermore, a lack of standardization in protocols for the *ex vivo* generation and maturation of DCs, study design as well as patient selection, made it unfeasible to analyse and define more clearly the criteria required to design an optimal DC vaccine.

4.3.2. Optimal DC quality with TLR ligands and PGE_2 for DC-based immunotherapy

Suboptimal phenotypic maturation and reduced migratory capacity have been proposed to be drawbacks of single TLR-derived DC vaccination (Dauer et al., 2008). There is substantial evidence that the combined activation of immature DCs with TLR-ligands, T-cell derived signals and PGE_2 is optimal for the large-scale generation of clinical grade migratory T_H1-polarizing DCs (Zobywalski et al., 2007; Boullart et al., 2008). These findings have been paralleled by our own observations in the *Fast*DC protocol. Furthermore, TLR-matured *Fast*DCs with PGE_2 and CD40L as well as IFN-γ showed an enhanced tendency to expand CTLs in the Melan-A model as compared with conventional DCs (Dauer et al. 2008). Interestingly, the supplementation of PGE_2 in TLR-activated DCs was associated with a reduction of IL-10 secretion (Boullart et al., 2008). Hence, it does not only improve the efficiency in terms of enhanced antitumour cell-mediated reponse but also acts as an opponent of tumour-induced immunosuppression. This new *Fast*DC population opens new routes for improving the efficacy of DC vaccines in antitumour therapy.

4.3.3. Future perspectives

Referring to recent studies on the efficacy of DC-based vaccines, hereunder is a re-evaluation and definition of the criteria essential to exploit DCs in anti-tumour regimes:

- Viability and purity of DC should exceed 75%
- High-quality DC : Fully mature phenotype, $CCR7^+$ expression with migration
- Generation of Th1 polarizing DCs with high IL-12 secretion
- Counteracting antitumour immunosuppression
- Standardization of culture protocols and immunomonitoring techniques
- Standardization in study design and patient selection

4.3.4. DC-based therapy and adjuvant antitumour therapy

Traditional vaccines prevent the outbreak of infectious diseases by inducing long-lived immunity against specific microbial antigens. In contrast, DC-based cancer vaccines are based on the exploitation of DC to induce immunity specifically against tumour antigens to treat a clinically established cancer. Cell-mediated immunity is a powerful non-toxic and selective line of defence against cancer, acting complementarily to the traditional antitumour therapy regimes including surgery, chemotherapy, and radiotherapy.

5. Summary

Monocyte-derived DCs generated with a standard 7-day protocol have contributed largely to the knowledge available about their biology. Nevertheless, increasing evidence shows that the short-term *Fast*DC protocol represents a rapid assay, resembling more closely physiological instances, superior in generating *ex vivo* DCs.

Summarising our results, under our experimental settings, mature *Fast*DCs could be generated under the concerted influence of microbial-derived stimuli, the TLR agonists R848 and LPS, combined with T-cell derived stimuli, CD40L and IFN-γ, in the presence of PGE_2. While exhibiting a mature phenotype, such *Fast*DC generated could, for the first time, induce high levels of IL-12p70 and show high migratory capabilities in the presence of PGE_2. Extrapolating these findings at the physiological level, *Fast*DCs can differentiate along two pathways. The first subset may represent DCs found at inflammatory sites sampling for "passerby-T cells" in the presence of PGE_2, or activating cells of the immune system with killing properties, thereby providing “primary immediate care” and restraining the infection at the inflammatory site. On the other hand, the second subset characterises those migrating to the lymph nodes for the expansion and differentiation of T helper cells. In addition to their efficient T_H1 polarizing potential, they were capable of activating and expanding selectively $CD8^+$ T cells into cytotoxic effector cells, and enhancing high levels of secretory IFN-γ. Taken together, the *Fast*DC protocol including the above combinatory maturity cocktail offers a new gold standard for the large-scale generation and exploitation of DCs for DC-based immunotherapies and DC-research.

