HUMAN DENDRITIC CELLS AND HEPATITIS C VIRUS

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ABSTRACT

Dendritic cells (DCs) constitute a large family of immune cells with a dendritic morphology and a critical role in all aspects of an immune response and immune regulation, from immunogenicity to tolerance. One of the important characteristic of DCs is maturation, during which DCs undergo significant changes in their phenotypic and functional properties and change from phagocytic cells to highly efficient antigen presenting cells (APCs). Dendritic cells have recently been at the centre of attention as a promising tool in treatment or control of cancer and infectious diseases. Accordingly, DCs have been generated, matured, and loaded with tumor-associated or microbial antigens *ex vivo*, to be subsequently used as therapeutic tools or vaccine carriers.

Hepatitis C virus (HCV) is a hepatotropic virus, which infects the liver in humans and results in a chronic infection in most cases. The persistent infection of the liver eventually results in cirrhosis and/or hepatocellular carcinoma in 15-20 years. Chronic hepatitis C (CHC) has recently become a serious health concern and the leading cause of liver transplantation. The mechanism of persistence of the virus is not clear yet, but as a Th1-type immune response is strongly correlated with elimination of HCV *in vivo*, it is evident that insufficient cellular immunity is a contributing factor. Non-cytopathic viruses such as HCV may infect immune cells to modify and evade a protective immune response. Dendritic cells, which are the most potent APCs, and uniquely capable of initiating a primary immune response, have been considered as a target for HCV. Inhibition of DC maturation by HCV has been suggested as a potential contributing factor in immune evasion; however, this issue remains controversial as many contradictory results have been reported.

To investigate this contention, we initially planned to evaluate the effects of HCV on DCs of CHC patients; however, due to limited access to patients' blood, we instead elected to examine the effects of HCV genes products on *in vitro* generated DCs from healthy volunteers. Specific attention was paid to the generation, maturation, and transfection of DCs *in vitro*, as variability in procedures might have been responsible for the controversial reports. Viral vectors have generally been used to transfect DCs; however, a vector and HCV genes might have synergistic effects on DC maturation. Thus, our first objective was to develop an efficient non-viral transfection method while retaining high viability of the DCs, as previous efforts in this regard resulted either in low efficiency or in low viability of DCs after transfection. In order to improve the viability of DCs after transfection, we established a new method for fast generation of monocyte-derived DCs (Mo-DCs) in two to three days. By performing a comprehensive study on transfection reagents, electroporation, and nucleofection with DNA or *in vitro* transcribed (IVT) RNA, we successfully established a new, highly efficient non-viral method for transfection of DCs with long-term viability. This method is based on the use of the X1 program of a nucleofection device with IVT RNA and results in high transfection efficiency of 93%, with 75% viability of DCs 72 h after transfection.

Subsequently, we performed a comprehensive study on the effects of different maturation methods on the phenotype, function and gene expression profile of DCs. Three commonly used treatments, TNF-α, LPS and a maturation cocktail (MC) consisting of IL-1β, IL-6, TNF-α, and prostaglandin E2 (PGE2) were compared. Our results showed that there is a significant difference in the level of maturity between these treatments, and MC generated more functionally competent mDCs than TNF-α or LPS. In addition, MC induced Th1-promoting changes in the transcriptional profile of mDCs. This observation was important, as the presence

of PGE2 in MC was previously challenged based on the potential induction of Th2-biased immune responses. However, our results suggest retaining PGE2 in the cocktail because of the fact that MC generated highly competent and functional mDCs with a Th1-promoting transcriptional profile.

Finally, Mo-DCs were transfected with IVT HCV RNAs, individually or in combination. While HCV genes had no inhibitory effect on DC maturation, transfection of DCs with IVT core RNA appeared to result in changes compatible with maturation. To investigate this in more detail, the transcriptional profiles of DCs transfected with IVT core, NS3 or green fluorescent protein (GFP) RNA were examined using a DC-specific membrane array. Of the 288 genes on the array, 46 genes were distinctively up- or down-regulated by transfection with IVT core RNA in comparison to NS3 or GFP RNA treatments, 42 of which are involved in DC maturation. The effects of core on maturation of DCs were further confirmed by a significant increase in surface expression of CD83 and HLA-DR, a reduction of phagocytosis, as well as an increase in proliferation and IFN-γ secretion by T cells in a mixed lymphocyte reaction assay. These results show that HCV core does not have an inhibitory effect on human DC maturation, but could be a target for the immune system.

The use of a non-viral method of transfection combined with confirmed transcriptional profiles of DCs in this study may make these results conclusive for *in vitro* generated DCs from healthy volunteers. However, further investigations are required to confirm the effects on DCs from CHC patients.

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DEDICATION

To Shima, for supporting me during easy and hard times

To Neusha, for bringing happiness to our life

To my mother and my late father, for devoting their lives to their children

To my sisters / brothers and their families, for making me proud by being a part of my identity

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2ME	β or 2-mercaptoethaonl
AIDS	acquired immune deficiency or acquired immunodeficiency syndrome
APCs	antigen presenting cells
ARFP	alternative reading frame protein
BTG1	B cell translocation gene 1 (gene name)
CARD	caspase recruitment domain
CARDIF	CARD adaptor inducing IFN-β
CD	cluster of differentiation
cDCs	conventional dendritic cells
CFSE	carboxyfluorescein diacetate succininidyl ester
CHC	chronic hepatitis C
CLDN1	claudin-1
CLPs	common lymphoid progenitors
CMPs	common myeloid progenitors
CMV	cytomegaluvirus
CpG ODN	CpG oligonucleotides
cRNA	complementary RNA
CRPMI	complete RPMI
CST3	cystatin 3 (gene name)
CTLs	cytotoxic T cells
DC	dendritic cell
DC-SIGN	dendritic cell specific intercellular adhesion molecule-3-grabbing non-integrin
dsRNA	double strand RNA
DX	dextran
E1 and E2	envelope protein 1 and 2
EBI3	Epstein-Barr virus induced gene 3
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmic reticulum
FACS	fluorescent activated cell sorting
Fas	tumor necrosis factor receptor superfamily member 6 (TNFRSF6)
FBS	fetal bovine serum
FCER1G	high affinity immunoglobulin E receptor, gamma chain (gene name)
FCGR3B	low affinity immunoglobulin gamma Fc region receptor III-B (gene name)
FDCs	follicular dendritic cells
FITC	fluorescein isothiocyanate
FITC-DX	dextran conjugated to fluorescein isothiocyanate
Flt3L	fms-like tyrosine kinase 3 ligand
fms	feline McDonough sarcoma (a family of DNA sequences)
FSC	forward scatter
gC1qR	globular domain of C1q (a complement soluble molecule) receptor
GFP	green fluorescent protein
GM-CSF	granulocyte macrophage-colony stimulating factor
GVHD	graft-versus-host disease

HBV	hepatitis B virus
HCC	hepatocellular carcinoma
HCV	hepatitis C virus
HLA	human leukocyte antigen
HSP90AB1	heat shock 90kDa protein 1 alpha, class B (gene name)
HVR1	hypervariable region 1
ICAM1	intercellular adhesion molecule 1 (gene name)
iDCs	immature dendritic cells
IDO	indoleamine 2-3 dioxygenase (an enzyme involved in T-cell tolerance)
IFN	interferon
Ig	immunoglobulin
IL	interleukin
IPCs	interferon producing cells
IRES	internal ribosome entry site
ISGs	interferon stimulated genes
IVT RNA	in vitro transcribed RNA
Jak	Janus Kinase (Jak)-1
JFH-1	Japanese fulminant hepatitis virus 1 (2a JFH-1 isolate)
LCs	Langerhans cells
LDL	low-density lipoprotein
LIPA	lipase A
LNs	lymph nodes
LPS	lipopolysacharide
LPS-mDCs	dendritic cells matured by lipopolysacharide
L-SIGN	liver specific intercellular adhesion molecule-3-grabbing non-integrin
LSM	lymphocyte separation medium
LY	lymphocyte antigen
MACS	magnetic activated cell sorting
MC	maturation cocktail
MCM	monocyte-conditioned medium
MC-mDCs	dendritic cells matured by maturation cocktail
mDCs	mature dendritic cells
MFI	mean fluorescent intensity
miRNAs	micro RNAs
MLR	mixed leukocyte reaction
MMR	macrophage mannose receptor
Mo-DCs	monocyte-derived dendritic cells
Mo-iDCs	monocyte-derived immature dendritic cells
MΦs	macrophages
mRNA	messenger RNA
MTT	3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide
MyD88	myeloid differentiation protein 88
NFKB	nuclear factor-kappa B
NKs	natural killer cells
NKTs	natural killer T cells
NS	non-structural

OCLN	occludin
ORF	open reading frame
PBMCs	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pDCs	plasmacytoid dendritic cells
PE	phycoerythrin
PEG	poly ethylene glycol or pegylated
PGE2	prostaglandin E2
PI	propidium iodide
PIM2	serine/threonine-protein kinase (gene name)
PKR	protein kinase R
PMNs	polymorphonuclear cells
PNRC1	proline-rich nuclear receptor coactivator 1 (gene name)
PRRs	pattern regonition receptors
PSCDBP	pleckstrin homology, Sec7 and coiled-coil domains, binding protein (gene name)
PTB	polypyrimidine tract binding protein
RdRp	RNA-dependent RNA polymerase
RELB	reticuloendotheliosis viral oncogene homolog B
rh	recombinant human
RIG-1	retinoic acid-inducible gene 1
RNAi	RNA interference
RPMI	Roswell Park Memorial Institute (a culture medium)
R-TTR	Transfection with TransMessenger transfection reagent using IVT RNA
RT	room temperature
SCID	severe combined immunodeficiency
SDS	sodium dodecyl sulfate
siRNA	small (short) interfering or silencing RNA
SOD2	superoxide dismutase 2
SR-BI	scavenger receptor class B type I
SREBP-1c	sterol regulatory element-binding protein 1c
SRPMI	supplemented RPMI
SSC	side scatter
ssRNA	single strand RNA
STAT	signal transducers and activators of transcription
TAAs	tumor-associated antigens
TCR	T cell receptor
TGF-β1	transforming growth factor beta-1
Th	T helper
Th1	T helper type 1
Th2	T helper type 2
TIR	Toll/IL1 receptor
TLRs	toll-like receptors
TM7SF4	transmembrane 7 superfamily member 4 (gene name)
TNF-mDCs	dendritic cells matured by tumor necrosis factor alpha
TNF-α	tumor necrosis factor alpha

Tregs	regulatory T cells
TRIF	TIR domain-containing adaptor protein inducing IFN-β
TTR	TransMessenger transfection reagent
Tyk	tyrosine kinase 2
UNAIDS	United Nation AIDS
UTRs	untranslated regions
VLPs	virus-like particles
WHO	world health organization
XRPMI	chemotaxis RPMI

Introduction and literature review

1.1 Dendritic cells

Dendritic cells (DCs) are composed of a family of immune cells with a dendritic morphology, which was the basis for their designation. The first member of DC was discovered by Paul Langerhans in 1868 and thought to be a kind of nerve cell; however, the family of DCs was identified and described by Ralph Steinman in 1973 as a part of the immune system (Steinman and Cohn 1973; Steinman, Lustig et al. 1974). Over the past few decades, a large number of studies established a role for DCs as one of the most important cell types in the immune system. DCs are potent antigen-presenting cells (APCs), which play a critical role in all aspects of an immune response and immune regulation resulting in immunity or tolerance. Accordingly, they became a promising tool for treatment or control of infectious diseases, cancers, allergies, and autoimmune disorders. DCs are also of interest in transplantation medicine.

1.1.1 Biology of dendritic cells and role in the immune system

DCs constitute a complex system of cells, with different phenotypes and specific functions. They originate from diverse lineages of bone marrow cells, and dispersed throughout the body, but localize more specifically at the sentinel barriers, such as skin and respiratory or gastrointestinal mucosa, thus providing protection from pathogens. DCs are non-dividing cells and need to be continuously re-generated as their *in vivo* life span is short (Kamath, Pooley et al.

2000; Matsuno and Ezaki 2000; O'Keeffe, Hochrein et al. 2002). Dendritic cells residing in non-lymphoid organs such as lung, intestine, liver, kidney, and heart are called interstitial DCs; while those in the skin are known as Langerhans cells (LCs). In general, DCs remain at an immature status in peripheral tissues, where they are highly exposed to potentially harmful non-self antigens (Cella, Sallusto et al. 1997; Banchereau and Steinman 1998; Mellman and Steinman 2001) (Fig. 1.1). They take up antigens, change their morphology and marker expression, and start the maturation process resulting in different functional capabilities and a directional motility toward local lymph nodes (LNs). On their way through afferent lymphatic vessels, they are called veiled cells based on their dendritic appearance. Their migration to LNs eventually results in effective contact with T cells (Mellman and Steinman 2001), which are also attracted selectively to the LNs (Savina and Amigorena 2007). In the T zone area of LNs, DCs are known as interdigitating cells where they present antigens to T cells.

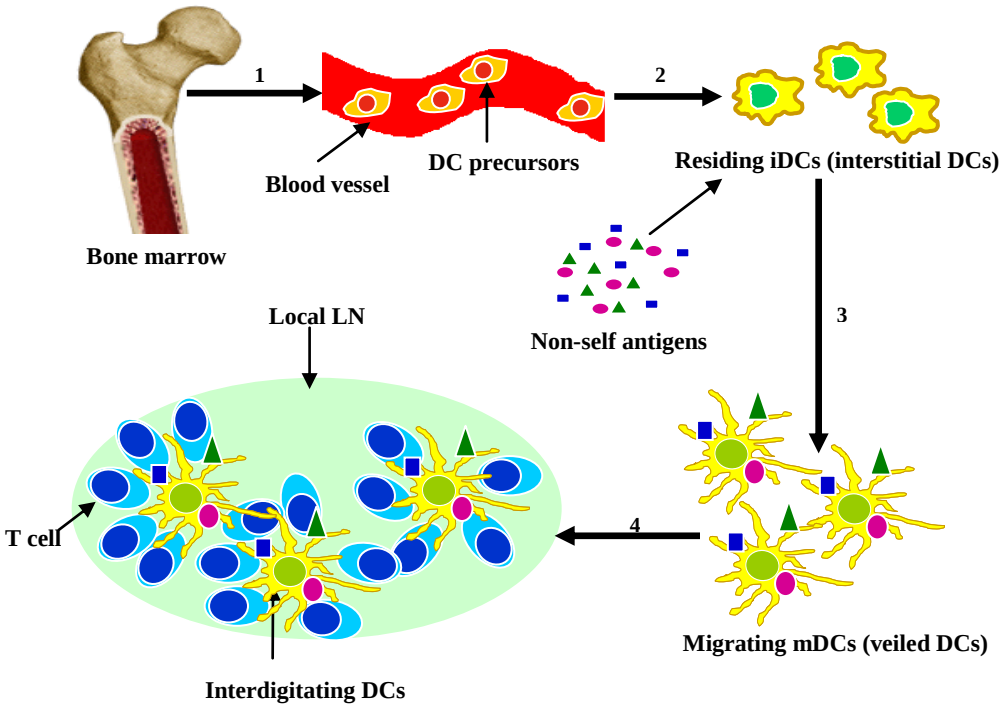

Figure 1.1. The biology and life cycle of dendritic cells.

Dendritic cells were originally considered to be of myeloid lineage and closely related to monocytes, macrophages (MΦs) and granulocytes. However, later studies suggested that DCs can be generated along distinct developmental pathways and from different haematopoietic precursors. There are at least two progenitors, which are called common myeloid progenitor (CMP) developing into all myeloid cells including DCs, granulocytes, and MΦs (Lotze M. T. 2001), and common lymphoid progenitor (CLP) developing into DCs, natural killer cells (NKs), B cells, and T cells. In most tissues, DCs are normally of CMP origin, whereas in the thymus they are of CLP origin and in the spleen they are presumed to be derived from both progenitors. However, other studies have questioned this restriction (Manz, Traver et al. 2001; Adolfsson, Mansson et al. 2005). There also is a low percentage (less than 1% of peripheral blood mononuclear cells [PBMCs]) of DCs in the circulating blood known as circulating DCs (Van Voorhis, Hair et al. 1982). Recently, four specialized features have been ascribed for DCs (Steinman and Banchereau 2007), including location of DCs (briefly explained above), maturation of DCs, antigen presentation by DCs, and subtypes of DCs.

1.1.2 Maturation of dendritic cells

Maturation is one of the most important features described for the DC family. It indicates the conversion of these cells from immature sentinels to mature immunostimulatory cells. In the process of maturation, DCs lose most of their endocytotic ability (although it is increased in the first few hours of this process), gain a higher capacity of antigen presentation, and switch their migratory potential toward the LNs in response to a high concentration of specific chemokines for mature DCs (mDCs) (Willimann, Legler et al. 1998; Ohl, Mohaupt et al. 2004). These chemokines consist of CCL19, CCL21, and CXCL12, which are constitutively secreted in LNs,

where DCs interact with naïve T cells. During maturation, DCs reduce the expression of uptake molecules and immature (i)DC-specific chemokine receptors such as CCR5 and CCR6. In contrast, the cell surface expression of antigen-presenting molecules such as CD83, co-stimulatory molecules such as CD80 and CD86, MHC molecules, and mDC-specific chemokine receptors such as CCR7 and CXCR4 is increased, and the secretion of cytokines and chemokines enhanced. Cluster of differentiation 83 molecule (CD83) is the best known and studied functional marker for mDCs as it is critical for antigen presentation (Lechmann, Krooshoop et al. 2001; Lechmann, Berchtold et al. 2002; Lechmann, Zinser et al. 2002; Fedele, Frasca et al. 2004; Kotzor, Lechmann et al. 2004; Zinser, Lechmann et al. 2004). Following contact with T cells, DCs are licensed to express cytokines such as IL-12 to presumably determine the type of immune response and polarize the response based on the characteristics of the antigen. Finally, T cells need to be released or de-attached from mDCs in order to be activated and to proliferate. DCs also play a role in this de-attachment step by expression of pleckstrin homology, Sec7 and coiled-coil domains, binding protein (PSCDBP), which mediates this process. As mDCs are not seen to leave LNs via efferent lymphatic vessels (Matsuno and Ezaki 2000), the maturation process appears to be irreversible and presumably ends with apoptotic death after antigen presentation within LNs. Nowadays, maturation is a subject of controversy as impairment of DC maturation may result in chronicity of infectious diseases or escape of tumoral cells from the immune response. However, it is clear that in order to achieve strong immunity against non-self antigens, DCs need to be mature at the time of antigen presentation to T cells. Indeed, it is well known that iDCs expressing low levels of MHC class I and II and co-stimulatory molecules are involved in peripheral tolerance (Sallusto, Cella et al. 1995), and by presenting antigens may

induce tolerance instead of immunity (Jonuleit, Schmitt et al. 2001; Moser 2003). In general, three functional characteristics of DCs dramatically change during DC maturation.

1.1.2.1 Phagocytosis

Phagocytosis is a process by which particles, microorganisms, or dead cells are taken up, usually through receptors on the cell surface, and then destroyed within the phagocytic cell. This is part of the innate immune response facilitating the clearance of pathogens. The most important phagocytic cells are MΦs and neutrophils, which are components of the innate immune system. Dendritic cells were originally assumed to be a type of MΦs. However, subsequent studies showed that the phagocytosis is quite different in DCs, as they are not directly involved in the clearance of pathogens. The key difference is that MΦs degrade the pathogen to the smallest particles possible to be destroyed and cleared from the body, whereas DCs process the pathogen in a strictly controlled manner to specific peptides, which are then presented to T helper (Th) cells. This is called antigen processing, which makes DCs unique among phagocytic cells. Although other phagocytes are also capable of antigen presentation, this is an inefficient process. Most importantly, as they cannot present antigens to naïve T cells, other phagocytes are not capable of initiating a primary immune response. Phagocytosis is one of the characteristics that are lost during DC maturation, and its loss is considered an indicator of the level of maturity in DCs.

1.1.2.2 Antigen presentation

Antigen presentation is likely the most important function of DCs, being the final stage in immune recognition and the start of an immune response. It determines the outcome of a non-self invasive or self-compromising response. DCs are very well programmed for this function and most of the immune-related diseases presumably correlate to unsuccessful or improper antigen presentation. For this purpose, DCs process and present the antigens through different intracellular pathways, eventually resulting in presentation of the target antigen on MHC class I or II molecules resulting in a Th1- and Th2-type immune response, respectively. While iDCs are not efficient APCs, antigen presentation becomes much more efficient during DC maturation and thus is the most important functional property of mDCs. Consequently, maturation is probably the most important characteristic of DCs resulting in proper antigen presentation, which in turn, is considered another specialized feature of DCs.

1.1.2.2.1 Th1 and Th2 decision

Appropriate antigen presentation is assumed to be accomplished through three signals. The first signal is delivered through contact between MHC molecules on the APCs and the T cell receptors (TCRs) which provides information about the identity of the pathogen. The second signal is facilitated by the co-stimulatory molecules (CD80 and CD86) on the APCs through contact with CD28 on T cells; this provides the activation signal for T cells (Lotze M. T. 2001), while the absence of this signal results in T cell anergy (Sallusto, Cella et al. 1995). The third signal, which presumably determines the type of the immune response (Sad and Mosmann 1994; Dustin and Shaw 1999; Kalinski, Hilkens et al. 1999; Lanzavecchia, Lezzi et al. 1999), depends on the polarizing cytokines and microenvironment at the time of antigen presentation in a step

called licensing. Successful antigen presentation by DCs results in an optimal combination of Th1- and Th2-type immune responses based on the pathogen involved. It could be Th1-biased for intracellular pathogens such as viruses and some types of bacteria or Th2-biased for extracellular pathogens such as helminth.