Furthermore, this work illustrates the elaborate and flexible programs that DCs are having at their disposal, generating different subsets “on demand”, instructed by the stimuli and signals found at the inflammatory sites. Migration and IL-12p70 secretion have been two crucial objectives in DC studies. Following to improvements in isolating procedures, recent findings are exemplifying how mediators can modulate the development of a plethora of DC subsets. The physiological correlation between “artificial” DC subsets and *in vivo* DCs is unpredictable, and critical interpretation of the results is essential.

6. Zusammenfassung

Die Verwendung dendritischer Zellen zu experimentellen oder klinischen Zwecken war anfänglich insbesondere durch die suboptimalen Bedingungen in Bezug auf ihre Isolierung und Kultur erschwert. Erst durch die Entwicklung der Isolierungsmethode nach Sallusto (Sallusto et al. 1994 Romani et al., 1994) konnte vieles über DC aufgeklärt werden. Diese Methode wird noch heute als Goldstandard verwendet. Dabei werden Monozyten aus dem peripheren Blut isoliert, 5-7 Tage mit GM-CSF und IL-4 inkubiert, und anschließend mit pro-inflammatorischen Mediatoren (Jonuleit et al., 1997) stimuliert.

In den letzten Jahren konnte gezeigt werden, dass die Isolierung DC aus Monozyten auch in einer kürzeren Zeitspanne möglich ist. Czerniecki et al., 1997 verwendeten Kalzium-Ionophoren während Dauer et al. IL-4 oder IFN-α (Dauer et al., 2003) zur Aktivierung benutzen. Das besondere Interesse an solchen „short-term“ Protokollen liegt in der Annahme, dass eine schnellere *in vitro* Differenzierung die physiologischen Prozesse *in vivo* besser widerspiegelt. Außerdem ist die Methodik nicht nur ökonomischer, sondern die Kontaminationsgefahr konnte ebenfalls gesenkt werden.

In unserer eigenen Arbeitsgruppe wurde ein Protokoll zur Generierung von DC aus Monozyten innerhalb von 48 Stunden, die sogenannte *Fast*DC, beschrieben (Dauer et al. 2003). Mit dem Stimulationscocktail PGE_2/TNF-α/IL-1β konnten reife stabile *Fast*DC generiert werden, die einen reifen Immunophänotyp aufwiesen, ein migrierendes Potenzial zeigten, jedoch nur wenig IL-12p70 sezernierten.

In unseren aktuellen Ergebnissen konnten wir zeigen, dass anhand von mikrobiellen Stimuli wie dem TLR Agonisten R848 und LPS kombiniert mit T-Zell-abhängigen Faktoren wie CD40L und IFN-γ in Anwesenheit von PGE_2, reife dendritische Zellen, sogenannte *Fast*DC, generiert werden konnten. Diese wiesen einen reifen Phänotyp auf, sezernierten erstmalig hohe Mengen an IL-12p70 und zeigten gleichzeitig ein hohes migratorisches Potenzial in Gegenwart von PGE_2. Die *Fast*DC übten einen signifikanten polarisierenden Effekt auf T-Helfer Zellen, T_H1, aus. Außerdem waren die *Fast*DC in der Lage $CD8^+$ T- Zellen in zytotoxischen T- Effektorzellen zu differenzieren, welche hohe Mengen an IFN-γ sezernieren.

Betrachtet man diese Ergebnisse auf physiologischer Ebene, so können *Fast*DC in zwei Subtypen differenziert werden: Die erste Subpopulation repräsentiert die DC, die man in entzündetem Gewebe findet und die in Gegenwart von PGE_2 die Einwanderung von T-Zellen fördern oder Zellen des Immunsystems mit zytotoxischen Eigenschaften aktivieren. Sie stellt

somit eine Art „primäre Soforthilfe“ dar. Die andere Subpopulation beinhaltet die DC, die in Lymphknoten migrieren und dort die Differenzierung und Expansion von *T-Helfer* Zellen induzieren, und somit die zelluläre Immunantwort fördern.