The flexibility and heterogenicity of DCs play a unique role in this process, which may be mediated by the type of cytokine secreted or present in the microenvironment (Macatonia, Hosken et al. 1995; Heufler, Koch et al. 1996). Interleukin (IL)-12 and IL-4 are the two main cytokines responsible for Th1- and Th2-type immune responses, respectively. In the presence of IL-12, emerging Th1-type cells secrete interferon (IFN)-γ, which promotes additional secretion of IL-12 by DCs eventually resulting in a cellular immune response. There are not enough studies on Th2-type immune responses, although IL-4 is known as the main cytokine driving the response toward a Th2 bias. In the presence of IL-4 developing Th2-type cells secrete IL-4, IL-5 and IL-13 facilitating a humoral immune response (Kopf, Le Gros et al. 1993; Magram, Connaughton et al. 1996; Murphy and Reiner 2002; O'Garra and Robinson 2004). Type 1 Th cells are directly cytotoxic against infected or transformed cells, and more importantly, provide helper signals to cytotoxic $CD8^+$ T lymphocytes (CTLs), and non-specific killer cells, such as NKs, natural killer T cells (NKTs), and MΦs. In contrast, Th2 cells produce B cell-stimulatory factors and promote the proliferation, survival, cytokine production and immunoglobulin (Ig) class switch in B cells.

Generally, the effect of DCs on the type of immune response is mainly based on their capability to secrete IL-12, which is associated with a Th1 response (Cella, Scheidegger et al. 1996; Cella, Jarrossay et al. 1999; Kalinski, Hilkens et al. 1999). In addition, since DCs are not documented to be IL-4 secreting cells, it is believed that the absence of IL-12 secretion by DCs

may, by default, drive the immune response toward Th2. However, DCs are capable of expressing the ligand for the OX40 molecule (a member of TNF receptor family), which has been suggested to be involved in promoting a Th2-type response through induction of IL-4 secretion upon stimulation of the OX40 receptor (CD134) on naïve T cells (Flynn, Toellner et al. 1998; Ohshima, Yang et al. 1998). There also is evidence based on *in vitro* experiments that the DC:T cell ratio determines the type of immune response, as high ratios of DC to T cells favor a Th1 response (Tanaka, Demeure et al. 2000). Furthermore, the antigen dose presented to T cells has been reported to play a role in the type of immune response induced; however, linking the dose level of an antigen to a specific type of immune response is controversial (Hosken, Shibuya et al. 1995; Menon and Bretscher 1998; Power, Wei et al. 1998; Ruedl, Bachmann et al. 2000; Boonstra, Asselin-Paturel et al. 2003). Recently, another type of T cells has been described as regulatory T cells (Tregs). The balance between Tregs and T effector cells may determine the outcome of an immune response. Regulatory T cells are mainly involved in tolerance against self antigens (natural Tregs) (Levings, Sangregorio et al. 2002; Bach 2003) or harmless non-self antigens (adaptive Tregs) such as intestinal flora (Khoo, Proctor et al. 1997; Bluestone and Abbas 2003). However, whether Tregs have any role in Th1 and Th2 decision is not yet clear.

In summary, as a non-optimal combination of cellular and humoral immunity may result in chronic disease, the balance between Th1- and Th2-type immune responses is likely the most critical aspect of immunity. To prevent chronic disease due to uncontrolled spread of the pathogen or autoimmune damage to the host tissues and immunopathology, the Th1:Th2 ratio of the immune responses needs to be properly matched to a particular type of pathogen.

1.1.2.2.2 Cross-presentation

In general, extracellular antigens are processed and presented through the MHC class II pathway driving a Th2 response, which results in antibody production (humoral immunity), whereas processing and presentation of intracellular antigens is handled via the MHC class I pathway driving a Th1 response, which results in a CTL response (cellular immunity). Cross-presentation could be defined as the potential of an APC to switch this process to a pathway that is not the default for that antigen; for instance, presentation of an extracellularly obtained antigen through the MHC class I pathway. The switch to the MHC class I pathway could happen by destruction of endocytic compartments and release of an extracellular antigen to the cytoplasm favoring cytosol delivery resulting in cross-presentation. The other potential mechanism is the entry and involvement of MHC class I molecules in endosomes and lysosomes during recycling in the cells, as these cellular compartments are carrying antigens, which are usually processed through the MHC class II pathway (Gromme, Uytdehaag et al. 1999; Kleijmeer, Escola et al. 2001). Cross-presentation is a critical aspect of immunity as it provides flexibility for APCs to properly present antigens of pathogens such as cytomegalovirus (CMV), which escape the usual presentation pathways as an immune evasion mechanism (Lutz 2006). Moreover, if an intracellular pathogen does not normally infect immune cells, APCs would still be able to present antigens obtained from an extracellular source to the proper pathway. It is also important for the presentation of tumor-associated antigens (TAAs) in the MHC class I pathway (Berard, Blanco et al. 2000; Nouri-Shirazi, Banchereau et al. 2000; Russo, Tanzarella et al. 2000), as tumoral cells usually break down outside the APCs. Although the capability of cross-presentation was initially assumed to be a characteristic of all APCs, it was later shown to be mainly a DC-specific property (Yrlid and Wick 2000; Jung, Unutmaz et al. 2002). This has given DCs a unique role in

the immune system as decision-making cells. Detailed knowledge of this capacity of DCs may lead to innovation of *in vitro* techniques in order to manipulate DCs for clinical purposes, in order to switch undesired types of immune responses into appropriate ones.

1.1.2.3 Migration

Migration primarily refers to the movement of mDCs toward the regional LNs, in order to present antigens to T cells, which then initiate an immune response. DC motility and migration is a strictly controlled process depending on the expression of chemokine receptors and chemokine production by DCs. Studies in mice and rats have shown that DCs constitutively migrate toward LNs even in the absence of pathogens or any damage to tissues (Pugh, MacPherson et al. 1983; Wu, Vremec et al. 1995; Liu, Zhang et al. 1998; Kamath, Pooley et al. 2000). In fact, this is part of their role in inducing T cell tolerance to self-antigens. However, when this motility is induced due to maturation of DCs after detection of non-self antigens, it results in immunogenicity.

In theory, without migration to the LNs, an immune response would be aborted or ended, followed by presentation of antigens in the periphery, which may result in tolerance. Chemokine receptors CCR7 and CXCR4 are two important receptors as their ligands, CCL19, CCL21, and CXCL12, are present in high concentrations in the LNs. In general, mDCs without these receptors fail to reach the LNs to deliver their message, resulting in inappropriate or inefficient immune responses.

1.1.3 Subtypes of dendritic cells

Different subtypes of DCs have been described based on their location, the type of pathogens they are exposed to, the type of immunity expected, or tolerance as their main function. It has been suggested that the existence of diverse subtypes of DCs could explain dissimilar or sometimes controversial functions of DCs observed in the immune system, as each subtype could behave differently in promoting or regulating an immune response.

Studies on DC subtypes have been largely performed in mice; however, murine DCs are not necessarily comparable to human DCs. A simplified and useful categorization of human DCs, based on limited studies on blood DCs or *in vitro* generated DCs, includes two types of conventional myeloid DCs (cDCs) originated from CMPs, and plasmacytoid lymphoid DCs (pDCs) originated from CLPs. Although there is considerable similarity and flexibility in these two subtypes of DCs, they show important differences in their phenotypes, surface markers and functions.

Conventional DCs are considered classic DCs with dendritic morphology and primarily located at sentinel barriers in an immature state with high phagocytic ability. They patrol tissues for pathogens, endocytose them, proceed with the maturation process, migrate to LNs, and introduce the antigens to T cells to initiate an immune response. They are capable of producing normal amounts of IFN-α/β and high amounts of IL-12 (Ito, Amakawa et al. 2004), and of inducing Th1- as well as Th2-type immune responses. Conventional DCs express most myeloid lineage receptors such as CD11b, CD11c, CD13, and CD33 and most TLRs (TLR1-6, and TLR8) with exception of TLR9, and are dependent on both GM-CSF and IL-4 for *in vitro* differentiation. In contrast, pDCs are round cells without dendrites and similar to plasma cells, more resembling a DC-precursor than a DC. In contrast to cDCs, pDCs are not phagocytic cells,

mainly located in lymphoid tissues and not normally seen in peripheral non-lymphoid tissues except at a time of inflammation. This occurrence is reported to be associated with inflammatory situations seen in autoimmune, allergic, or tumoral diseases (Farkas, Beiske et al. 2001; Wollenberg, Wagner et al. 2002; Hartmann, Wollenberg et al. 2003; Vermi, Bonecchi et al. 2003; Lande, Giacomini et al. 2004). Plasmacytoid DCs have limited potential for IL-12 secretion (Kadowaki, Ho et al. 2001; Krug, Towarowski et al. 2001) and high capacity of type I IFN production, as they were originally known as IFN-producing cells (IPCs) (Svensson, Johannisson et al. 1996; Grouard, Rissoan et al. 1997; Siegal, Kadowaki et al. 1999). Plasmacytoid DCs were first considered to be Th2-biased DCs (Rissoan, Soumelis et al. 1999); however, it was later shown that they can normally mature and induce both Th1- and Th2-type immune responses, although the Th1 response was IL-12-independent (Kadowaki, Antonenko et al. 2000; Fonteneau, Gilliet et al. 2003). In contrast to cDCs, pDCs do not express the surface markers related to the myeloid lineage; instead, they express CD2, CD4, CD5, CD7, and $CD123^{+}$ (IL-3Rα), which is the characteristic marker for isolation of pDCs from peripheral blood. They also express TLR1, 6, 10 and specifically TLR 7 and 9, which makes them responsive to single-strand (ss)RNA and CpG oligonucleotides (ODN) (Kadowaki, Ho et al. 2001). The differentiation of pDCs in *in vitro* cultures is highly dependent on IL-3, while addition of IL-4 to the culture may kill them. Of the two major subtypes of DCs in humans, cDCs seemed to be more efficient in cross-presentation than pDCs (Albert, Pearce et al. 1998; Albert, Sauter et al. 1998; Schnurr, Chen et al. 2005).

Langerhans cells are a well-characterized subtype of DCs. They are located in the epidermis with a specific structural characteristic of rod- or tennis racket-shaped Birbeck granules (Wolff 1967). Their most selective marker is CD207 or langerin (Valladeau, Ravel et al.

2000; Valladeau, Clair-Moninot et al. 2002). In comparison to other DCs, LCs have a longer life span, replenish themselves, and do not constitutively migrate toward LNs under steady state (Lucas and MacPherson 2002; Merad, Manz et al. 2002). Because of their similarities to residing iDCs in peripheral tissues, they are considered to be a member of the cDC family, although there are some arguments for recognizing them as a local variant of cDCs (Wolff 1972; Rowden 1981; Romani, Holzmann et al. 2003; Wilson and Villadangos 2004). Langerhans cells initially known as a nervous system-related cell, is the oldest known member of the DC family, and was placed in this family by Schuler and Steinman (Schuler and Steinman 1985; Romani, Holzmann et al. 2003).

There also is a population of DC-like cells in the germinal centre and primary B-cell follicles of all secondary lymphoid tissues known as follicular DCs (FDCs). Although FDCs share some aspects of morphology with DCs, they are different from T cell-priming DCs, belong to the stromal cells of lymphoid organs, and are closely related to fibroblast-like cells.

Tolerogenic DCs are suggested to be a subtype of DCs with a role in tolerance against self-antigens. However, they could be considered as a double-edge sword, because they can be used as a target by pathogens to evade immunogenicity or as a tool in the clinic to control autoimmune diseases (Steinman, Hawiger et al. 2003). It is still questionable whether tolerogenic DCs exist as an intrinsic subtype of DCs or are just generated by inhibition of DC maturation and modification to tolerogenic DCs under certain environmental signals.

1.1.4 Cytokine secretion by dendritic cells

Cytokine secretion is an important aspect of DCs and may determine the outcome of an immune response. As the component of the third signal in antigen presentation, cytokines can

switch the immune response toward Th1 or Th2 type of immunity or even create tolerance. Although the secretion of other cytokines has been reported for DCs, the most important cytokines secreted by DCs are type I IFN α/β, IL-1β, IL-6, IL-12, IL-23, IL-27, IL-18, TNF-α, IL-15, and IL-10.

Type I IFNs are a part of the innate antiviral response and have immunomodulatory capacities (Muller, Steinhoff et al. 1994; Roberts, Liu et al. 1998; Oritani, Medina et al. 2000; Tough 2004). Most nucleated cells can secrete type I IFNs but pDCs have a high natural capacity to secrete IFNs, and they were previously known as IPCs. Other types of DCs are also capable of secreting type I IFNs, but at lower levels, in response to TLR ligands such as LPS and poly I:C. Secretion of type I IFNs may promote the maturation of DCs toward Th1-polarized DCs.

Interleukin-1 consists of two different molecules, IL-1a and Il-1β, which both are pro-inflammatory cytokines with similar biological activities conducted via the IL-1 receptor (IL-1R) (Dinarello 1998; Dinarello 1998; Sims 2002). Although the role of IL-1 secreted by DCs is not understood yet, there is evidence that the administration of IL-1α may result in increased Th1- and strikingly decreased Th2-cytokine production in mice (Von Stebut, Ehrchen et al. 2003). In combination with other agents, IL-1 induces maturation and migration of DCs *in vitro* (Heufler, Koch et al. 1988; Roake, Rao et al. 1995; Cumberbatch, Dearman et al. 1997; Cumberbatch, Dearman et al. 1997; Stoitzner, Zanella et al. 1999; Kruse, Meinl et al. 2001; Guo, Zhang et al. 2003).

Interleukin-6 is a cytokine secreted by DCs in response to several maturation factors (Hope, Cumberbatch et al. 1995; Verhasselt, Buelens et al. 1997; Josien, Wong et al. 1999; Morelli, Zahorchak et al. 2001). It was demonstrated that it could have completely opposite roles in promoting or inhibiting a Th1-type immune response (Dodge, Carr et al. 2003; Pasare and

Medzhitov 2003; Fehervari and Sakaguchi 2004; Kubo, Hatton et al. 2004). Interleukin-6 can also affect the role of DC in antigen presentation as suggested elsewhere (Ming, Steinman et al. 1992; Akira, Taga et al. 1993; Gajewski, Renauld et al. 1995; Jonuleit, Kuhn et al. 1997; Drakesmith, O'Neil et al. 1998).

Interleukin-12 cytokine family consists of three structurally-related cytokines (Trinchieri, Pflanz et al. 2003), which play a role in the induction of Th1-type responses by promoting the production of IFN-γ by T cells. Each cytokine consists of two subunits (IL-12 [p35 and p40], IL-23 [p19 and p40], and IL-27 [p28 and EBI3]). Interleukin-12 induces both naïve and memory T cells while IL-23 and IL-27 have selective effects on stimulation of memory T cells and naive T cells, respectively. Interleukin-12 secretion by DCs is tightly correlated to induction of Th1-type immune responses (Macatonia, Hosken et al. 1995; Koch, Stanzl et al. 1996; Berberich, Ramirez-Pineda et al. 2003). Most DC maturation stimuli, such as LPS, poly I:C, CD40L and TLR ligands, are inducing secretion of IL-12 (Cella, Scheidegger et al. 1996; Verhasselt, Buelens et al. 1997; Thoma-Uszynski, Kiertscher et al. 2000; Morelli, Zahorchak et al. 2001; Doxsee, Riter et al. 2003). Dendritic cells also produce high levels of IL-23 and IL-27 in response to induction of maturation (Oppmann, Lesley et al. 2000; Pflanz, Timans et al. 2002; Nagai, Devergne et al. 2003; Sheibanie, Tadmori et al. 2004; Smits, van Beelen et al. 2004; Veckman, Miettinen et al. 2004). Overall, it seems that these three cytokines may play a similar role in the induction of a Th1-type immune response. However, the involvement of each may depend on the targets.

Interleukin-18 is a member of the IL-1 family and plays role in regulation of the type of immune response. Like IL-12, IL-18 activates NKs, stimulates T cells to proliferate and secrete IFN-γ and is considered a Th1-type cytokine (Zhang, Kawakami et al. 1997; Stoll, Jonuleit et al.

1998; Okamoto, Kohno et al. 1999; Kawakami, Qureshi et al. 2000; Pien, Satoskar et al. 2000). Interleukin-18 is secreted by DCs activated by microbial agents and DC maturation stimuli (Gardella, Andrei et al. 1999; Demeure, Tanaka et al. 2000; Giacomini, Iona et al. 2001; Dreher, Kok et al. 2002; Kolb-Maurer, Kammerer et al. 2003; Nagai, Devergne et al. 2003).

Tumor necrosis factor alpha is a well-known powerful cytokine having profound effects on both the innate and adaptive arms of the immune system (McDevitt, Munson et al. 2002). It is one of the most common agents used to mature DCs *in vitro* (Jonuleit, Kuhn et al. 1997; Rieser, Bock et al. 1997; Zhang, Mukaida et al. 1997; Schnurr, Then et al. 2000; Yu, Gu et al. 2003), while it is also a product of the maturation process in *in vitro* generated DCs (Lotze M. T. 2001).

Interleukin-15 is a cytokine structurally and functionally similar to IL-2, which binds to IL-2 receptors on T cells (Burton, Bamford et al. 1994; Grabstein, Eisenman et al. 1994). It has been reported to be secreted by LCs (Blauvelt, Asada et al. 1996) and *in vitro* generated mDCs (Jonuleit, Wiedemann et al. 1997) and promotes Th1-type immune responses through induction of CTL activity (Kuniyoshi, Kuniyoshi et al. 1999).

Interleukin-10 is a Th2-type cytokine, which is not usually secreted by DCs. However, there are reports showing the secretion of IL-10 by some DCs specifically localized in mouse bronchi and Peyer's patches corresponding to induction of a Th2-type immune response (Stumbles, Thomas et al. 1998; Iwasaki and Kelsall 1999).

1.1.5 Chemokine and chemokine receptors in dendritic cells

Chemokines represent a family of low-molecular weight cytokines involved in directional movement of leukocytes under physiological and pathological conditions. One group of chemokines is constitutively secreted in the LNs under physiological conditions and is

responsible for regular trafficking of lymphocytes such as mDCs and T cells toward LNs, whereas others are secreted in peripheral tissues to retain resident immune cells (such as iDCs) responsible for immune surveillance. Moreover, there are inflammatory chemokines, which are expressed in response to damage in tissues in order to recruit cells involved in inflammation such as neutrophils. Each chemokine acts as a ligand delivering its effect on target cells via a specific chemokine receptor; however, with exception of a few chemokine receptors, there is a high level of overlap and flexibility in this specificity. Different tissues secrete basal levels of diverse sets of chemokines, and the chemokine surface receptors on the cells influence the type of cells entering tissues.

Because of the nature of their life cycle, the function of DCs is highly dependent on the expression of different chemokine receptors mobilizing them toward the required location. Immature DCs express high levels of CCR1, CCR2, CCR4, CCR5, and CCR6 (Sozzani, Allavena et al. 1999; Sozzani, Mantovani et al. 1999; Allavena, Sica et al. 2000; Cavanagh and Von Andrian 2002), which direct them toward peripheral tissues. Upon infection and tissue damage, followed by DC maturation, the secretion of iDC ligands (CCL2, CCL3, CCL4, CCL5, CCL20, and IL-8) is increased by other cells in tissues, such as MΦs, resulting in attraction of more iDCs in order to retain the density of iDCs in the periphery. This helps with the phagocytosis process and compensates for the number of maturing and migrating iDCs. Throughout the maturation process, iDCs down-regulate the expression of CCR1, CCR2, CCR4, CCR5, and CCR6 (particularly in lung iDCs and LCs), and instead, up-regulate the expression of CCR7 and CXCR4 (Lin, Suri et al. 1998; Sallusto, Schaerli et al. 1998; Sozzani, Allavena et al. 1998; Yanagihara, Komura et al. 1998) facilitating the continuous flow of mDCs from peripheral tissues toward the constitutive secretion of CCL19, CCL21, and CXCL12 in the LNs

(Willimann, Legler et al. 1998; Forster, Schubel et al. 1999; Saeki, Moore et al. 1999; Arai, Yasukawa et al. 2000; Baekkevold, Yamanaka et al. 2001; Blades, Manzo et al. 2002; Scimone, Felbinger et al. 2004). Once in the LNs, mDCs themselves have the capability of secreting CCL19 to attract naïve T cells and central memory T cells, which both are also expressing high levels of CCR7. In contrast, iDCs secrete a profile of inflammatory chemokines such as IL8, CCL3, CCL4, and CCL5 in the periphery to recruit more immune cells to the site of infection (Foti, Granucci et al. 1999; Sallusto, Palermo et al. 1999; Padovan, Spagnoli et al. 2002). However, this profile is switched to chemokines such as CCL17, CCL18, CCL19, and CCL22, a mDC-specific profile attracting T cells, NKs, and B cells to the LNs (Imai, Nagira et al. 1999; Sallusto, Palermo et al. 1999; Vulcano, Albanesi et al. 2001).

With respect to circulating DCs, cDCs and pDCs both express high levels of chemokine receptors CCR2 and CXCR4, while the expression of CCR5 and CXCR3 (receptor for CXCL9, CXCL10, and CXCL11 chemokines) is high in pDCs and low in cDCs (Penna, Sozzani et al. 2001; Kohrgruber, Groger et al. 2004). However, in contrast to cDCs, pDCs do not respond to the chemoattractant effects of CXCL12 through CXCR4 unless in the presence of CXCR3 ligands (Krug, Uppaluri et al. 2002; Vanbervliet, Bendriss-Vermare et al. 2003).

In brief, chemokines play a critical role in DC motility, a crucial characteristic of DCs. As a result, a detailed understanding of chemokines and their receptors on DCs would improve the use of DCs in medicine as therapeutic tools.

1.1.6 Dendritic cells and tolerance

In general, there are two types of central and peripheral tolerance, which are mediated through four different mechanisms. Dendritic cells were demonstrated to play important roles in

both types of tolerance using different mechanisms (Finkelman, Lees et al. 1996; Adler, Marsh et al. 1998; Heath and Carbone 2001; Steinman, Hawiger et al. 2003). Central tolerance is mainly achieved in the thymus; resting DCs in the medulla of the thymus are involved in this process through a mechanism called negative clonal selection of auto-reactive T cells (Brocker, Riedinger et al. 1997; Volkmann, Zal et al. 1997; Fujimoto, Tu et al. 2002). However, tolerance against escaped self-antigens or harmless non-self antigens such as intestinal flora needs to be achieved in the periphery. The continuous migration of iDCs in steady state in absence of any inflammatory signal plays an important role in peripheral tolerance as they express low levels of MHC class I and II and co-stimulatory molecules resulting in T cell anergy, which is another mechanism for tolerance. With their cross-presentation potential, DCs could also induce tolerance by skewing the immune response toward a Th2 type as the third tolerance mechanism. Finally, DCs may promote peripheral tolerance by a mechanism called ignorance, where they keep the organ antigens away from their specific T cells in the periphery. Recently, it was suggested that DCs might also participate in suppression of immune responses and tolerance by inducing the generation of Tregs.

1.1.7 Dendritic cells and disease

The role of DCs in presentation of microbial antigens is obvious. They recognize diverse pathogens via pattern recognition receptors (PRRs) such as TLRs and undergo a distinct process of maturation based on the type of microorganism they are exposed to, leading to antigen presentation. In contrast, infectious agents may develop different mechanisms to evade the immune system through modulating DC functions. They can directly destroy DCs by injecting toxin (Steinman and Banchereau 2007), inducing apoptosis (Albert, Sauter et al. 1998) or

affecting different aspects of DC biology. One of the most prevalent mechanisms used to impair DCs is to completely or partially inhibit the maturation process in DCs. This mechanism has been reported for several viruses such as HCV, herpes simples virus, vaccinia virus (VV), human immunodeficiency virus (HIV), respiratory syncytial virus, CMV, varicella zoster, ebola, measles, and a number of non-viral pathogens such as *Coxiella burnettii*, *Salmonella typhi*, *Bacillus anthracis*, *Mycobacterium tuberculosis*, and *Borrelia garinii* (Salio, Cella et al. 1999; Granelli-Piperno, Golebiowska et al. 2004; de Graaff, de Jong et al. 2005; Khader, Partida-Sanchez et al. 2006; Coutanceau, Decalf et al. 2007; Hartiala, Hytonen et al. 2007; Steinman and Banchereau 2007; Yao, Li et al. 2007; Munir, Le Nouen et al. 2008). Several pathogens only affect and prevent the proper migration of mDCs toward the LNs by hampering the expression of CCR7 (Khader, Partida-Sanchez et al. 2006). They may also switch a Th1 protective immune response to a Th2 non-protective response (Steinman and Banchereau 2007).

With improvement in human life expectancy around the world, cancer is becoming one of the most significant health concerns. In parallel with a better understanding of the role of the immune system in controlling the growth of tumor cells, DCs as the main players in the initiation and coordination of an immune response, are a promising tool in cancer immunotherapy. In addition, as the number of circulating DCs may be reduced, the maturation process inhibited, or the expression of co-stimulatory molecules on DCs affected (Nestle, Burg et al. 1997; Troy, Davidson et al. 1998; Almand, Resser et al. 2000; Hoffmann, Muller-Berghaus et al. 2002; Della Bella, Gennaro et al. 2003; Wojas, Tabarkiewicz et al. 2004), DCs might be a target in cancer pathogenesis. In other studies, the migration of mDCs to the tumor site was reported to be inhibited, while iDCs were attracted to the site due to secretion of iDC-specific chemokines (Tsuge, Yamakawa et al. 2000; Vicari, Treilleux et al. 2004). Conclusively, mDCs loaded with

TAAs appear to be an efficient tool in cancer immunotherapy; however, several barriers have limited these efforts.

DCs are responsible for inducing tolerance to harmless environmental antigens such as pollens and foods. In allergic reactions, a Th2-type immune response develops involving Th2 cells expressing IL-4, IL-5, and IL-13, which in turn results in secretion of IgE. The generation of this complex is responsible for most allergic symptoms. The mechanism by which this immune response pathway is activated is not clear, but inhibitory effects of pollen on the secretion of IL-12 by DCs has been suggested to be a contributing factor (Traidl-Hoffmann, Mariani et al. 2005). If this mechanism would be elucidated, it might lead to manipulation and application of DCs as an approach to control allergies.

Since DCs are responsible for both immunity against pathogens and tolerance to self-antigens, they have been at the center of attention with respect to autoimmune diseases. Impairment of DCs in generating tolerance may potentially cause auto-reactions due to excessive presentation of self-peptides by DCs or defects in down-regulation of activated DCs (Lutz 2006). In addition, other studies recently suggested overproduction of certain cytokines by DCs such as TNF-α, type I IFNs, and IL-23, to be a contributing factor (Asselin-Paturel and Trinchieri 2005; Lowes, Chamian et al. 2005; Banchereau and Pascual 2006; Hue, Ahern et al. 2006). However, more studies should be designed to clarify the exact process, in which DCs may be involved. This might eventually lead to a treatment for autoimmune disorders by manipulation of DCs.

Dendritic cells from a graft can migrate to the recipient and activate an allo-stimulatory immune response; in contrast, DCs from the recipient can also induce a graft-versus-host disease by presenting antigens to the donor organ's T cells. The focus in transplant immunology could be to deplete graft DCs, which would avoid rejection, or to induce tolerance against the donor's

antigens by the recipient's DCs. In other words, if the host's DCs could induce tolerance against the donor antigens similar to self-antigen, a graft can be protected; however, DCs from the donor organ may need to be manipulated or depleted as well to protect the host. One approach might be the *in vitro* generation of tolerogenic DCs by using the recipient's iDCs carrying the donor's antigen, which could result in anergy following antigen presentation to the recipient's T cells. Another strategy can be the generation of iDCs with reduced capacity for production of cytokines such as IL-12.

1.1.8 Dendritic cell applications in medicine and challenges

In an era when immunology plays a significant role in almost any disease, DCs as the main player in the initiation of an immune response have attracted remarkable attention. Indeed, they are responsible for most of the adaptive response outcomes such as specific immunogenicity, tolerance, and allergy. In addition, their unique role in cross-switching antigens between class I and class II pathways has given them the capability of skewing the immune response toward a Th1- or Th2-type response as required.

With respect to cancer and infectious diseases, the goal is to generate an enhanced Th1-type immune response against TAAs and microbial targets, which have escaped from or weakly presented to the immune system, resulting in unsuccessful destruction of the tumor or infected cells. However, in autoimmune diseases and transplant rejection, manipulation of DCs in order to switch back or induce tolerance toward self and donor or recipient's antigens is the objective. Finally, DCs could be a useful tool to control the Th2-type immune response in allergic diseases.

The use of DCs in immunotherapy of cancer has been at the centre of attention in recent years. However, the clinical application of DCs, regardless of all their capabilities, has been a big challenge and encountered numerous barriers and obstacles, which are discussed below.

1.1.8.1 Dendritic cell generation and maturation

By introducing a specific antigen from tumor cells or infectious pathogens to DCs, we expect to see proper antigen processing, optimal DC maturation, migration of DCs toward LNs, and finally effective antigen presentation leading to a qualitatively distinct immune response.

To achieve this goal, the first step would be the *in vitro* generation of DCs, which still needs to be improved and evaluated in more detail. In the context of DC generation, maturation is important and crucial in DC therapy against cancer and infectious diseases as mDCs induce stronger immune responses than iDCs.

In humans, there are four sources for *in vitro* generation of DCs. The most commonly used cells are circulating $CD14^+$ monocytes in peripheral blood typically converting to cDCs. The other source is $CD34^+$ stem cells, which are primarily present in cord blood and considered precursors for pDCs. Circulating DCs consisting of both kinds of cDCs ($CD11c^+$, $CD123^-$) and pDCs ($CD11c^-$, $CD123^+$) (MacDonald, Munster et al. 2002) are the third constitutive source, but represent a very small pool of DCs. They are usually characterized and isolated based on surface marker expression. The recently used source for generating DCs in cancer patients is bone marrow-originated $CD34^+$ cells, which can be differentiated into both types of cDCs and pDCs.

To generate DCs *in vitro* different cytokines have been used. Granulocyte macrophage-colony stimulating factor (GM-CSF) in combination with IL-4 is the most common cocktail applied to generate human cDCs from monocytes (Rossi, Heveker et al. 1992; Sallusto and

Lanzavecchia 1994; Akagawa, Takasuka et al. 1996). Granulocyte macrophage-CSF is a cytokine produced by many cell types such as MΦs, T cells, and fibroblasts. It promotes the development and differentiation of murine bone marrow stem cells to DCs (Inaba, Inaba et al. 1992); however, it has to be combined with IL-4 to generate cDCs from human monocytes. Interleukin-4 is a multifunctional cytokine, which is crucial in generating human cDCs, as it inhibits differentiation to MΦs and skews the process towards generation of DCs (Sallusto and Lanzavecchia 1994). Interleukin-3 is a cytokine used for generation of pDCs; however, in monocyte culture it induces the generation of Th2-biased DCs (Ebner, Hofer et al. 2002). Interleukin-15 and transforming growth factor beta-1 (TGF-β1) are two cytokines generating Mo-DCs with LC features; nevertheless, LC-like cells generated by IL-15 lack the Birbeck granules (Mohamadzadeh, Berard et al. 2001; Guironnet, Dezutter-Dambuyant et al. 2002). Of other cytokines involved in DC generation, feline McDonough sarcoma (a family of DNA sequences; fms)-like tyrosine kinase ligand (Flt3L) is generally used *in vivo* to expand the population of circulating DCs in human blood before isolation of DCs (Maraskovsky, Daro et al. 2000; Pulendran, Banchereau et al. 2000) or in *in vitro* culture of human $CD34^+$ progenitors to pre-expand DC precursors (Arrighi, Hauser et al. 1999). Plasmacytoid DCs can be isolated from PBMCs as $CD11c^-$ $CD123^+$ cells; however, they can be generated *in vitro* by culturing $CD34^+$, isolated from cord blood or bone marrow, with a cytokine cocktail mainly including GM-CSF, Flt3L, and IL-3. As fully matured DCs are required for clinical applications, TNF-α is the most common cytokine added to DC cultures in order to mature iDCs *in vitro*. TNF-α is involved in many common immune reactions *in vivo* such as inflammation, apoptosis, and stress response.

Finally, specific attention should be paid to the protocol of DC generation and maturation, as subtype, level of maturity, and cytokine profile of generated DCs may determine the type of immune response they induce.

1.1.8.2 Dendritic cell transfection

In theory, if microbial antigens or TAAs are properly presented to T cells such that a Th1-type immune response is induced, the pathogen or tumor cells are specifically eliminated by the immune system. Based on this fact, DCs were generated *in vitro*, loaded by target antigens, and re-injected to the host to elicit a specific cellular immune response. However, loading DCs with nucleic acids was a serious challenge and although several new protocols were established, it is still a significant obstacle. Viral vectors initially thought to be the best tools to transfect DCs, as they mimic the natural course of infection. However, their use in clinical applications was hampered by the need to avoid introducing viral genes into individuals. Even with respect to *in vitro* studies of specific target genes, the presence of viral vector genes may synergistically enhance or reduce the intrinsic effect of the target protein. In contrast, the use of non-viral methods such as chemical reagents in combination with DNA plasmids generally resulted in unacceptable transfection efficiency in DCs. When electroporation in combination with plasmid DNA was used, the viability of DCs was significantly reduced, while with other protocols insufficient transfection efficiency was reported. To date, the highest non-viral transfection efficiency of plasmid DNA has been achieved by nucleofection. This method resulted in up to 56% efficiency but only 37% viability of the transfected DCs after 24 h (Lenz, Bacot et al. 2003) to almost 0% after 48 h. In contrast, several studies have demonstrated efficient transfection of DCs with mRNA (Van Tendeloo, Ponsaerts et al. 2001; Lundqvist, Noffz et al. 2002; Mu,

Gaudernack et al. 2003; Ueno, Tcherepanova et al. 2004; Michiels, Tuyaerts et al. 2005). However, protein expression levels were generally either low or not discussed, and little information was provided with respect to short- and long-term cell viability; as was recently concluded in an extensive review on this subject, strategies for efficient transfection still need to be established for human DCs (Grunebach, Muller et al. 2005), and in particular Mo-DCs.

The *in vitro* generation and transfection of DCs can also determine the type of immune response induced; thus these manipulations should be well designed to achieve the appropriate type of the immune response. If a Th1-type immune response is dominant and protective, the antigen should be generated endogenously, by transfecting DCs with nucleic acids. Conversely, for a Th2-type immune response, *in vitro* produced proteins should be transferred into DCs, usually resulting in a better chance of processing through the MHC class II pathway.

1.1.8.3 Dendritic cell delivery

The ultimate goal for DCs applied in the clinic would be the successful delivery to the local LNs. The route of administration is an important variable in this regard for *in vitro* generated DCs. In addition to other standard routes of vaccine administration, intranodal delivery or release of DCs to the LN-rich areas could have particular significance for the efficiency of immunotherapy. Indeed, many studies are currently focused on *in vivo* migratory capacities of DCs injected into animals (Bedrosian, Mick et al. 2003; Yoneyama, Matsuno et al. 2004; Huck, Tang et al. 2008).

In spite of all challenges, DC immunotherapy has already been tested in the clinic because of an urgent need for treatment of cancer; however, not many promising results have been achieved yet, with exception of occasional tumor regression, particularly in patients with

melanoma (Steinman and Banchereau 2007). This indeed led researchers to reassess the barriers to be overcome; the key ones are the problems discussed above (Figdor, de Vries et al. 2004; Melief and van der Burg 2008). Finally, it is important to state that in infectious diseases, a DC vaccine can only be applied for prevention or at early stages of infection, as the induction of a strong Th1 response in a chronic disease may result in massive destruction of the chronically infected organ.

1.2 Hepatitis C

1.2.1 General considerations and epidemiology

Hepatitis C is a blood-borne infection of the liver that was known as a non-A, non-B transfusion-associated hepatitis until 1989, when the causative agent was cloned and named hepatitis C virus (HCV) (Choo, Kuo et al. 1989). Acute HCV infection is usually a benign asymptomatic disease with no or non-specific symptoms. However, a low percentage of infected cases (20-25%) clears the virus (Alter 2005) and in more than 70%, the infection becomes persistent (Liang, Rehermann et al. 2000; Lauer and Walker 2001) and continues as a chronic-active hepatitis causing a progressive liver fibrosis. Chronic hepatitis C (CHC) infection results in cirrhosis in 15-30% of cases (Tremolada, Casarin et al. 1992; Tong, el-Farra et al. 1995) and/or hepatocellular carcinoma (HCC) in 1-5% of cases (Lauer and Walker 2001). Hepatocellular carcinoma is responsible for half of the liver cancers, which is the third killer cancer in the world (8.8% of all cancers) (WHO) (Alter 1997; Alter and Seeff 2000). After the development of a hepatitis B vaccine, HCV, one of the confirmed oncogenic viruses is becoming the leading cause of HCC and liver transplantation (El-Serag and Mason 2000; Hassan, Hwang et al. 2002; Rosen 2002; Koike 2005). According to a WHO report in 1999 (http://www.who.int/en/), at least 3.1% of the world population is chronically infected with this virus. The prevalence rate differs from 0.1% in Iceland and Norway to 18.1% in Egypt (WHO). It is estimated that each year 3 to 4 million people are newly infected (WHO, 2000 report), and then play important roles as carriers and reservoirs of the disease (Major and Feinstone 1997; Moradpour, Brass et al. 2002; Meylan, Curran et al. 2005). In Canada, it is estimated that about

250,000 people are currently infected with HCV, with an estimated 8000 new cases each year, of which only 30% know they have the virus (http://www.hc-sc.gc.ca); thus, virus transmission remains a significant health concern.

Hepatitis C virus spreads primarily by direct contact with human blood. Transfusion-associated infection was the main cause of infection before HCV testing became available. However, the major causes of infection worldwide are different today. In developed countries, use of injection drugs and much more rarely, high-risk sexual activities or medical (acupuncture) and cosmetic (tattoos and piercing) procedures are important sources of infection (Craxi and Licata 2006), while in developing countries, unsafe therapeutic injections and contaminated blood are the most common cause of new infections (2004). The other lower-risk methods of transmission are needle sticks in health workers and perinatal or vertical transmission (children born from HCV positive mothers; 4-7%).

Difficulties in *in vitro* culture of the virus and lack of a good animal model have hampered research on HCV. Chimpanzee is the only good animal model available; however, costs and ethical issues have limited the use of this model (Bartenschlager and Lohmann 2000; Moradpour, Brass et al. 2002). Another animal model is the severe combined immunodeficiency (SCID) mouse engrafted with human liver tissue (Ilan, Burakova et al. 1999) and transgenic mice; however, the replication of the virus in these models is limited (Moradpour, Brass et al. 2002).

Overall, with a five times higher prevalence (3.1%) in comparison to HIV infection (0.6%; UNAIDS 2008 Report), lack of vaccine, inefficient therapy, and limitations in research, a further increased rate in mortality from HCC associated with CHC can be expected during the next 20-25 years.

1.2.2 Hepatitis C virus

Hepatitis C virus is a hepatotropic virus, which primarily attacks the liver and hepatocytes. Other extrahepatic compartments are also presumed to be potential infection sites, which could act as hidden locations for the virus. Hepatitis C virus is the sole member of the genus Hepaciviruses within the family of Flaviviridae. It is a small (30-80 nm), enveloped plus-sense ssRNA virus, with a narrow host range consisting of humans and chimpanzees.

The virus life cycle is entirely cytoplasmic and starts by entering the cytoplasm through a receptor binding process. The RNA is then uncoated and all viral proteins are translated using the cellular machinery. The viral RNA replicates to a negative-strand RNA, and non-structural (NS) proteins promote the generation of a new positive strand, resulting in more viral RNA via double strand (ds)RNA intermediates (Moradpour, Brass et al. 2004). Subsequently, the viral RNA is packed into core protein forming viral capsids, enveloped, and released from the endoplasmic reticulum (ER)-Golgi system into the cytoplasm. Finally, the packaged virus is released from the cells.

1.2.2.1 Virus genome

The genome is about 9.6 kb in length and contains an open reading frame (ORF) flanked by 5'- and 3'-untranslated regions (UTRs) (Choo, Kuo et al. 1989). The highly conserved 5'UTR contains an internal ribosomal entry site (IRES), which mediates cap-independent translation of the ORF (Brown, Zhang et al. 1992; Rijnbrand and Lemon 2000). The 3'UTR is also a highly conserved RNA element and is essential for viral replication *in vivo* (Kolykhalov, Feinstone et al. 1996). It interacts with several cellular proteins including polypyrimidine tract binding protein (PTB), which may contribute to the enhanced translational efficiency observed for RNAs

containing 5'IRES and the HCV 3'UTR; the X-tail portion at the very 3' end appears to enhance IRES-dependent translation (Cheng, Chang et al. 1999; Oh, Sheu et al. 2000). The HCV ORF codes for a polyprotein of 3011-3022 amino acids that is co- and post-translationally cleaved by viral and cellular proteases (Major and Feinstone 1997) into four structural (core, E1, E2, and p7) and six non-structural (NS2, NS3, NS4A, NS4B, NS5A, and NS5B) proteins (Shimotohno, Tanji et al. 1995; Tanji, Kaneko et al. 1995; Lohmann, Koch et al. 1996; Major and Feinstone 1997; Penin, Dubuisson et al. 2004) (Fig. 1.2). The proteolytic process at the junctions between structural proteins and p7-NS2 is mediated by cellular signal peptidase, while NS2/NS3 protease activity is responsible for cleavage between NS2 and NS3 (Hussy, Langen et al. 1996; Pallaoro, Lahm et al. 2001; Lemberg and Martoglio 2002; Weihofen, Binns et al. 2002; Okamoto, Moriishi et al. 2004). Finally, NS3 cleaves the remaining four junctions between NS proteins (Bartenschlager, Ahlborn-Laake et al. 1994; Lin, Pragai et al. 1994). It has later been reported that one protein can also be translated in a second reading frame by a frameshift event in the core region of the HCV genome (Walewski, Keller et al. 2001; Xu, Choi et al. 2001). This protein was named alternative reading frameshift protein (ARFP) and might be considered a structural protein.

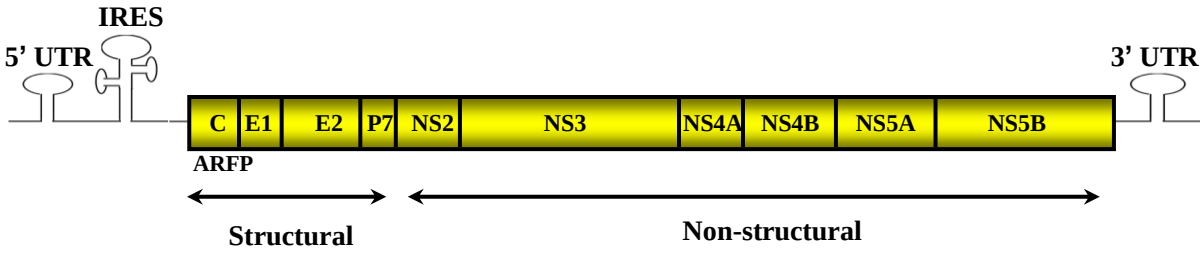

Figure 1.2. The schematic orientation of the HCV genome and proteins.