Zusammenfassend stellt das *Fast*DC-Protokoll mit dem oben dargestellten Stimulationscocktail einen neuen überlegenen Goldstandard zur Generierung und Verwendung von funktionsfähigen DC für DC- basierte Immunotherapien und DC-Forschung dar.

Diese Arbeit zeigt wie DC komplexe und flexible Programme zur Verfügung besitzen, um verschiedene Subklassen von DC „bei Bedarf“ zu generieren, gesteuert von Stimuli und Signalen am Inflammationsort. Migration und IL-12p70-Sekretion stellen die zwei wichtigsten Ziele in DC-Studien. In Folge der Verbesserungen der Isolierungsprozeduren konnten neue Erkenntnisse gewonnen werden wie Mediatoren die Generierung einer Fülle von DC-Subpopulationen bewirken. Die Korrelation zwischen „artifiziellen“ DC Subpopulationen und *in vivo* DC ist jedoch schwer einschätzbar und eine kritische Interpretation der Datenergebnissen ist daher unerlässlich.

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8. Abbreviations

APC	antigen-presenting cell(s)
CCL	chemokine ligand
CCR7	chemokine receptor 7
CD	cluster of differentiation
CD40L	CD40 ligand
CLP	common lymphoid progenitors
CMP	common myeloid progenitors
CTL	cytotoxic T lymphpcytes
cpm	counts per minute
DC	dendritic cell
DCs	dendritic cells
EDTA	ethylen-Diamin-Tetra-Acetic acid
ELC	EBI ligand chemokine
ELISA	enzyme linked immunosorbent assay
EP	prostaglandin E receptor
ER	endoplasmatic reticulum
FACS	fluorescence activated cell sorting
FCS	fetal calf serum
FOXP3	forkhead box P3
FSC	forward scatter
Flt	FMS-like tyrosine kinase
GM-CSF	granulocyte macrophage - colony stimulating factor
h	hour(s)
HEV	high endothelial venules
HPV	human papilloma virus
HSA	human serum albumin
IFN	interferon
IL	interleukin
IRF	interferon gamma regulator factors
LPS	lipopolysaccharide
MACS	magnetic activated cell sorting
MAPK	mitogen activated protein kinases

MFI	mean fluorescence intensity
MHC	major histocompatibility complex
min	minute(s)
MIP	macrophage inflammatory protein
moDCs	monocyte-derived dendritic cells
MyD88	myeloid differentiation factor 88
NF-KB	Nuclear factor „kappa-light-chain-enhancer“ of activated B cells
NK	natural killer
NOD	nucleotide-binding oligomerization
PAMP	pathogen-associated molecular pattern
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
pDC	plasmacytoid DC
PGE2	prostaglandin E2
PRR	pattern recognition receptor
RANTES	Regulated on activation normal T cell expresssed and secreted
RIG	retinoid acid-inducible gene
RLR	RIG-like receptor
SEM	standard error of the mean
SSC	sideward scatter
SLC	secondary lymphoid tissue chemokine
TAA	tumour-associated antigens
TAP	transporters associated with antigen processing
TCR	T cell receptor
TGF-β	transforming growth factor beta
TIR	Toll/IL-1R
TIRAP	Toll-like receptor adaptor protein
TLR	Toll-like receptor
TNF	tumour necrosis factor
VEGF	vascular endothelial growth factor

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10. Publications

Dauer M, Lam V, Arnold H, Junkmann J, Kiefl R, Bauer C, Schnurr M, Endres S, and Eigler A. Combined use of toll-like receptor agonists and prostaglandin E(2) in the FastDC model: rapid generation of human monocyte-derived dendritic cells capable of migration and IL-12p70 production. *J Immunol Methods* 337: 97-105, 2008.