1.2.2.2 Structural proteins

Core: The core protein located at the N-terminus of the polypeptide is one of the most conserved proteins of HCV. It interacts with viral RNA and constitutes the viral capsid (Baumert, Ito et al. 1998; Yasui, Wakita et al. 1998). The C-terminal end of core is critical for correct folding of protein. Core protein in mature form may contain secondary structures and exists as a large multimer, which is likely involved in the virus assembly process (Matsumoto, Hwang et al. 1996; Nolandt, Kern et al. 1997; Irshad and Dhar 2006). Core can be efficiently self-assembled *in vitro* along with envelope proteins to form virus-like particles (VLPs) (Nolandt, Kern et al. 1997), while in combination with the viral genome it is encapsulated in a lipid envelope containing two envelope proteins 1 and 2 (E1 and E2) to form the infective virion (Kunkel, Lorinczi et al. 2001; Acosta-Rivero, Rodriguez et al. 2004). In addition, core interacts with several cellular proteins including the cytoplasmic domain of the TNF receptor, lymphotoxin-β receptor, protein 53, and RNA helicase (Chen, You et al. 1997; Zhu, Khoshnan et al. 1998; Lu, Lo et al. 1999; Mamiya and Worman 1999). It also plays a regulatory role in viral and cellular gene expression resulting in steatosis and oncogenesis through a number of cellular signalling pathways (Ray, Lagging et al. 1996; Moriya, Yotsuyanagi et al. 1997; Chang, Yang et al. 1998; Marusawa, Hijikata et al. 1999; Hahn, Cho et al. 2000; Lai 2002; Yamaguchi, Tazuma et al. 2005). The lipid droplet formation in hepatocytes of transgenic mice expressing core protein is compatible with observations in HCV-infected livers with HCC (Knipe D. M. 2001). This fact proposes a potential role for core protein in HCC through interference with the lipid metabolism pathway. Core also has dual roles with opposite actions on apoptosis favouring viral immune evasion (Marusawa, Hijikata et al. 1999; Hahn, Cho et al. 2000; Siavoshian, Abraham et al. 2004; Nguyen, Sankaran et al. 2006), and plays an inhibitory role in production of IFN-α by

pDCs (Dolganiuc, Chang et al. 2006). Furthermore, the presence of high levels of core protein circulating in peripheral blood during the early stage of the infection suggests a role for core in suppression of the immune response (Urushihara, Sodeyama et al. 1994; Aoyagi, Ohue et al. 1999).

Alternative reading frame protein (ARFP) or frameshift (F) Protein: Alternative reading frame protein (ARFP) or F protein is encoded by a reading frame overlapping the core sequence and expressed by a frameshift during translation (Walewski, Keller et al. 2001; Xu, Choi et al. 2001; Varaklioti, Vassilaki et al. 2002; Xu, Choi et al. 2003). It is a labile protein, consisting of up to 160 amino acids. Similar to core, ARFP protein is associated with the ER, suggesting that it may participate in HCV morphogenesis (Xu, Choi et al. 2003). The ARFP is a potential target for cell-mediated immunity in hepatitis C patients (Bain, Parroche et al. 2004).

Envelope proteins: Envelope proteins 1 and 2 are highly glycosylated type-1 transmembrane proteins on the virions' surface and responsible for binding and entry of the virus (Knipe D. M. 2001). They form a non-covalent heterodimer (Deleersnyder, Pillez et al. 1997), and in contrast to most viral envelope proteins, both have an ER retention signal (Cocquerel, Meunier et al. 1998). This feature may help the virus escape from immune attack. There is evidence that E2 modulates the IFN-α response (Taylor, Shi et al. 1999) and mediates viral attachment (Rosa, Campagnoli et al. 1996). The N-terminus of E2 contains a hypervariable region (HVR1) (Kato, Ootsuyama et al. 1992), which is likely to reflect adaptation of the virus to the host immune responses and selection of immune escape variants (Weiner, Geysen et al. 1992). The HVR1 region of E2 is not identical in any of the virus isolates, and is a target for neutralizing antibodies (Knipe D. M. 2001).

To date, several receptors for virus entry have been identified, which are involved in virus entry mostly through interaction with envelope proteins, especially E2. This list includes CD81 (Pileri, Uematsu et al. 1998), low density lipoprotein (LDL) receptor (Agnello, Abel et al. 1999), claudin-1 (CLDN1) (Evans, von Hahn et al. 2007), occludin (OCLN), scavenger receptor class B type I (SR-BI) (Scarselli, Ansuini et al. 2002), mannose-binding lectins DC-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) for DCs and liver SIGN (L-SIGN) for hepatocytes (Lozach, Lortat-Jacob et al. 2003). In the blood stream, virions combine with LDL, which covers their surface, protects them against antibodies and facilitates a viral glycoprotein-independent entry process through cellular LDL receptors (Agnello, Abel et al. 1999). Claudin-1 is highly expressed in the liver and essential for HCV entry. Occludin is an essential HCV cell entry factor as its expression in non-permissive murine cells enhances the uptake of HCV-VLPs, whereas its silence in permissive cells inhibits the infection by HCV-VLPs (Ploss, Evans et al. 2009). The interaction between the highly selective human SR-BI and E2 has been shown to be specific (Barth, Cerino et al. 2005); however, direct interaction between SR-BI and the E1/E2 heterodimer has not been reported yet.

Protein 7: Protein 7 is a small membrane-spanning protein, the exact role of which is not known; however, it may function as an ion channel (Griffin, Beales et al. 2003). In addition, it may mediate cation permeability across membranes, an important event for virus release or maturation (Carrere-Kremer, Montpellier-Pala et al. 2002), which is essential to viral infectivity (Sakai, Claire et al. 2003).

1.2.2.3 Non-structural proteins

Non-structural proteins play an important role in viral RNA replication and proteolytic processing of the polyprotein (De Francesco, Neddermann et al. 2000). There are six NS proteins in HCV.

Non-structural proteins 2 and 3: The NS2/NS3 complex is a metalloprotease that cleaves NS2 from NS3, which subsequently is responsible for cleavage of other NS proteins (Grakoui, McCourt et al. 1993; Hijikata, Mizushima et al. 1993; Pieroni, Santolini et al. 1997). This cleavage is shown to be essential for *in vivo* replication of HCV and persistent infection of HCV in chimpanzees (Kolykhalov, Mihalik et al. 2000). Non-structural protein 2, an integral membrane protein targeted to the ER, is inserted into the membrane only when expressed in the form of NS2/NS3 and subsequently cleaved from NS3 (Santolini, Pacini et al. 1995; Yamaga and Ou 2002). Although it is not required for RNA replication, *in vitro* interaction of NS2 with other HCV proteins has suggested a potential role of the NS2 complex in RNA replication (Dimitrova, Imbert et al. 2003; Welbourn, Green et al. 2005; Kiiver, Merits et al. 2006; Welbourn and Pause 2007). Recently, a role for NS2 in up-regulation of IL-8 through the nuclear factor-kappa B (NFKB) pathway was described (Oem, Jackel-Cram et al. 2008). In addition, the effect of NS2 in up-regulation of sterol regulatory element-binding protein 1c (SREBP-1c) and fatty acid synthase (a well established target gene of SREBP-1c) transcription has been suggested to be a contributing factor in liver steatosis (Oem, Jackel-Cram et al. 2008).

Non-structural protein 3 is a highly conserved 630-amino-acid protein, which has two domains with serine protease (N-terminal) and helicase activity (C-terminal). The NS3 protease activity is most efficient when combined with NS4A and is responsible for all of the downstream cleavages of the NS proteins (Grakoui, McCourt et al. 1993). The helicase activity is presumably

involved in unwinding of duplex RNA during replication (Kim, Morgenstern et al. 1998). The NS3 enzymatic activities are used as targets to develop anti-HCV drugs.

Non-structural proteins 4A and 4B: Non-structural protein 4A is a small protein containing three distinct domains. The N-terminal and central domains of NS4A form a dimer with NS3 and are a co-factor for NS3 serine protease activity as well as recruitment of NS3 to membranes (Failla, Tomei et al. 1994; Glenn 2005). The C-terminal acidic domain of NS4A has been recently shown to be critical in viral replication (Lindenbach, Pragai et al. 2007). It has also been suggested that NS4A in a complex with NS4B may be involved in inhibition of transport of newly synthesized MHC class I molecules to the cell surface (Konan, Giddings et al. 2003). The NS4B is a larger protein in comparison to NS4A but its role in the HCV life cycle was unknown for a long time. However, a role for NS4B in membrane-associated replication of virus was suggested (Hugle, Fehrmann et al. 2001). This observation was then supported by reporting a critical role for NS4B in inducing the formation of an intracellular membrane, called membranous web, as potential site for virus assembly (Gosert, Egger et al. 2003). In addition, NS4B, in combination with NS3, may indirectly influence the function of NS5B and regulate HCV replication, according to the stage of the viral life cycle (Piccininni, Varaklioti et al. 2002; Gosert, Egger et al. 2003; Einav, Elazar et al. 2004).

Non-structural proteins 5A and 5B: Non-structural protein 5A is a serine phosphoprotein (Koch and Bartenschlager 1999) localized to the periplasmic membrane fraction (Tanji, Kaneko et al. 1995; Tarr, Lin et al. 1996). A role in membrane-associated RNA replication was suggested for NS5A, which is likely involved in resistance against IFN therapy via inhibition of protein kinase R (PKR) (Koch and Bartenschlager 1999). NS5B is a well-studied protein known as the viral RNA-dependent RNA polymerase (RdRp), which lacks a

proof-reading function (Oh, Ito et al. 1999). It has a hydrophilic domain at its C-terminal mediating insertion into membranes (Yamashita, Kaneko et al. 1998). Since the specific viral polymerization activity of NS5B does not exist in humans, this protein is another target for anti-HCV drugs. Regardless of the unique enzymatic activity of NS5B, *in vivo* studies in chimpanzees have shown a combined requirement of the NS2/NS3 protease, NS3/NS4A serine protease, NS3 NTPase and helicase, and NS5B polymerase activities for viral infectivity. These reports agreed with *in vitro* studies (Knipe D. M. 2001).

The core, E1, and NS5 proteins, which are all well-conserved, have been used as the basis for classification of HCV isolates to six distinct genotypes, 1 (a, b, c), 2 (a, b, c), 3 (a, b), 4a, 5a, and 6a, and more than fifty subtypes (Simmonds, Alberti et al. 1994; Bukh, Miller et al. 1995; Major and Feinstone 1997).

1.2.3 Immunovirology and immune evasion strategies

A viral infection could result in elimination of the host, elimination of the virus, or persistence of the infection. From a philosophical point of view, the latter could be the ideal situation for the virus in order to continue its presence while the host also survives, although not in an entirely healthy condition with long-term complications. In general, in the case of a cytopathic virus, one of the first two happens, whereas for a non-cytopathic virus (such as HCV) persistence is the choice.

The first line of defense to a viral infection is a non-specific innate antiviral immune response. This step involves IFNs, NKs, MΦs, and apoptosis. In the next step, as the adaptive responses require specific antigen presentation to effector cells, DCs, monocytes, MΦs, B cells, and T cells are involved. The end result of antigen presentation to T cells would be the secretion

of antibodies (humoral immunity) and/or a broad multi-specific $CD4^+$ and $CD8^+$ T cell response (cellular immunity). Concerning HCV infection, the interaction of the host's immune response and HCV is very complex resulting in an acute resolving or a chronic persistent infection. For the first two weeks, the virus replicates quickly followed by a period of slower or stable replication (Hoofnagle 2002; Bertoletti and Ferrari 2003). The specific humoral and cellular responses start 1-3 months after exposure to the virus (Bertoletti and Ferrari 2003; Rehermann and Nascimbeni 2005), while at the same time virus disappears from the serum, followed by viral clearance from the liver (Heller and Rehermann 2005). However, virus clearance might never happen even in so-called resolved infection (Bertoletti and Ferrari 2003; Pham, MacParland et al. 2004). The cellular immune response usually starts earlier than the antibody response and it is believed that early and strong responses by $CD4^+$ and $CD8^+$ T lymphocytes is correlated to viral clearance (Gruner, Gerlach et al. 2000; Orland, Wright et al. 2001; Thimme, Oldach et al. 2001; Wodarz 2003; Heller and Rehermann 2005; Sarobe, Lasarte et al. 2006). Accordingly, Th1 cytokine secretion is associated with recovery from infection, as it stimulates a CTL response (Afdhal 2004; Mondelli, Cerino et al. 2005). Overall, the main strategy that HCV uses to evade the immune system is not clear yet, but there is evidence that most immune defense components are compromised.

1.2.3.1 Innate immunity

As a component of innate immunity, NKs are capable of eliminating virus through direct killing and cytokine production. Inhibition of NKs due to the binding of HCV E2 protein to CD81 on the cell surface was one of the earliest reported possible mechanism resulting in the failed immune response to HCV (Corado, Toro et al. 1997; Crotta, Stilla et al. 2002; Tseng and

Klimpel 2002). Accordingly, the decrease of NKs and NKTs in the liver was shown to favor histological progression of disease (Kawarabayashi, Seki et al. 2000; Deignan, Curry et al. 2002). The role of NKs and NKTs has been highlighted as HCV-infected chimpanzees eliminate the virus through an innate immune response without any detectable T-cell response (Thomson, Nascimbeni et al. 2003). However, the *in vitro* cytolytic function of NKs does not appear to be impaired in CHC patients, while higher levels cytolytic activity of NKs are associated with less liver fibrosis (Morishima, Paschal et al. 2006). In contrast, impaired NKs derived from CHC patients could modify DCs to generate IL-10 producing $CD4^+$ T cells, which in turn, skews the immune response toward a Th2 type, enabling virus to evade cellular responses (Jinushi, Takehara et al. 2004). By taking their role in activation of DCs (Piccioli, Sbrana et al. 2002) into account, it seems that dysfunction of NKs is involved in evasion of HCV and not just because of its role in innate immunity, but also due to its role in the modulation of adaptive immunity.

Interferons constitute another important component of the innate anti-viral defense system. Type I IFNs are secreted by almost every nucleated cell but most commonly by pDCs. After binding to their receptor, IFNs promote the phosphorylation of tyrosine kinase (Tyk)-2 and Janus Kinase (Jak)-1 (Honda, Yanai et al. 2005). This, in turn, causes phosphorylation of signal transducers and activators of transcription (STAT)-1 and STAT-2, which after translocation to the nucleus and combination with other proteins leads to activation of IFN-stimulated genes (ISGs)(Taniguchi, Ogasawara et al. 2001). Interferon-stimulated gene products impart antiviral and immunomodulatory activities that limit virus replication and spread (Sen 2001; Katze, He et al. 2002). Interferons also play a role in activation of other cells such as MΦs and NKs, maturation of DCs, bias of the immune response toward Th1, and enhancement of MHC-I and II expression on the cell surface.

Hepatitis C virus directly interferes with intracellular pathways and inhibits the activation of cellular genes including type I IFN genes (Foy, Li et al. 2003; Duong, Filipowicz et al. 2004). The HCV NS3/4A can attenuate the signaling mediated through retinoic acid-inducible gene 1 (RIG-1) and TLR-3 pathways by cleaving their adaptor proteins, caspase recruitment domain adaptor inducing IFN-β (CARDIF) and Toll/IL1 receptor (TIR) domain-containing adaptor protein inducing IFN-β (TRIF) (Li, Foy et al. 2005; Meylan, Curran et al. 2005). It can also disrupt the process of STAT1 phosphorylation (Helbig, Yip et al. 2008). Inhibition of these pathways inhibits the expression of IFN-α and IFN-β as well as IFN-independent activation of ISGs. Core protein is reported to have an inhibitory effect on the Jak-STAT signaling pathway through inducing the expression of suppressor of cytokine signaling-3 (SOCS-3) (Bode, Ludwig et al. 2003). Likewise, an inhibitory role of core in production of IFN-α by pDCs was suggested in one study (Dolganiuc, Chang et al. 2006). In a normal course of viral infection, IFNs shut off the translation of viral proteins through a protein kinase called PKR. PKR is one of the ISGs, which is induced by IFN and can be activated by ds-RNA. After activation, it undergoes an autophosphorylation process and then phosphorylates other substrates such as eukaryotic initiation factor 2-α (eIf2-α) and the inhibitor of NFKB (IκB). This effect results in the inhibition of protein translation. Hepatitis C virus E2 and NS5A can bind to PKR and inhibit this function leading to production of viral proteins (Taylor, Shi et al. 1999; Podevin, Sabile et al. 2001; Xiang, Martinez-Smith et al. 2005). Non-structural protein 5A also induces production of IL-8, which, in turn, partially inhibits the IFN-induced antiviral response (Polyak, Khabar et al. 2001; Girard, Shalhoub et al. 2002). In addition, it binds to myeloid differentiation protein 88 (MyD88; the major TLR adaptor molecule) and modulates the TLR signaling pathways in immune cells (Abe, Kaname et al. 2007).

Apoptosis is another innate defense mechanism occurring when host cells are infected with a non-cytopathic virus. Although different reports on the HCV core protein effects indicate controversial anti-apoptotic or pro-apoptotic consequences, it seems that both are in favor of viral evasion, as anti-apoptotic effects are reported in liver cells or cell lines (Marusawa, Hijikata et al. 1999; Nguyen, Sankaran et al. 2006) and pro-apoptotic effects are observed in immune cells such as DCs and T cells (Hahn, Cho et al. 2000; Siavoshian, Abraham et al. 2004). Similar observations for HCV NS3 have been reported (Fujita, Ishido et al. 1996; Nguyen, Sankaran et al. 2006). Thus, HCV may affect this defense mechanism in opposite directions, depending on the type of the infected cells.

Dendritic cells as an intermediate element between innate and adaptive immunity are considered another target for HCV leading to impairment of effective antigen presentation. The effects of HCV on DCs as the main base for the hypothesis of this study are discussed in more detail in section 1.3.

1.2.3.2 Adaptive immunity

The effects on some components of the innate arm of the immune system could indirectly affect the adaptive arm of the immune response, as described above. However, HCV may also directly affect the adaptive immune response to evade the immune defense.

The presence of anti-HCV antibodies during CHC (Farci, Alter et al. 1992; Logvinoff, Major et al. 2004; von Hahn, Yoon et al. 2007) indicates that antibodies do not play a significant role in viral clearance and that HCV has developed very effective strategies to escape neutralizing antibodies. This capability is most likely due to the high rate of mutation resulting in large genetic heterogenicity and quasispecies of the virus. The lack of proofreading activity in

HCV NS5B protein (RdRps) combined with a high viral replication rate could be the reason for the high mutation rate. As a result, HCV is presented as a pool of viruses with diverse B and T cell epitopes, which makes it difficult for the adaptive immune response to overcome the rapidly changing epitopes. One of the regions frequently changing is HVR1 located on glycoprotein E2. If the pattern of quasispecies in HVR1 during the acute phase of infection continues to evolve, chronic infection occurs (Farci, Shimoda et al. 2000). There is evidence that the target epitopes of the virus escape from neutralizing antibodies through association of HCV with lipoproteins (Thomssen, Bonk et al. 1992; Nielsen, Bassendine et al. 2006). Shielding of epitopes by glycosylation of defined amino acids of E2 protein could also be responsible for virus escape (Falkowska, Kajumo et al. 2007; Helle, Goffard et al. 2007). And finally, direct cell to cell passage is suggested to be a mechanism to avoid exposure of the virus to neutralizing antibodies (Timpe, Stamataki et al. 2008). In summary, it seems that HCV has developed several efficient strategies to escape the neutralizing effects of antibodies.

Similar to humoral immunity, a high rate of viral mutation and diversity of CTL epitopes plays an important role in viral evasion from cellular immunity, as the virus is under selective pressure and continually modifies its CTL epitopes. In chronic patients, a CTL response was observed in the liver but not in the peripheral circulation, and this seems to be insufficient to eliminate the infected cells (He, Rehermann et al. 1999). Since HCV-specific CTLs are rather inactive in comparison to HBV-specific CTLs this raises the possibility of low antigenicity of HCV as an evasion mechanism (Kita, Hiroishi et al. 1995). The newly discovered natural subgenomic deletion mutants of HCV (Iwai, Marusawa et al. 2006; Bernardin, Stramer et al. 2007; Noppornpanth, Smits et al. 2007; Pacini, Graziani et al. 2009) may be involved in the low antigenicity of HCV, since they do not express the structural proteins. However, they contain all

regions of the viral genome that are essential for RNA replication, including the 5'UTR, the 3'UTR, and the region coding from NS3 to NS5B. This fact may indeed be similar to the core effect in reducing the viral load to maintain a low steady state of HCV in infected cells in order to escape from antigen presentation (Kato 2001).

There is evidence that HCV core protein plays a role in switching the immune response toward Th2 as the production of IL-12 by MΦs may be suppressed due to HCV core expression (Lee, Choi et al. 2001). In addition, core could limit the induction of Th1 responses by interaction with the globular domain of the C1q (a complement soluble molecule) receptor (gC1qR) on DCs leading to inhibition of TLR-induced IL-12 production (Waggoner, Hall et al. 2007). Through ligation to gC1qR on T cells, core also suppresses T cell responses by lowering IL-2 production and IL-2 receptor expression (Yao, Nguyen et al. 2001; Yao, Waggoner et al. 2005) and down-regulates T cell proliferation (Kittlesen, Chianese-Bullock et al. 2000; Yao, Nguyen et al. 2001). Moreover, other extensive studies also revealed defects in the HCV-specific $CD8^+$ cell differentiation from central memory $CCR7^+CD8^+$ cells to effector $CCR7^-CD8^+$ cells due to HCV core protein (Accapezzato, Francavilla et al. 2004; Francavilla, Accapezzato et al. 2004). Nonstructural protein 4A and NS5B also showed potential to down-regulate the surface expression of MHC class I molecules, preventing the elimination of infected cells by CTLs (Tu, Gao et al. 1999).

A direct association was reported between the frequency of Tregs and CHC (Sugimoto, Ikeda et al. 2003; Accapezzato, Francavilla et al. 2004; Cabrera, Tu et al. 2004); however, it is not clear if this is a cause or a consequence of the disease. In addition, involvement of viral proteins in this mechanism has not been reported yet. Depletion of Tregs *in vitro* increases virus-specific $CD4^+$ and $CD8^+$ T cells, and addition of purified Tregs in cell culture suppresses T cell

effector mechanisms (Boettler, Spangenberg et al. 2005; Chang 2007; Manigold and Racanelli 2007; Ebinuma, Nakamoto et al. 2008); however, this effect was not observed in *in vivo* studies (Manigold, Shin et al. 2006). Nevertheless, considering the higher number of Tregs in CHC patients (Dolganiuc, Paek et al. 2008; Smyk-Pearson, Golden-Mason et al. 2008), there may be a contributing role for Tregs in the viral immune evasion.

Finally, it is worth mentioning that HCV can hide in extra-hepatic locations as a possible evasion mechanism. Although this may not be a specific immune evasion mechanism, it could still be the reason for recurrence of infection in newly transplanted livers (Araya, Rakela et al. 1997; Garcia-Retortillo, Forns et al. 2002). One of these locations is assumed to be the PBMCs such as monocytes or DCs.

Taken together, although defects in the innate immune response play an important role, it could be the magnitude and quality of the adaptive immune response that determines the outcome of HCV infection. Indeed, many evasion strategies affecting innate immunity indirectly result in defects in adaptive cellular immunity. However, if the immune system has not been broadly compromised by HCV, infection might simply resolve by the potency of either the innate or the adaptive arm of the immune system.

1.2.4 Vaccine

At present, there is no vaccine to prevent HCV infection. However, the presence of protective immunity against re-infection in previously infected people or chimpanzees (Bassett, Guerra et al. 2001; Mehta, Cox et al. 2002; Nascimbeni, Mizukoshi et al. 2003) has kept this promise alive. In addition, recent studies showed different levels of protection against HCV in chimpanzees by using different approaches such as T-cell-based vaccines (Capone, Meola et al.

2006; Capone, Zampaglione et al. 2006; Folgori, Capone et al. 2006), or VLPs containing HCV structural proteins (Elmowalid, Qiao et al. 2007). Interestingly, transfer of DCs loaded with HCV proteins in mice have shown protection against VV expressing HCV NS3/4a or NS5a proteins (Yu, Huang et al. 2006; Yu, Babiuk et al. 2007; Yu, Babiuk et al. 2008). A good candidate for this purpose can be core protein, which has proven to be a well-conserved HCV protein inducing both humoral and cellular immunity (Iwata, Wakita et al. 1995; Lasarte, Garcia-Granero et al. 1998; Davis 1999). A HCV vaccine, if it becomes available, is considered to be used as a therapeutic tool besides being a preventive means.

1.2.5 Therapy

1.2.5.1 Existing therapy

Treatment of CHC has been a challenge for a long time, since IFN-α was first used in an attempt to cure the disease in 1986 (Hoofnagle, Mullen et al. 1986). With not very much change in content, the most effective therapy available today is still a combination of pegylated (a large poly ethylene glycol molecule; PEG) IFN-α-2a or -2b with ribavirin (Strader, Wright et al. 2004; Yee, Currie et al. 2006; Farrell 2007). This treatment is expensive, and not effective against all subtypes of HCV, and has serious side effects in many cases. With respect to the clinical and para-clinical effects of the treatment, after IFN therapy the level of HCV RNA in the serum rapidly decreases, followed by complete disappearance of the virus from serum and liver and healing of the liver (Lau, Kleiner et al. 1998).

The other component of the existing therapy for HCV, the broad-spectrum antiviral effect of ribavirin, has been already demonstrated to be effective in other viral diseases (Hruska, Bernstein et al. 1980; Hruska, Morrow et al. 1982; 1983). Ribavirin, a synthetic purine

nucleoside, which is an analog to guanosine ribonucleoside, was added to the HCV therapy in 1998 (McHutchison, Gordon et al. 1998; Poynard, Marcellin et al. 1998), and enhanced the antiviral response via inhibition of HCV replication (Pawlotsky, Dahari et al. 2004) and prevention of recurrence of infection (Dusheiko, Nelson et al. 2008). Interestingly, ribavirin enhances the production of Th1 cytokines, while it suppresses the production of Th2 cytokines, resulting in a polarizing T cell response toward Th1 (Hultgren, Milich et al. 1998; Ning, Brown et al. 1998; Tam, Pai et al. 1999).

The use of PEG-IFN started in 2000 (Manns, McHutchison et al. 2001; Fried, Shiffman et al. 2002), and improved the pharmacokinetics of IFN therapy, since it has a lower renal clearance (Rajender Reddy, Modi et al. 2002) and longer half-life, which results in sustained exposure leading to a more effective antiviral response. This combination also showed fewer side effects (Kozlowski, Charles et al. 2001), making it useful for patients who were not tolerant to the side effects of the conventional IFN-α therapy.

In addition to virus genotype as a major determinant for the success rate, there are several host factors such as race, age, gender, obesity, and the level of hepatic fibrosis, which are important to a lesser extent for the success rate of treatment.

Finally, for end-stage liver disease due to HCV, liver transplantation is the treatment of choice. However, in spite of a relatively acceptable survival rate of 65% for seven years, the new grafts are always re-infected.

1.2.5.2 Future therapies

Because of the disadvantages of the current therapy, there is an urgent need for discovery of new drugs. Several approaches are targeting the NS3 helicase activity (Borowski, Deinert et

al. 2003; Kim, Seo et al. 2003; Lamarre, Anderson et al. 2003; Perni, Pitlik et al. 2004) or NS5B enzymatic properties (Shim, Larson et al. 2003; Beaulieu, Bos et al. 2004; Chan, Das et al. 2004; Chan, Pereira et al. 2004; Eldrup, Allerson et al. 2004; Eldrup, Prhavc et al. 2004). Other approaches are based on synthetically produced nucleic acids in order to target the translation process. The use of small interfering RNAs (siRNAs) to target the IRES (Sioud and Iversen 2005) or synthesized oligonucleotides (anti-sense technology) to interfere with translation of certain mRNAs (Crooke 2004) are two examples of these strategies. The use of RNA interference (RNAi) (Kapadia, Brideau-Andersen et al. 2003; Wilson, Jayasena et al. 2003), and micro RNAs (miRNAs) are examples of other potential therapeutic tools for the future. Immunomodulatory molecules like those stimulating TLRs such as CpG ODN are promising as well. However, immunotherapy strategies including preventive and therapeutic vaccine strategies (Houghton and Abrignani 2005) such as DC-vaccines are still potential choices for this purpose.

1.3 Effects of hepatitis C virus on the function and maturation of human dendritic cells

In general, viruses use multiple mechanisms, such as infection of immune cells, to evade the host's immune defense to become persistent. Dendritic cells have been suggested to be major targets for viruses. Viruses can infect DCs and induce apoptotic cell death, inhibit the maturation process, or interfere with antigen processing and presentation resulting in inhibition or switch of the immune response to a non-protective one (Jenne, Schuler et al. 2001). In addition, they may indirectly inhibit maturation by synthesis of homologs for receptors involved in the induction of danger signals by viruses (Alcami and Koszinowski 2000). I summarized different strategies used by HCV to evade the immune system in section 1.2.3. One of the most important strategies HCV presumably uses to establish chronic disease could be the infection of DCs. Although pro-apoptic effects due to HCV core, NS3, NS5A and NS5B have been observed in DCs (Siavoshian, Abraham et al. 2005), most of the effects of HCV on DCs were suggested to be related to inhibition of DC activation and maturation. Dendritic cell activation can be inhibited or the type of immunity induced can be switched to Th2, because of impairment in DC activation by NKs as mentioned previously (Piccioli, Sbrana et al. 2002; Jinushi, Takehara et al. 2004); in turn, defective DCs expressing low level of MHC class I molecules on the surface are incompetent to activate the NKs (Jinushi, Takehara et al. 2003), resulting in a vicious cycle. Interference by HCV proteins with production of IFNs (Dolganiuc, Chang et al. 2006) is another possible mechanism, which may inhibit DC maturation. In addition, core can inhibit TLR-induced IL-12 secretion by DCs, as well as their ability to stimulate T cells to proliferate and secrete IFN-γ in an allo-stimulatory mixed leukocyte reaction (MLR) assay (Waggoner, Hall et al. 2007).

Although the role of DCs in generation of Tregs is suggested to be an evasion mechanism during HCV infection (Pachiadakis, Pollara et al. 2005), it may be a consequence of inhibition of DC maturation as it could be due to the presentation of HCV antigens by non-matured iDCs (Jonuleit, Schmitt et al. 2001).

The first studies on the effects of HCV on human DCs reported a diminished ability of DCs from chronic HCV carriers to properly mature and stimulate allogenic MLR in comparison to DCs from healthy individuals or patients who cleared viral infection (Kanto, Hayashi et al. 1999; Auffermann-Gretzinger, Keeffe et al. 2001; Bain, Fatmi et al. 2001). These observations were later supported by reports on a reduced number and functional impairment of circulating cDCs and pDCs, or *in vitro* generated Mo-DCs in CHC patients (Kanto, Inoue et al. 2004; Tsubouchi, Akbar et al. 2004; Tsubouchi, Akbar et al. 2004; Szabo and Dolganiuc 2005; Kanto, Inoue et al. 2006; Averill, Lee et al. 2007; Della Bella, Crosignani et al. 2007; MacDonald, Semper et al. 2007; Miyatake, Kanto et al. 2007; Miyazaki, Kanto et al. 2008). These DCs were also reported to be impaired in secreting IL-12 and inducing IFN-γ secretion by T cells. Furthermore, inhibition of maturation or allostimulatory ability of *in vitro* generated Mo-DCs from healthy donors transfected with HCV genes or exposed to HCV proteins was demonstrated (Sarobe, Lasarte et al. 2002; Dolganiuc, Kodys et al. 2003; Waggoner, Hall et al. 2007; Saito, Ait-Goughoulte et al. 2008; Zimmermann, Flechsig et al. 2008). These observations were confirmed in murine models (Hiasa, Horiike et al. 1998; Large, Kittlesen et al. 1999; Kim, Lee et al. 2002; Sarobe, Lasarte et al. 2003) and supported by evidence of infection of PBMCs (Sarih, Bouchrit et al. 2000), monocytes (Muratori, Gibellini et al. 1996; Lerat, Rumin et al. 1998; Garcia, Garcia et al. 2000), circulating DCs (Navas, Fuchs et al. 2002; Goutagny, Fatmi et al. 2003; Pham, MacParland et al. 2004), and Mo-DCs by HCV (Bain, Fatmi et al. 2001; Navas,

Fuchs et al. 2002). In most of theses studies, the focus was on the maturation process and impairment of mDC-specific functions such as antigen presentation. In several studies, this impairment was suggested to be specifically related to the function of pDCs, based on evidence that the decrease in the number and ability of pDCs to secrete IFN-α is a more prominent effect than that on cDCs (Szabo and Dolganiuc 2005; Ulsenheimer, Gerlach et al. 2005; Dolganiuc, Chang et al. 2006; Decalf, Fernandes et al. 2007).

Regardless of the type of affected DCs, other studies produced contradictory results showing normal functional properties of DCs in chronic HCV patients or DCs expressing HCV genes (Sun, Bodola et al. 2001; Longman, Talal et al. 2004; Longman, Talal et al. 2005; Piccioli, Tavarini et al. 2005; Li, Krishnadas et al. 2006; Li, Li et al. 2006; Zhou, Lukes et al. 2007; Barnes, Salio et al. 2008; Thumann, Schvoerer et al. 2008). Inhibition of maturation and function of DCs has also been suggested to be a consequence of HCV chronic infection, not the cause (Rollier, Drexhage et al. 2003). In addition, the inhibition of function of pDCs in CHC patients has recently been argued, although the number of pDCs might reduce (Albert, Decalf et al. 2008). Furthermore, DCs transfected with HCV genes were shown to be good candidate vaccines because of their *in vitro* and *in vivo* stimulatory effects (Li, Krishnadas et al. 2006; Li, Li et al. 2006; Yu, Huang et al. 2006; Yu, Babiuk et al. 2007; Yu, Babiuk et al. 2008). However, the inhibition of DCs in CHC might be indisputable as it is evident that the protective Th1-type immune response, a decision mediated by DCs, is weak in chronic HCV infection. Indeed, the inhibitory effects on DCs may merely happen *in vivo* during the normal course of infection. Accordingly, reduced expression and functional impairment of TLR2 was recently reported in CHC patients (Yakushijin, Kanto et al. 2006). Based on this fact and a report of impairment of DCs in cross-presentation due to the systemic activation of DCs via TLR2 (Wilson, Behrens et

al. 2006), HCV may use this mechanism to skew the immune response away from a Th1 bias. The impaired IL-15 production by DCs is also supportive of a possible defect in generation of a Th1-type immune response, as IL-15 is required for type I IFN-mediated expression of MHC class I molecules on DCs (Jinushi, Takehara et al. 2003). Taken together, while the effect of HCV on the inhibition of DC maturation remains controversial, the fact that in contrast to MHC class II molecules, MHC class I synthesis and transportation to the surface of the DCs is continued after maturation (Rescigno, Citterio et al. 1998; Cella, Salio et al. 1999) may still support this mechanism as a possible hypothesis to be more extensively investigated.

2 Hypothesis and objectives

Our hypothesis was that “HCV inhibits maturation of DCs leading to impairment of their functional capacity” based on evidence suggesting inhibitory effects of HCV on human DC maturation.

According to the hypothesis, we performed a comprehensive study of the effects of HCV gene products on DCs focusing on the maturation process, based on marker expressions, functional properties, and gene expression profiles of DCs.

To achieve this goal, the first objective was to optimize the generation of DCs and study the maturation process in detail. In order to achieve this objective, the progression of the maturation process needed to be carefully monitored and evaluated in detail by using different maturation agents such as TNF-α, LPS, and a maturation cocktail containing IL-1β, IL-6, TNF-α, and prostaglandin E2.

The second objective was to efficiently transfect DCs while retaining high viability, since transfection of DCs was problematic and the originally planned nucleofection method had an efficiency rate of 45%, while having significant negative effects on DC viability.

The third objective was the evaluation of the effects of expression of HCV genes on marker expression, phagocytosis, and antigen presentation of DCs, and the evaluation of the effects of some of these genes at the molecular level by gene expression profiling.

3 High transfection efficiency, gene expression and viability of monocyte-derived human dendritic cells after non-viral gene transfer

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3.1 Abstract

Dendritic cells (DCs) are bone marrow-originated professional antigen-capturing and -presenting cells, which can function as vaccine carriers. Although efficient transfection of human DCs has been achieved with viral vectors, viral gene products may influence cellular functions. In contrast, non-viral methods have generally resulted in inefficient gene transfer, low levels of gene expression and/or low cell viability. Monocyte-derived DCs (Mo-DCs) are the most common source of DCs both for *in vitro* studies and for *in vivo* applications. We hypothesized that reduction of the time to generate immature DCs (iDCs) might result in higher viability after transfection. Therefore, we established a protocol to generate human iDCs from $CD14^+$ monocytes within three days. These "fast" iDCs were phenotypically and functionally indistinguishable from conventional iDCs, showing high endocytic ability and low antigen-presenting capacity. Furthermore, the "fast" iDCs matured normally and had similar antigen presenting capacity to conventional mDCs. To optimize transfection of iDCs, we compared non-viral transfection of plasmid DNA and *in vitro* transcribed (IVT) RNA with transfection reagents, electroporation and nucleofection. Nucleofection of IVT RNA with the X1 program of

an Amaxa Nucleofector resulted in the most efficient transfection with an average of 93% transfected iDCs, excellent long-term viability and strong protein expression. Furthermore, the IVT RNA transfected iDCs retained all phenotypic and functional characteristics of iDCs. This method is applicable to most purposes including *in vitro* functional assays, *in vivo* DC immunotherapy and DC-based vaccines.

3.2 Introduction

Dendritic cells (DCs) are bone-marrow-originated professional antigen-capturing and -presenting cells, which initiate primary immune responses *in vivo.* DCs have unique functions, as they are capable of priming naïve T cells and cross-presenting antigens. One of the characteristics of the DC family is maturation, a process during which they change both morphologically and functionally. Immature DCs (iDCs) are phagocytic cells capable of sampling antigens at the site of injury or infection. Phagocytosis by DCs is a process that is much more complicated than that of other phagocytic cells and allows them to present the correct antigenic peptides on the MHC-I and II molecules. After processing the antigen, they mature, migrate toward local lymph nodes, and present the antigen to naïve T cells. This central role in cell-mediated immunity has made them attractive targets for cancer immunotherapy (Koido, Kashiwaba et al. 2000; Rughetti, Biffoni et al. 2000; Heiser, Maurice et al. 2001; Heiser, Maurice et al. 2001; Heiser, Coleman et al. 2002; Grunebach, Muller et al. 2003). DCs loaded with TAAs and injected into patients induce anti-tumor responses, which do not occur under natural conditions because of low tumor antigen expression or inaccessibility of the antigen to DCs. DCs are also potential vectors for the treatment of chronic infectious diseases, which evade

the immune system. Moreover, recent advances in generating DCs have provided strategies for the design of DC-based vaccines (Grunebach, Muller et al. 2005).

Because of their unique properties, DCs are of interest for studies on the mechanisms of antigen processing and presentation. Since DCs are professional cells and strictly control the trafficking of molecules across their cell membranes, it is difficult to transfer genes into the nucleus. Many studies have been performed on loading DCs with different antigens for *in vitro* assays or cancer immunotherapy and efficient transfection has been achieved with viral vectors (Hiasa, Horiike et al. 1998; Jooss, Yang et al. 1998; Ponnazhagan, Mahendra et al. 2001; Dullaers, Breckpot et al. 2004; Siavoshian, Abraham et al. 2005). However, the viral vectors may influence DC functions and interact with the host immune system (Engelmayer, Larsson et al. 1999; Salio, Cella et al. 1999; Drillien, Spehner et al. 2000; Jenne, Hauser et al. 2000; Kruse, Rosorius et al. 2000; Tortorella, Gewurz et al. 2000), which may affect the interpretation of *in vitro* studies. Furthermore, there is a need for DC-based immunotherapy without risk of exposing individuals to other viral genes or integration of the viral genome, which supports the importance of establishing an effective and safe non-viral method for DC transfection. However, inefficient gene transfer to DCs, low levels of gene expression or low cell viability by non-viral transfection methods have limited studies on DCs (Van Tendeloo, Snoeck et al. 1998; Irvine, Trinder et al. 2000; Haines, Irvine et al. 2001; Kalady, Onaitis et al. 2002; Lundqvist, Noffz et al. 2002; Chamarthy, Jia et al. 2004; Dullaers, Breckpot et al. 2004; Erhardt, Gorschluter et al. 2005; Tan, Beutelspacher et al. 2005). To date, the highest non-viral transfection efficiency of plasmid DNA has been achieved by nucleofection. This method resulted in up to 56% efficiency but only 37% viability of the transfected DCs after 24 h (Lenz, Bacot et al. 2003). In contrast, several studies have demonstrated efficient transfection of DCs with mRNA (Van Tendeloo, Ponsaerts et al.

2001; Lundqvist, Noffz et al. 2002; Mu, Gaudernack et al. 2003; Ueno, Tcherepanova et al. 2004; Michiels, Tuyaerts et al. 2005). However, protein expression levels were generally either low or not discussed, and little information was provided with respect to short- and long-term cell viability. As was recently concluded in an extensive recent review on this subject, strategies for efficient transfection still need to be established for human DCs (Grunebach, Muller et al. 2005), and in particular monocyte-derived DCs (Mo-DCs).

In this study, we first established a protocol to generate monocyte-derived iDCs (Mo-iDCs) in three days and then carried out an extensive comparison of non-viral transfection methods, including transfection of plasmid DNA and *in vitro* transcribed (IVT) RNA with transfection reagents, conventional electroporation, and nucleofection. Finally, we optimized some protocols as the methods of choice for transfecting human DCs for *in vitro* studies and *in vivo* trials.

3.3 Materials and methods

3.3.1 *In vitro* generation of monocyte-derived dendritic cells

Human venous blood was obtained following informed consent through the protocol approved by the Biomedical Research Ethics Board at the University of Saskatchewan. Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient separation using Lymphocyte Separation Medium (LSM) (MP Biomedicals, LLC, Irvine, CA, USA). Briefly, 4 ml blood diluted 1:2 in endotoxin-free phosphate buffered saline pH 7.2 (PBS; Invitrogen Canada Inc., Burlington, ON, Canada) was layered on 3 ml of LSM and centrifuged at 400 x g for 25 min. PBMCs were collected, washed and incubated with human CD14-specific antibody

conjugated to paramagnetic MicroBeads (Miltenyi Biotech, Auburn, CA, USA). The $CD14^+$ monocytes were isolated on LS columns (Miltenyi). Conventional iDCs were generated by resuspending monocytes at 1×10^6 cells/ml in complete RPMI (CRPMI; RPMI 1640 [Invitrogen] supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 100 μM non-essential amino acids, 1 mM sodium pyruvate, 50 μM 2ME, 10 mM HEPES, and 50 μg/ml gentamycin). Recombinant human (rh)GM-CSF (100 ng/ml, Peprotech Inc., NJ, USA) and rhIL-4 (≤100 ng/ml, Peprotech) were added and then the $CD14^+$ monocytes were dispensed into the wells of a 24- or 12-well plate (Corning Incorporated, Corning, NY, USA). To generate "fast" iDCs, the monocytes were resuspended at 1×10^6 cells/ml in CRPMI made with phenol-red-free RPMI 1640 [Invitrogen], a lower concentration of heat-inactivated FBS (5%) and a higher amount of L-glutamine (4 mM). In addition, the concentration of IL-4 was increased to 200 ng/ml. In conventionally generated iDC cultures the media was replaced with fresh media containing cytokines every second day. Cultured $CD14^+$ cells were checked daily for marker expression by flow cytometry. The phagocytic and antigen-presentation capacities of the monocytes and DCs were examined at different time points to confirm the exact times of differentiation and maturation. Conventional iDCs collected on day 7 and fast iDCs collected on day 3 were evaluated in functional assays, and the fast iDCs were used for transfections. To mature the iDCs, a cocktail of 10 ng/ml rhIL-1β (Peprotech), 10 ng/ml rhIL-6 (Peprotech), 20 ng/ml rhTNF-α (Peprotech) and 500 ng/ml PGE2 (Sigma-Aldrich Canada Ltd., Oakville, ON, Canada) was used for 16-48 h.

3.3.2 Flow cytometry

PBMCs, monocytes and DCs were washed twice in PBS and resuspended in FACS buffer (PBS, pH 7.2 with 0.2% gelatin and 0.03% sodium azide) at 10^5 cells/100 µl in a round-bottom 96-well plate (Corning Incorporated). Subsequently, 2 µl of FITC- or PE-conjugated monoclonal antibodies (Becton Dickinson Biosciences, Oakville, ON, Canada) were added to each well and the plate was incubated on ice for 15-30 min. After incubation, cells were washed three times with FACS buffer, fixed with 2% formaldehyde in PBS and analyzed by FACScan (BD Biosciences).

To check the transfection efficiency and intensity, iDCs transfected with plasmid encoding GFP (pmaxGFP, Amaxa Company, Köln, Germany) were washed once in PBS and analyzed by flow cytometry. The cells were gated electronically according to the control, non-transfected cells for Forward Scatter (FSC) and Side Scatter (SSC) properties to include the main population of the cells and exclude dead cells. To check the cell viability, the cells were stained with Propidium Iodide (PI) (Sigma-Aldrich) and more than 10,000 events were analyzed by FACScan without gating. Cell Quest software (BD Biosciences) was used for analysis of flow cytometry data.

3.3.3 Phagocytosis assay

A phagocytosis assay was performed as described elsewhere (Sallusto, Nicolo et al. 1996) with minor modifications. Immature and mature DCs were collected, washed once in PBS, and resuspended in CRPMI at 1×10^6 cells/ml. Two hundred µl of cell suspension were cultured in a 24-well plate and 200 µl of FITC-conjugated dextran (FITC-DX, Sigma-Aldrich) in PBS (1 mg/ml) was added to the DCs. The treatment plate was incubated at 37ºC and the control plate

was incubated on ice for 1-2 h in the dark. After incubation, the cells were collected, washed three times with cold PBS, and checked by flow cytometry for FITC-DX fluorescence as an indicator of phagocytosis.

3.3.4 Allostimulatory mixed leukocyte reaction (MLR)

Immature and mature DCs were collected, washed in PBS once, and resuspended at 1×10^6 cells/ml in CRPMI. One hundred µl of cell suspension was dispensed in the wells of a flat-bottom 96-well ELISA plate (Corning Incorporated). Subsequently, 3×10^5 CD14-negative cells (70-80% $CD3^+$ cells) in 100 µl CRPMI from a different, HLA-mismatched healthy donor was added to each well followed by addition of 50 µl of CRPMI. The plate was incubated at 37ºC and 5% CO_2. After 48-72 h, 20-40 µl of MTT (Sigma-Aldrich) at a concentration of 5 mg/ml in PBS was added to each well and the cells were incubated for an additional 30-60 min until the uptake of dye by the cells was visible under the light microscope. Subsequently, the plates were centrifuged at 400 x g for 10 min and 150 µl of supernatant was replaced with 100 µl of acidified isopropanol (0.375% HCl in isopropanol). The plates were mixed for 4 min and read by an ELISA Reader (Molecular Devices Corporation, Sunnyvale, CA, USA) at a wavelength of 595 nm.

3.3.5 Transfection with plasmid DNA

ExGen 500 (Fermentas, Burlington, ON, Canada), FuGene 6 (Roche Diagnostics, Laval, QC, Canada), GeneJuice (EMD Biosciences, Novagen Brand, Madison, WI, USA), SuperFect (QIAGEN Inc., Mississauga, ON, Canada), and TransFast Transfection Reagent (Promega

Corporation, Madison, WI, USA) were used to transfect DCs with pmaxGFP. Since no protocols were available for human DCs, optimized protocols were developed. Electroporation of DNA was performed according to a previously described protocol (Strobel, Berchtold et al. 2000).

3.3.6 Transfection with IVT RNA

The pGEM4Z-5'UT-eGFP-3UT-64A vector was kindly provided by Dr. E. Gilboa (Duke University Medical Centre, Durham, NC, USA). This plasmid contains the GFP gene flanked by the 5' and 3' un-translated regions and a poly A tail. There is a *SpeI* site after the poly A stretch to allow linearization of plasmid for *in vitro* transcription. *In vitro* transcription and purification was performed by using the mMESSAGE mMACHINE Kit (Ambion, Austin, TX, USA). This kit uses a T7 RNA polymerase to transcribe RNA from the plasmid, which has a T7 RNA polymerase promoter site before 5' untranslated region. *In vitro* transcribed IVT RNA was stored at -70ºC until use.

For electroporation, iDCs were collected on day 3 and washed with PBS followed by serum-free Opti-MEM. Subsequently, 3×10^6 cells were resuspended in 200 µl serum-free Opti-MEM. IVT RNA was added to the cell suspension at different concentrations and the mixtures were transferred into a 4 mm cuvette and electroporated at different conditions (250-400 V voltage, 125-960 µF capacitance, and 100-1000 Ω resistance) in an electroporation apparatus (Bio-Rad Laboratories, GenePulser^{TM} Model 1652076, Mississauga, ON, Canada). Immediately after transfection, the DCs were resuspended at 5×10^5/ml in 37ºC CRPMI supplemented with 50 ng/ml rhGM-CSF and 100 ng/ml rhIL-4 and incubated at 37ºC until FACS analysis.

Transfection with TransMessenger Transfection Reagent (TTR) (Qiagen, Mississauga, ON, Canada) was performed according to the manufacturer's instructions with some

modifications. TTR is based on a lipid formulation and is used in conjunction with a specific RNA-condensing reagent (Enhancer R) and an RNA-condensing buffer (Buffer EC-R). Immature DCs were collected on day 3, and washed with PBS. The Enhancer R Solution and different amounts of IVT RNA were diluted in an appropriate amount of Buffer EC-R and incubated for 5 min at room temperature (RT). TTR was added to the mixtures and the complexes were incubated for 10 min at RT. RPMI 1640 was added to the IVT RNA-TTR complexes, which were then immediately added to the iDCs. Finally, the iDCs were transferred to a 24-well plate, and incubated at 37ºC. After 1 h the iDCs were washed with PBS, fresh CRPMI supplemented with 50 ng/ml rhGM-CSF and 100 ng/ml rhIL-4 was added, and the plate was incubated again until analysis by flow cytometry.

3.3.7 Nucleofection of DNA and IVT RNA

Nucleofection of iDCs was performed according to the manufacturer's instructions with some modifications, by using the Nucleofector Machine (Amaxa). The iDCs were collected on day 3, and washed twice in PBS. Subsequently, 2×10^6 cells were resuspended in 100 µl of Human Dendritic Cell Nucleofection Solution (Amaxa). Five µg pmaxGFP were added per 2×10^6 cells and the samples were transferred into certified cuvettes (Amaxa) and transfected by using programs K2, M2, Q2, T2, U1, U2, U8, X1, or X10. The same programs were used for transfection of IVT RNA at a concentration of 5-10 µg per 1×10^6 cells. RPMI-1640 supplemented with 10% heat inactivated FBS, 2mM L-glutamine, 1 mM sodium pyruvate, 100 µg/ml streptomycin and 100 µg/ml penicillin was warmed to 37ºC and 500 µl was added immediately after transfection to each cuvette. The iDCs were collected, dispensed in the wells of a 24-well plate containing 2.2 ml pre-warmed SRPMI supplemented with 50 ng/ml rhGM-

CSF and 100 ng/ml rhIL-4 and divided into four wells to be analyzed at 8, 24, 48 and 72 h. The plate was incubated at 37ºC until analysis by flow cytometry. All samples were checked for transfection efficiency and intensity as well as cell viability with an Axiovert 200M fluorescent microscope (Carl Zeiss Canada Ltd., Toronto, ON, Canada) and a FACScan.

3.3.8 Stimulation of dendritic cells and cytokine secretion

Four hours after nucleofection with GFP IVT mRNA, iDCs were stimulated for 20 h in the presence of LPS (Sigma-Aldrich) at a final concentration of 10 μg/ml and supernatants were checked for IL-12 (p70), IFN-γ and IL-10 production by using a BD OptEIA Human ELISA Set (BD Biosciences) according to the instructions provided by the manufacturer. Non-nucleofected iDCs as well as iDCs nucleoporated without genetic material were used as controls.

3.3.9 Statistical analysis

Data were analyzed using statistical software (GraphPad Prism Version 4.00). As outcome variables were not normally distributed, differences among all groups were examined using the Kruskal-Wallis test. If a significant difference was found among the groups, the medians between pairs of groups were compared using the Mann-Whitney U test. Differences were considered significant if the two-tailed *P* value was lower than 0.05 with the confidence intervals of 95%. All transfection results are shown as mean ± SEM of at least four independent experiments performed with different donors.

3.4 Results

3.4.1 *In vitro* generation of immature and mature dendritic cells

Transfection of Mo-iDCs generated over a period of 7 to 14 days as described previously (Sallusto, Cella et al. 1995; Zhou and Tedder 1996; Cella, Sallusto et al. 1997; Brossart, Grunebach et al. 1998; Yang, Howard et al. 1999) resulted in progressively reduced viability over time (data not shown). We hypothesized that generation of DCs in a shorter period of time would result in higher viability and thus we established a protocol to differentiate monocytes into iDCs more rapidly. $CD14^+$ monocytes were isolated from PBMCs by magnetic activated cell sorting (MACS), which routinely resulted in at least 95% pure populations (Fig. 3.1a). Subsequently, the $CD14^+$ monocytes were cultured with different concentrations of rhGM-CSF and rhIL-4 and checked daily for changes in CD1a, CD14, CD209 (DC-SIGN), CD83 and CD86 expression to identify the exact time of differentiation and maturation. When the $CD14^+$ cells were cultured in medium supplemented with 100 ng/ml rhGM-CSF and 200 ng/ml rhIL-4 on day 0, they differentiated into iDCs in three days (Fig. 3.1b) without further addition of cytokines. The expression of CD14, which is a specific monocyte marker, decreased to 5%, CD209 or DC-SIGN as an interstitial DC marker increased from 0% to 92%, CD1a a MHC-I-like molecule increased to 71%, CD86 as a co-stimulatory receptor decreased from 95% to 57% and CD83 was expressed at less than 4% confirming the immature status of the iDCs. When the iDCs were cultured with a cocktail of rhIL-1β, rhIL-6, rhTNF-α and prostaglandin E2, CD83, the well-defined marker for maturation (Lechmann, Krooshoop et al. 2001; Lechmann, Berchtold et al. 2002; Lechmann, Zinser et al. 2002), increased to 89%, so they became fully mature on day 5 (Fig. 3.1c). In addition, the morphology of these iDCs and mature (m)DCs was as expected (Fig. 3.2c,d). Similar but slower marker changes were observed with the conventional method (Fig.

3.1b,c). As shown in Figure 3.1, the expression of CD1a, CD209, CD83 and CD86 was very similar on “fast” iDCs and conventionally generated iDCs, as well as on the mDCs after maturation. To further characterize the iDCs and mDCs, their phagocytic ability, and antigen-presenting capacity were evaluated and compared to conventionally generated DCs. Fast iDCs showed strong phagocytic ability, which decreased upon maturation to mDCs, similar to conventionally generated iDCs (Fig. 3.3a). The mDCs, regardless of whether they were generated from onventionally or fast-generated iDCs, had increased antigen-presenting capacity (Fig. 3.3b). Overall, this approach generated iDCs more rapidly than previously reported methods using only one treatment with rhGM-CSF and rhIL-4. These data demonstrate that the conventional and fast DCs were phenotypically and functionally indistinguishable, both before and after maturation.

Due to the fact that GM-CSF alone drives monocytes toward macrophages, we hypothesized that IL-4 might play a key role in promoting differentiation toward DCs and that an increase in IL-4 concentration might be responsible for the expedited differentiation of fast DCs. To investigate this, we checked the effects of GM-CSF or IL-4 on expression of key phenotypic markers. The results indicate that IL-4 is the main cytokine for reduction of CD14 expression, which is a specific marker for monocytes also present on macrophages (Fig. 3.4a). Moreover, CD209 (DC-SIGN), an important marker for DCs, increased more dramatically in the presence of IL-4 than with GM-CSF (Fig. 3.4b). We also showed that IL-4 was responsible for maintaining stable levels of MHC-II expression during differentiation (Fig. 3.4c). These observations support the contention that IL-4 plays a major role in the differentiation of monocytes to iDCs and rationalize the use of high IL-4 concentrations during this process.

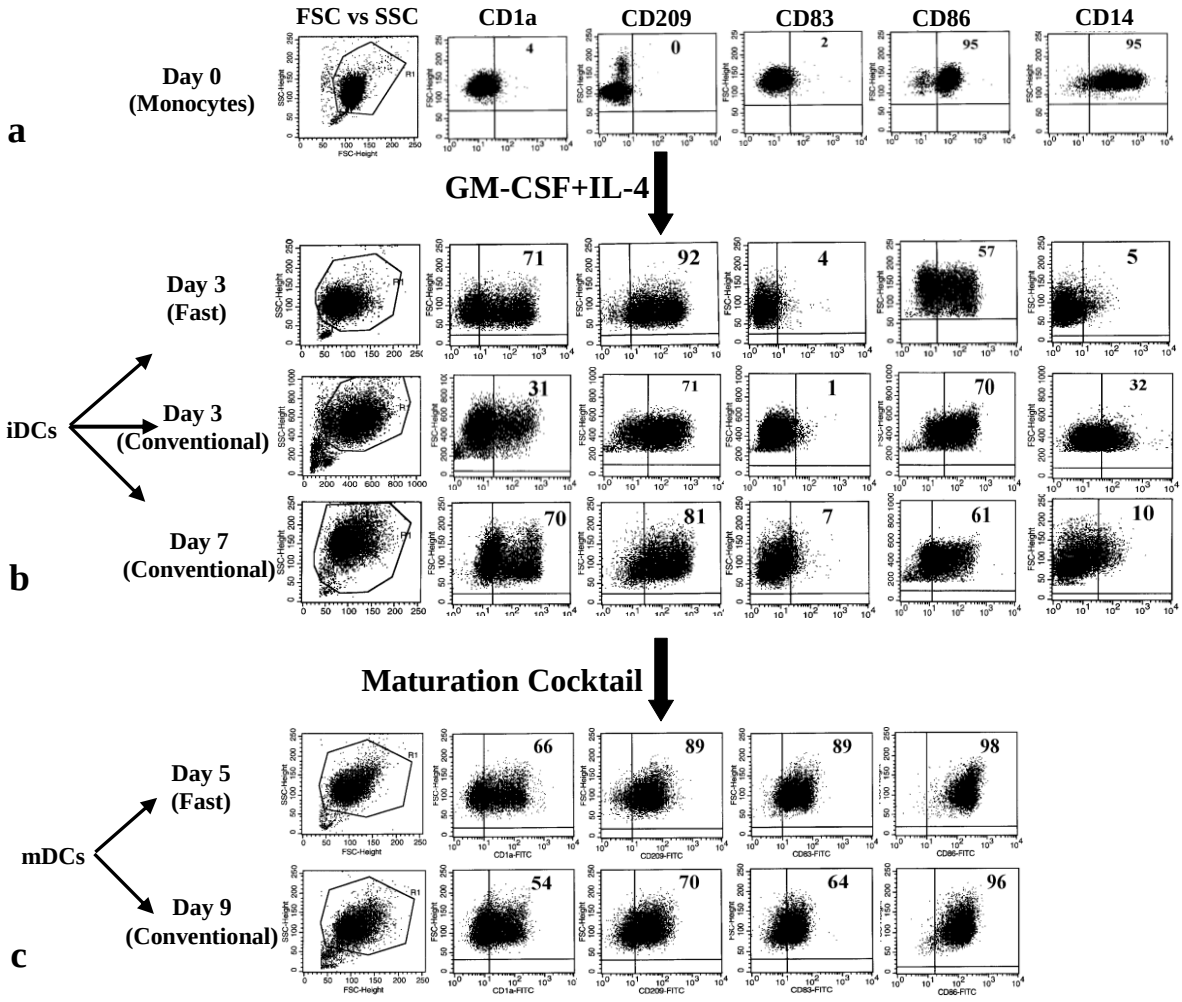


Figure 3.1. Phenotypic characterization of fast and conventionally generated monocyte-derived DCs. **a.** Expression of cell surface markers on monocytes isolated by anti-CD14 antibody conjugated to paramagnetic MicroBeads. **b.** Differentiation of monocytes to iDCs. **c.** Maturation of iDCs. Monocytes were differentiated into iDCs with 100 ng/ml rhGM-CSF and ≤100 ng/ml (conventional) or 200 ng/ml (fast) rhIL-4 and matured to mDCs by adding a maturation cocktail (IL-1β, IL-6, TNF-α, and PGE2) on day 3 (fast) or 7 (conventional). The phenotypes of monocytes, iDCs, and mDCs were monitored by flow cytometry. Numbers in the upper right quadrants represent the percentage of positive cells for each marker and are representative of eight experiments.

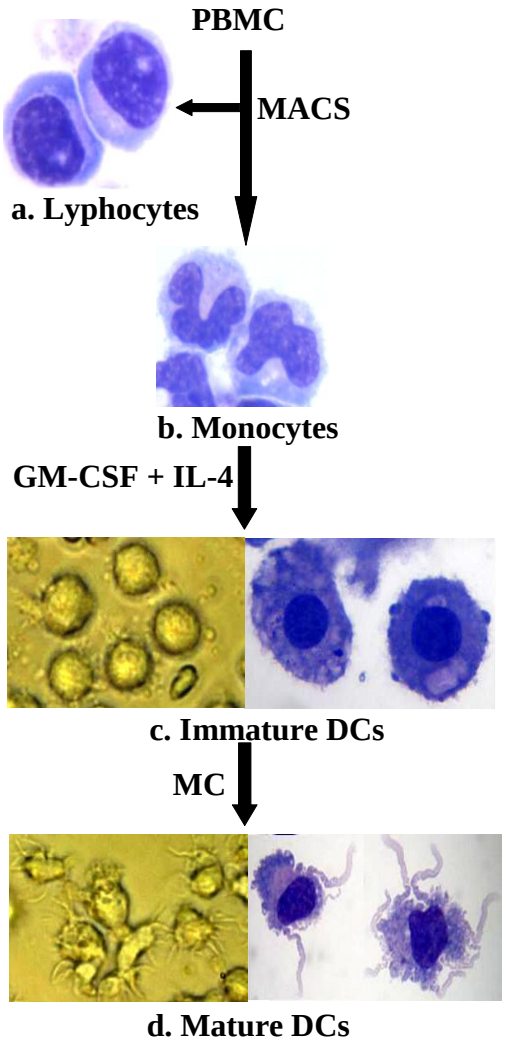
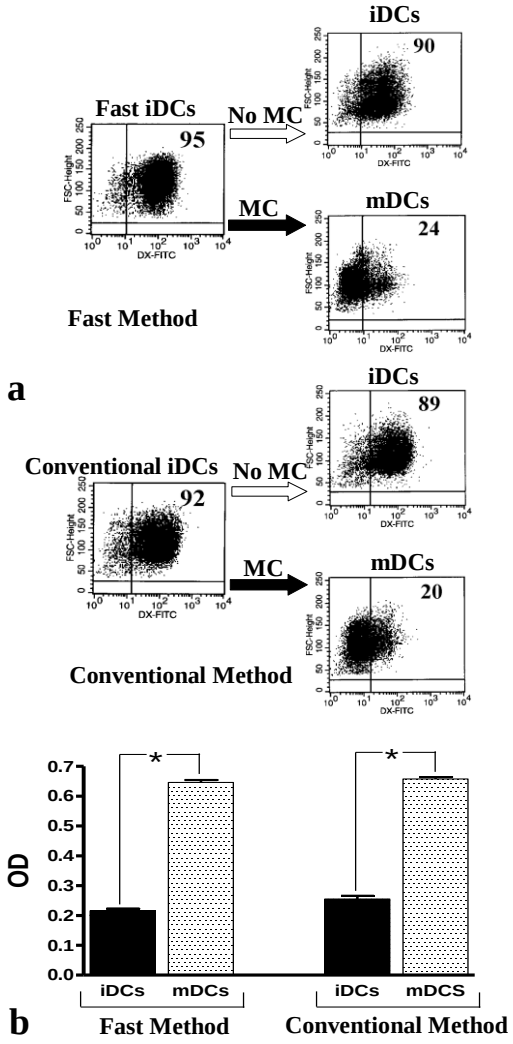


Figure 3.2. Morphology of monocytes, iDCs and mDCs after fast differentiation and maturation. **a.** Lymphocytes (80% of CD14-negative cells flowing through the MACS column); this population was used for MLR assays as responder cells; (× 100) **b.** Monocytes ($CD14^+$ cells) isolated from PBMCs by MACS; (× 100) **c.** iDCs three days after culturing monocytes with rhGM-CSF and rhIL-4, and **d.** mDCs one day after addition of maturation cocktail (IL-1β, IL-6, TNF-α, and PGE2) to the iDC cultures. Left iDC and mDC panels show the cells in the culture (× 40) and right iDC and mDC panels show the cells stained by modified Wright's stain (HemaTek Stain, Bayer Corporation, Tarrytown, NY, USA) (× 100). (MC = maturation cocktail).



Figiure 3.3. Functional evaluation of fast and conventional DCs **a.** Phagocytosis assay showing similar uptake of FITC-conjugated dextran (FITC-DX) in fast and conventional iDCs. Numbers in the upper right quadrants represent the percentage of phagocytic cells and are representative for three experiments. **b.** Antigen-presenting abilities in fast and conventional mDCs measured by MTT MLR assay (P<0.05 is shown as *). (MC = maturation cocktail, Black arrows = MC added, and white arrows = No MC).

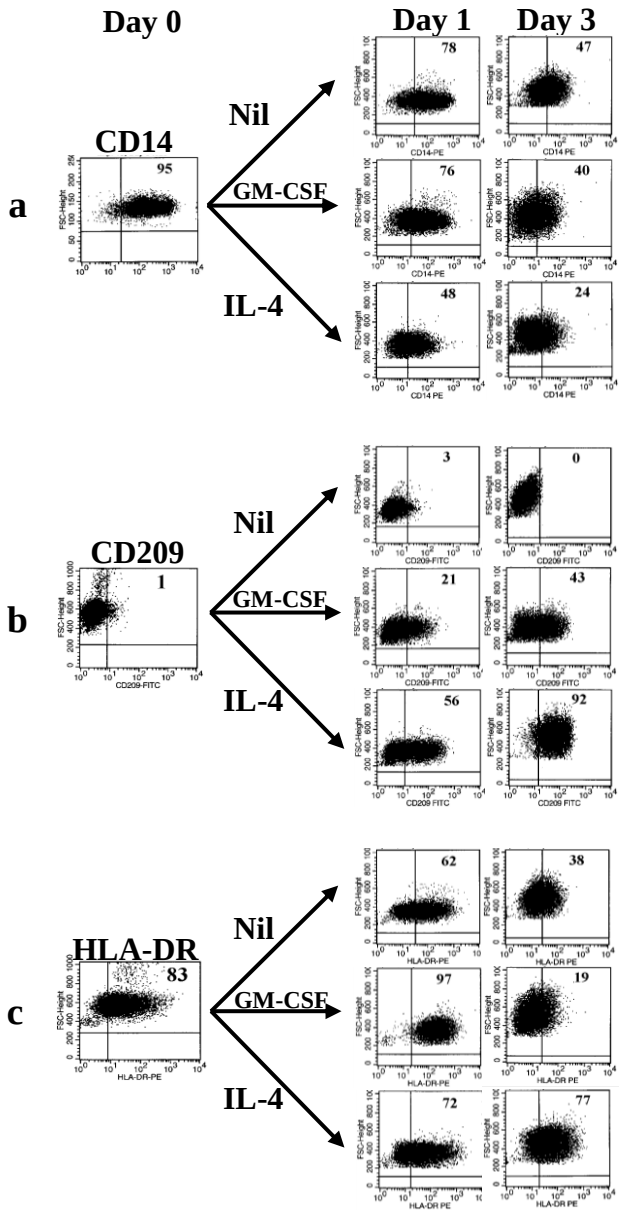


Figure 3.4. Effects of GM-CSF and IL-4 on expression of three key markers during differentiation from monocytes into iDCs. **a.** CD14. **b.** CD209. **c.** HLA-DR. Nil = Monocytes cultured without any cytokines. GM-CSF = Monocytes cultured with 100 ng/ml of GM-CSF. IL-4 = Monocytes cultured with 100 ng/ml of IL-4. Numbers in the upper right quadrants represent the percentage of positive cells for each marker and are representative of at least four experiments.

3.4.2 Transfection with plasmid DNA

To develop an optimal non-viral transfection method that would result in high gene expression levels and long-term viability and thus allow functional studies, the iDCs were transfected on day 3 with pmaxGFP and various transfection reagents or conventional electroporation. The transfection efficiency, GFP intensity, and cell viability were evaluated at different time points after transfection. Regardless of the reagent selected, the transfection efficiency was low. The highest efficiency was obtained with FuGene 6, with 20% of the iDCs transfected. However, although to our knowledge this was the highest efficiency reported for any transfection reagent with plasmid DNA, this was still not sufficient for either *in vitro* or *in vivo* studies. The other transfection reagents tested, ExGen 500, GeneJuice, SuperFect and TransFast Transfection Reagent, resulted in less than 10% efficiency and low intensity. Electroporation also resulted in less than 10% transfection efficiency, as well as low viability (data not shown). Overall, this demonstrated that regardless of the method or reagent used, transfection with plasmid DNA was inefficient.

3.4.3 Transfection with IVT RNA

Since we did not achieve acceptable transfection efficiencies with fore-mentioned methods, we considered IVT RNA as the next approach to transfect DCs. After generating IVT RNA from the pGEM4Z-5'UT-eGFP-3UT-64A vector, iDCs were transfected with the GFP IVT RNA (10 μg per 1×10^6 cells) by conventional electroporation with different settings in terms of voltage, capacitance, and resistance. In comparison to transfection with plasmid DNA, this method was less toxic to the DCs and more effective, resulting in more than 80% transfection efficiency (data not shown), which is comparable to previous reports on RNA electroporation

(Van Tendeloo, Ponsaerts et al. 2001). Some electroporation conditions resulted in high transfection efficiency, but the cell viability was not high enough for further *in vitro* studies or *in vivo* applications (data not shown). Five electroporation conditions resulted in both acceptable efficiency and viability after 72 h (Fig. 3.5a, c). However, the intensity was very low (Fig. 3.5b), so although electroporation could be useful for transfection with genes that need to be expressed at low levels, the protein expression was considered insufficient for most applications.

Since TTR is a novel transfection reagent recommended for use with IVT RNA, we subsequently tested the efficacy of this reagent with GFP IVT RNA. When the TTR and IVT RNA were incubated for 2 h, up to 61% of the iDCs became transfected, but the viability was very low (data not shown). Therefore, we reduced the incubation time to 1 h, which resulted in 40% efficiency, high levels of GFP expression and 69% viability after 72 h (Fig. 3.5). Interestingly, after transfection of iDCs with TTR the CD83 expression increased (Fig. 3.7a) and the cell morphology changed (Fig. 3.9c), indicating that TTR induced the iDCs to become mature. Thus, transfection of DCs with TTR may have effects on the functional properties of the DCs.

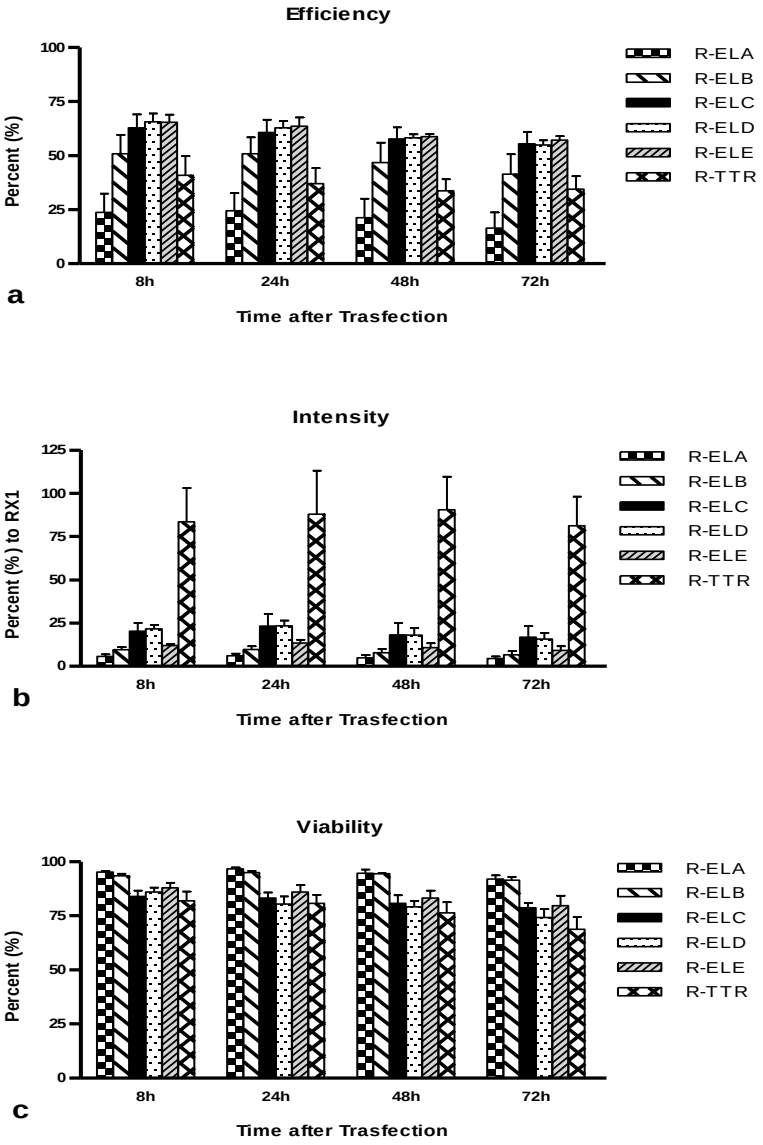


Figure 3.5. Transfection efficiency, transfection intensity, and cell viability after transfection of *in vitro* transcribed GFP IVT RNA. Immature DCs were either transfected by electroporation using five different settings (R-ELA = 300 V, 125 µF, and 100 Ω; R-ELB = 300 V, 250 µF, and 100Ω; R-ELC = 300 V, 500 µF, and 200 Ω; R-ELD = 300 V, 500 µF, and 400 Ω; and R-ELE = 350 V, 250 µF, and 200 Ω) or with TTR (R-TTR). **a.** Transfection efficiency expressed as percentage of iDCs expressing GFP. **b.** Intensity expressed as a percentage of the strongest fluorescence achieved with the most effective method, the Nucleofector program X1 with GFP IVT RNA. The geometric mean intensity was calculated based on FACS results and expressed as a percentage of the highest intensity obtained with program X1 and IVT RNA, which was calculated as 100%. **c.** Cell viability expressed as percentage of live iDCs.

3.4.4 Nucleofection of DNA

Since there is a report on nucleoporation of human DCs with a Nucleofector (Lenz, Bacot et al. 2003), we evaluated this approach as an alternative for transfection of iDCs with plasmid DNA. To optimize the protocol, we screened nine different programs and further evaluated the five most promising programs with respect to efficiency, level of protein expression, and viability after nucleofection with pmaxGFP. Reasonably high transfection efficiency and protein expression levels were achieved (Fig. 3.6a,b), as well as good viability for up to 24 h (Fig. 3.6c) with some of the programs, in particular U2, and X1, which led to significantly better transfection efficiencies than K2 and M2 ($P<0.05$). However, with the U2 and X1 programs, the viability decreased significantly between 24 and 48 h. Program X1 was comparable to program U2, which is recommended by Amaxa for nucleofecting DCs with DNA (Fig. 3.6a-c).

3.4.5 Nucleofection of IVT RNA

Since the transfection efficiency achieved by nucleofection of plasmid DNA was at most 50% and the viability of the iDCs decreased significantly between 24 and 48 h, we subsequently used the same programs with GFP IVT RNA. Nucleofection with IVT RNA resulted in higher efficiency regardless of the program used (Fig. 3.6d), as well as good viability after 72 h (Fig. 3.6f). The iDCs expressed higher levels of protein when transfected with program X1 than with any other transfection method (Fig. 3.6e), while the other programs resulted in comparable levels of protein expression to DNA nucleofection (Fig. 3.6b, e). The transfection efficiency obtained with program X1 varied between 90 and 98%, with a mean of 93% 8 h after transfection, while after 72 h the mean efficiency was 78% and the mean viability was 75%. Furthermore, high protein expression was observed 8 h after transfection and although there was some decrease, the

protein expression level was still reasonably high at 72 h after transfection. In fact, GFP could be detected as early as 1 h and as long as five days after transfection (data not shown). In addition, there was less variability in protein expression levels between cells within one experiment (Fig. 3.9a, b) and less variation between experiments (Fig. 3.6b, e) when iDCs were transfected with IVT RNA instead of DNA. Interestingly, at 8 h after transfection the GFP intensity in the RNA transfected iDCs was higher than that obtained after DNA nucleofection, whereas long-term protein expression was equivalent. To check if the nucleofection has any effect on the functional properties of DCs, iDCs were checked for their antigen presentation ability as well as CD83 expression after nucleofection and addition of maturation cocktail. Normal maturation similar to that of non-transfected DCs and comparable antigen presentation ability were observed after nucleofection with the X1 program (Fig. 3.7a, b).

Since DCs may lose their capacity to produce IL-12 p70 after electroporation, iDCs were stimulated with LPS at 10 μg/ml. Supernatants were collected and checked for the presence of IFN-γ, IL-12p70, and IL-10. The results demonstrate that DCs nucleofected with mRNA using the X1 program were capable of secreting significant amounts of cytokines in comparison to non-nucleofected DCs (Fig. 3.8a-c). Nucleofection had no effect on IFN-γ and IL-10 secretion, and although the ability of the nucleofected iDCs to secrete IL-12, was reduced, about 200 pg/ml was still detected, which is a significant amount.

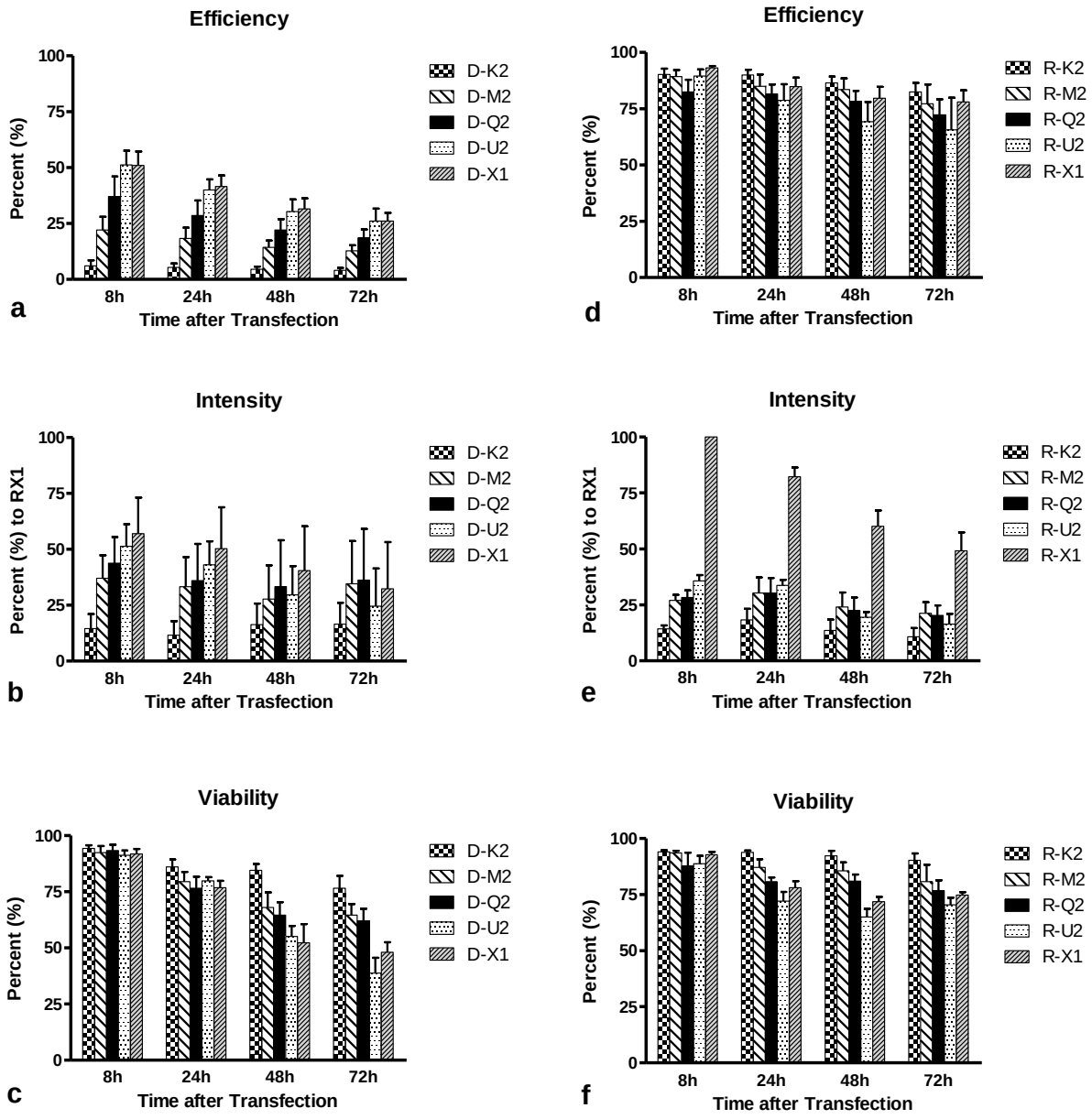
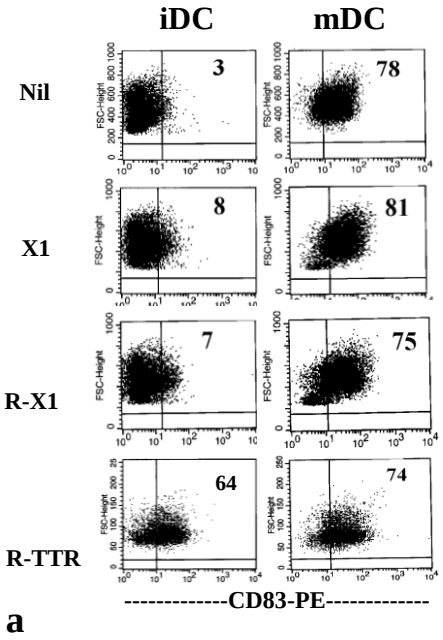


Figure 3.6. Transfection efficiency, transfection intensity, and cell viability after nucleofection of GFP plasmid DNA or GFP IVT RNA. **a, b, c:** Nucleofection with 2.5 µg of GFP plasmid DNA per 1×10^6 cells, using programs K2 (D-K2), M2 (D-M2), Q2 (D-Q2), U2 (D-U2) and X1 (D-X1). **d, e, f:** Nucleofection with 10 µg of GFP IVT RNA per 1×10^6 cells, using programs K2 (R-K2), M2 (R-M2), Q2 (R-Q2), U2 (R-U2) and X1 (R-X1). **a** and **d:** Transfection efficiency expressed as percentage of iDCs expressing GFP. **b** and **e:** Intensity calculated as for Fig. 3.4 and expressed as a percentage of the strongest fluorescence achieved with the Nucleofector program X1 with GFP IVT RNA. **c** and **f:** Cell viability expressed as percentage of live iDCs.



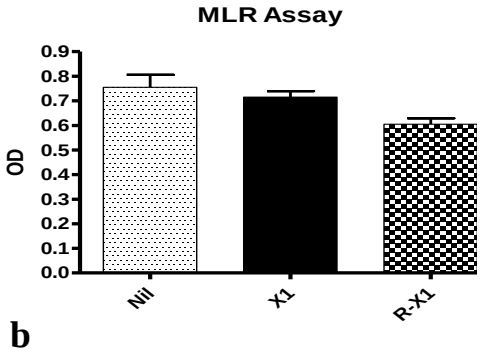


Figure 3.7. Investigation of the effects of transfection on the maturation and antigen presentation ability of DCs. **a.** Comparison of the expression of CD83 on non-transfected, nucleofected, and TTR transfected iDCs and mDCs. Numbers in the upper right quadrants represent the percentage of DCs expressing CD83. **b.** Antigen presentation capacity of nucleofected DCs. Non-transfected iDCs **(Nil)**. Nucleofected iDCs without RNA or DNA with program X1 **(X1)**. Nucleofected iDCs with GFP IVT RNA and program X1 **(R-X1)**. Transfected iDCs with GFP IVT RNA and TTR **(R-TTR)**.

3.4.6 Comparison of DNA and RNA transfection methods

To compare the different approaches for transfection with plasmid DNA and IVT RNA directly, we selected the best conditions for each method based on statistical analyses in terms of efficiency, intensity, and viability and compared them to IVT RNA nucleofection with the X1 program at 72 h after transfection (Fig. 3.10). The efficiency was higher after nucleofection of IVT RNA with the X1 program than after nucleofection of plasmid DNA ($P<0.01$), transfection of IVT RNA by conventional electroporation ($P<0.05$) or transfection of IVT RNA with TTR ($P<0.01$), but equivalent to nucleofection of IVT RNA with the U2 program (Fig 3.10a). In contrast, the protein expression level was equivalent to that achieved with nucleofection of plasmid DNA or transfection of IVT RNA with TTR, but higher than the level achieved by conventional electroporation or nucleofection of IVT RNA with the U2 program ($P<0.05$) (Fig. 3.10b). The viability after nucleofection of IVT RNA with the X1 program was higher than after nucleofection of plasmid DNA ($P<0.01$) but equivalent to the viability achieved with program U2, conventional electroporation or transfection with TTR (Fig. 3.10c). Overall, nucleofection of IVT RNA with the X1 program resulted in extremely efficient transfection, with good long-term viability and comparable or higher levels of protein expression to other methods. Although transfection with TTR was less efficient than nucleofection with IVT RNA with the X1 program, this method resulted in strong protein expression as well as maturation of iDCs.

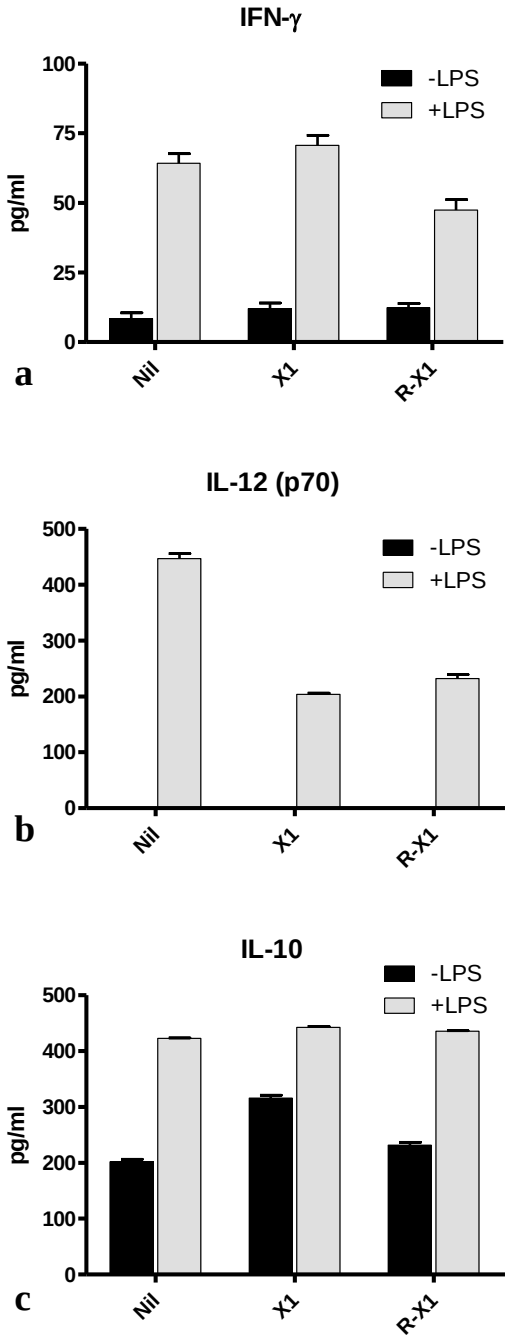


Figure 3.8. Evaluation of the capacity of fast-generated iDCs to secrete cytokines before and after nucleofection. **a.** IFN-γ. **b.** IL-12 (p70). **c.** IL-10. Day 3 iDCs were nucleoporated without or with GFP IVT mRNA using program X1 (X1 and R-X1 respectively) and non-nucleofected iDCs (Nil) were used as control. Four hours after nucleofection 10 μg/ml LPS was added to stimulate cytokine secretion and cells were incubated for an additional 20 h. Supernatants were checked for cytokine secretion (-LPS = No LPS, +LPS = LPS added).

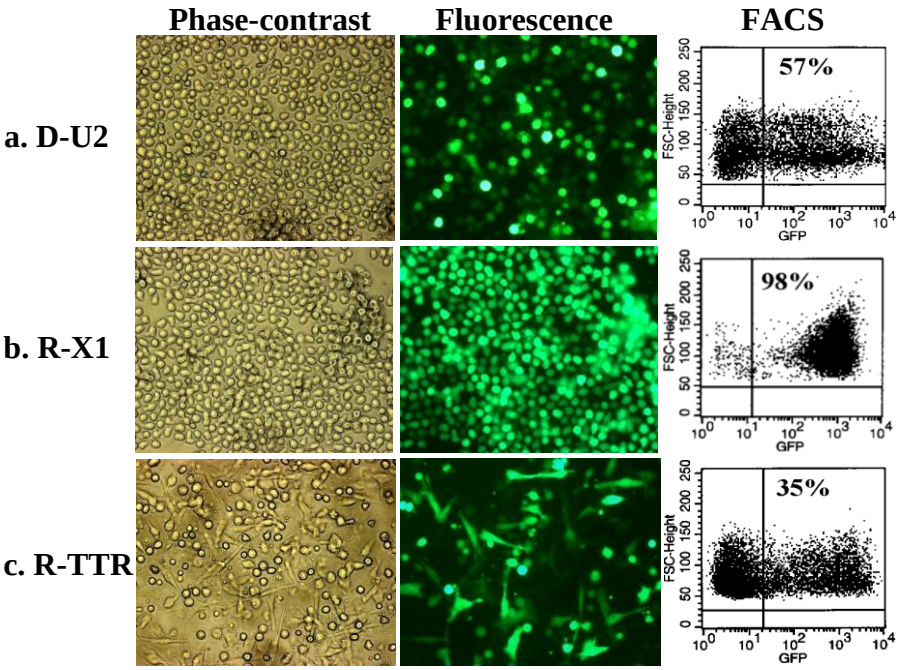


Figure 3.9. Human iDCs transfected on day 3 and analyzed 8 h after transfection: **a.** Nucleofection (program U2) with GFP plasmid DNA **(D-U2)**. **b.** Nucleofection (program X1) with GFP IVT RNA **(R-X1) c.** Transfection of GFP IVT RNA with TTR **(R-TTR)**. Left panels: Phase contrast (× 40). Middle panels: Fluorescence (× 40). Right panels: analysis of GFP expression by flow cytometry on the FL1 channel.

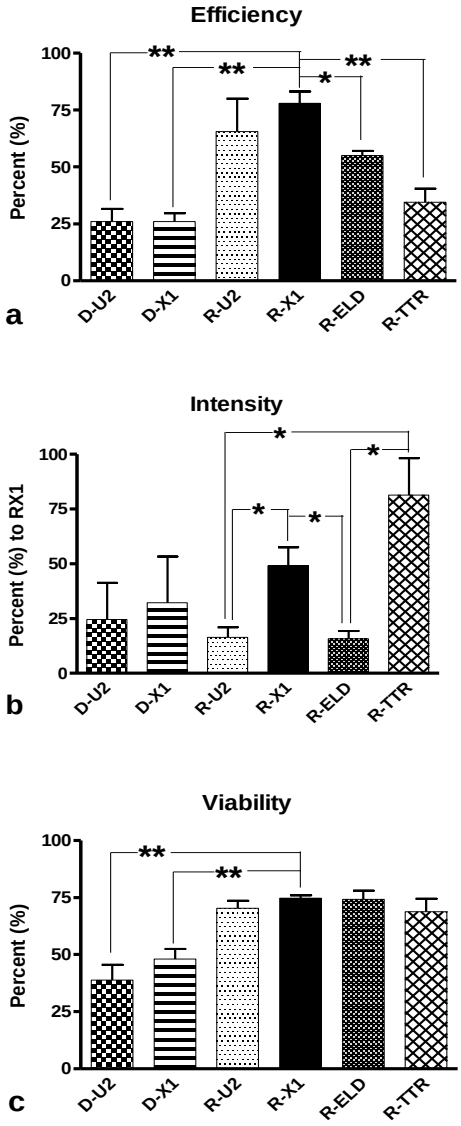


Figure 3.10. Comparison of transfection methods for DNA and RNA. The optimal conditions for the different transfection methods were selected and compared with respect to transfection efficiency, transfection intensity, and cell viability after 72 h. **D-U2** and **D-X1**: Nucleofection of GFP plasmid DNA using programs U2 and X1, respectively. **R-U2** and **R-X1:** Nucleofection of GFP IVT RNA using programs U2 and X1, respectively. **R-ELD:** electroporation of IVT RNA at 300 V, 500 μF, and 400 Ω. **R-TTR:** transfection of IVT RNA with TTR. **a:** Transfection efficiency expressed as percentage of iDCs expressing GFP. **b:** Intensity calculated as in Fig. 3.4 and expressed as a percentage of the strongest fluorescence achieved with the Nucleofector program X1 with GFP IVT RNA. **c:** Cell viability expressed as percentage of live iDCs (P<0.05 and P< 0.01 are shown as * and **, respectively).

3.5 Discussion

Transfer of genetic material into DCs has become a major focus of research having applications to the development of DC-based vaccines for treatment of cancer or chronic infectious diseases. It is also an important requirement for *in vitro* evaluation of the effects of different microbial genes on DCs. Although viral transduction is efficient, increased immunogenicity of transfected DCs due to viral proteins and the risk of oncogenesis from the insertion of the viral genome into host cells *in vivo* have encouraged efforts to establish non-viral transfection methods. We hypothesized that if we generate iDCs more rapidly, this may result in higher viability after transfection, so we established a new protocol to generate iDCs. Although there is one report on direct differentiation and maturation of monocytes into mDCs in 48 h (Dauer, Obermaier et al. 2003), this protocol is faster than previous reports in terms of generating iDCs. These "fast" iDCs showed high endocytic ability and low antigen-presenting capacity, comparable to conventional iDCs generated in 7-14 days. Furthermore, after addition of maturation cocktail the iDCs matured as expected, with an increase in antigen presenting ability.

Since GM-CSF alone drives monocytes toward macrophages (Xu, Roos et al. 2006; Xu, Schlagwein et al. 2007) we hypothesized that IL-4 plays a key role in differentiation toward DCs. Indeed, when we checked the individual effects of GM-CSF and IL-4 we observed that IL-4 was responsible for the reduction in CD14 expression, enhanced CD209 expression and maintained MHC-II expression on the iDCs, confirming that IL-4 is a critical component for the fast differentiation of monocytes to iDCs. This may be due to the fact that IL-4 operates through STAT6, (the primary STAT pathway activated in response to IL-4 (Nelms, Keegan et al. 1999)), which is critical in the activation of many IL-4 responsive genes, including those for MHC class II molecules. Since IL-4 has been demonstrated to enhance the survival of haematopoietic cells

(Dancescu, Rubio-Trujillo et al. 1992; Illera, Perandones et al. 1993; Zamorano, Wang et al. 1996) and to play a role in prolonging T and B cell survival in cultures (Hu-Li, Shevach et al. 1987), IL-4 may also be responsible for better viability of DCs after nucleofection. Activation of PI-3K pathways by IL-4 may enhance cell survival through the production of phosphoinositides and the subsequent activation of kinases critical for cell survival (Nelms, Keegan et al. 1999).

With respect to other culture conditions including higher glutamine, according to the Media Expert in Sigma-Aldrich glutamine is a very important amino acid for highly active cells and can replace serum in culture media. The use of glutamine could promote the differentiation because the cells need a high source of energy and better culture conditions due to being very active during the differentiation process. Finally, the use of higher glutamine and IL-4 resulted in healthier cells, which would also reduce the chance of driving monocytes toward macrophages.

In order to optimize the transfection efficiency of the "fast" iDCs with plasmid DNA we evaluated a number of transfection reagents. However, regardless of the reagent or conditions used, the transfection efficiency was low, whereas the reagents were frequently toxic to the DCs. Therefore, we selected electroporation as an alternative method. Conventional electroporation resulted in very low transfection efficiency and cell viability, whereas nucleofection with plasmid DNA was an effective method with good viability during the first 24 h (Lenz, Bacot et al. 2003). Unfortunately, the viability of the iDCs decreased progressively between 24 and 48 h. We hypothesized that the excess amount of DNA might be an important factor affecting cell viability, so we decreased the amount of DNA and indeed obtained better viability but significantly reduced efficiency. We concluded that using IVT RNA instead of DNA in combination with conventional electroporation or nucleofection might provide a solution. Moreover, since RNA does not need to be transferred through the large pores within the cell

membrane or across the nuclear membrane, gentler electroporation conditions can be used, which should result in better viability and fewer functional defects in the DCs. Transfection with IVT RNA would also be of benefit if the DCs are to be used for DC-based vaccination, either against infectious agents or in cancer patients, because there is no danger of possible integration in cellular genetic material. However, although transfection with IVT RNA by electroporation resulted in good efficiency and viability with some of the conditions, protein expression was low, so transfection of DCs with conventional electroporation and IVT RNA would only be useful for proteins that have to be expressed at low amounts.

Nucleofection of IVT RNA resulted in a mean transfection efficiency of 93%, which to our knowledge is the highest rate reported for a non-viral transfection method, as well as excellent protein intensity, and long-term viability. A concern with IVT RNA would be short stability in the cell cytoplasm; however, GFP was detected for at least five days after transfection with IVT RNA, which would be long enough for assays such as MLR. Although this may be due to high protein stability or high protein expression levels after transfection, there are some explanations supporting enhanced mRNA stability with the nucleofection method. First, there is a possibility that the reagent (patented) used for nucleofection may protect mRNA from degradation in the cytoplasm. Secondly, since the company claims that the nucleofection method transfers the genetic material directly to the nucleus, there is a possibility that mRNA will be directed to the nucleus, where it could be protected from degradation and released gradually to the cytoplasm. Although nucleofection with IVT RNA generally led to equivalent levels of protein compared to transfection with DNA, with program X1 higher amounts of protein were produced which is one of the major advantages of this method, especially where stable and high-enough antigen concentration of TAA is needed (Ochsenbein 2002; Breckpot, Heirman et al. 2004; Ribas 2005).

In addition, high protein expression was detected as early as two hours after nucleofection of IVT RNA, so the iDCs can be collected and used for further assays or immunotherapy very soon after transfection. This approach may provide a solution to poor protein production, which may result in a weak, not-sustained immune response leading to repeated vaccination in cancer immunotherapy trials (Batchu, Moreno et al. 2003; Nencioni and Brossart 2004). Another advantage of transfection with IVT RNA is the uniform and consistent level of protein expression in each cell and for each donor, which implies that all IVT RNA-transfected DCs have similar characteristics.

Importantly, the iDCs nucleofected with IVT RNA remained immature unless maturation cocktail was added. After maturation, the mDCs were capable of antigen presentation as shown in a MLR assay, which demonstrated that they were functional. In contrast, transfection of iDCs with IVT RNA and TTR induced maturation of the iDCs, so TTR may affect the functional properties of the DCs and would not be suitable for *in vitro* studies. However, as this method resulted in strong protein expression as well as maturation of iDCs, it is useful when mDCs, which appear to be more effective for cancer immunotherapy (Liao, Li et al. 2004) are required immediately after transfection without further induction of maturation.

In summary, we established a method for the generation of functionally active iDCs from $CD14^+$ monocytes in three days, after only one treatment with cytokines. This approach reduces the time required for *in vitro* functional assays and provides more viable iDCs for transfection. In addition, we developed two methods for transfection of human Mo-iDCs. Nucleofection led to high efficiency, as well as high protein expression and long-term viability, and thus can be applied to *in vitro* studies, cancer immunotherapy and DC-based vaccination. Transfection with IVT RNA and TTR was less efficient, but resulted in strong protein expression, good long-term

viability, and maturation of the iDCs, so this method might be useful in cancer immunotherapy or DC-based vaccines.

4 Dendritic cells matured by a prostaglandin E2-containing cocktail are phenotypically and functionally more mature and transcriptionally more Th1-biased than dendritic cells treated by TNF-α or LPS

In preparation for publication

4.1 Abstract

Dendritic cells (DCs) are an important component of the immune system and crucial in the initiation of an immune response. There currently is significant interest in the use of DCs in cancer immunotherapy. As maturation is critical for effective antigen presentation to naïve T cells, different methods have been used to generate mature DCs (mDCs) carrying target antigens *ex vivo*. The use of a maturation cocktail (MC) consisting of IL-1β, IL-6, TNF-α, and prostaglandin E2 (PGE2) initially showed promising results, but then was challenged based on the potential for induction of Th2-type immune responses. To investigate this contention, we compared MC with two of the most commonly used maturation factors, TNF-α and LPS. Maturation cocktail was superior to TNF-α and LPS with respect to enhancement of mDC-specific surface marker expression (CD83, CD86, HLA-DR), induction of T cell proliferation by mDCs, and directional motility of mDCs toward CCL19. These results were supported by increased expression of a significant number of additional maturation-related genes by MC in comparison to TNF-α and LPS. In addition, we did not observe a Th2-biased shift in the gene

expression profile of mDCs generated by MC. Conversely, MC induced more Th1-promoting transcriptional changes than LPS or TNF-α. This included increased transcript levels of Th1-type cytokines such as IL-15, IL-12β, EBI3 (IL-27β), which normally precedes the secretion of IL-12 during maturation, and an IL-12-induced gene, PSCDBP, as well as Th1-promoting changes in the transcripts of CXCL16, CCL13, and CCL18. Interestingly, these changes were accompanied by increased transcript levels of MHC class I molecules HLA-A and HLA-F, combined with a reduction in the expression of MHC class II molecules such as HLA-DQA1, HLA-DRB5, HLA-DRA, and HLA-DMA. Finally, MC treatment reduced the transcript levels of TLR2, which was recently suggested to be involved in Th2-type immune responses. These results provide support for the presence of PGE2 in the cocktail used for maturation of DCs.

4.2 Introduction

Dendritic cells (DCs) constitute a family of immune cells that play a central role in the induction of immune responses. They originate from bone marrow and are dispersed throughout the body, but localized more specifically at the sentinel barriers such as skin and respiratory or gastrointestinal mucosa, thus providing protection from pathogens. In these locations, where they are highly exposed to potential harmful non-self antigens, they remain immature (Cella, Sallusto et al. 1997; Banchereau and Steinman 1998; Mellman and Steinman 2001). Considering non-self recognition as the major and initial trigger for a specific immune response, the unique role of DCs to professionally identify and process the characteristic peptide for a specific target gives them a significant role in linking innate and adaptive immunity for an antigen-specific immune response.

Maturation is one of the important characteristics of the DC family and crucial for successful antigen presentation to naïve T cells. After taking up antigens, immature DCs (iDCs) change their morphology and surface marker expression and start the maturation process. In this process, they lose most of their endocytic ability, gain a high capacity for antigen presentation, and switch their migratory potential toward the lymph nodes (LNs) in response to a high concentration of CCL19 (Willimann, Legler et al. 1998) and CCL21 (Ohl, Mohaupt et al. 2004). This process eventually results in effective contact with T cells (Mellman and Steinman 2001), which are also selectively attracted to the LNs (Savina and Amigorena 2007).

Currently, maturation is becoming a more important concept as many studies suggest impairment of DC maturation as the main reason for infectivity or chronicity of several infectious agents, as well as escape of TAAs. When used for cancer immunotherapy, DCs need to have a sufficient level of maturation, as clinical data have shown that mDCs may provide greater therapeutic benefits than iDCs as cancer therapy (Chen, Moyana et al. 2001; Schuler-Thurner, Schultz et al. 2002; Schuler, Schuler-Thurner et al. 2003; Banchereau and Palucka 2005). Thus, the maturation protocol and level of maturity could in fact determine the efficacy and type of the immune response (Liao, Li et al. 2004; Gilboa 2007).

In previous studies, different maturation methods caused differences in the type and characteristics of mDCs generated; indeed, the maturation signal can determine the bias of the immune response (Krause, Singer et al. 2007; Savina and Amigorena 2007; Ten Brinke, Karsten et al. 2007; Xia, Dai et al. 2008). Among all methods, TNF-α and bacterial lipopolysacharide (LPS) at different concentrations are the most frequently used single agents for maturation of DCs *in vitro*. In addition to an increasing number of other single agents, different combinations of cytokines have been used for maturation of DCs as a means to provide highly competent

mDCs. The introduction of a cytokine combination of IL-1β, IL-6, and TNF-α in simulation of the originally used monocyte-conditioned medium (O'Doherty, Steinman et al. 1993) seemed to be more effective in DC maturation. Subsequently, addition of prostaglandin E2 (PGE2) to this cocktail provided good migration efficiency, another crucial mDC characteristic (Jonuleit, Kuhn et al. 1997). This maturation cocktail (MC) has become the most widely used method of maturation (Gilboa 2007; Boullart, Aarntzen et al. 2008), and is now considered the gold standard (Ten Brinke, Karsten et al. 2007). However, based on low production of IL-12 by the mDCs, the role of PGE2, which may result in the potential for Th2-biased immune responses has been challenged (Kalinski, Vieira et al. 2001; McIlroy, Caron et al. 2006; Ten Brinke, Karsten et al. 2007). Other studies, however, support retaining PGE2 in the MC because of several positive effects. For instance, a unique role for PGE2 was demonstrated in dissolution of podosomes and facilitation of mDC motility (van Helden, Krooshoop et al. 2006). The ability of PGE2-generated mDCs to stimulate an optimal T cell response, as well as chemokine and cytokine secretion, provides more support for the use of PGE2 in the MC (Jefford, Schnurr et al. 2003; Rubio, Means et al. 2005). In addition, it was shown that although PGE2 inhibits IL-12 production by LPS, it induces IL-12 secretion in synergy with TNF-α (Rieser, Bock et al. 1997). The importance of PGE2 in maturation of DCs is further supported by the fact that LPS, TNF-α, and IL-1β induce secretion of PGE2 during maturation (Harizi and Gualde 2005). In a recent study the effect of PGE2 in induction of tolerance due to expression of indoleamine 2-3 dioxygenase (IDO), an enzyme involved in T-cell tolerance (Braun, Longman et al. 2005), was contradicted by the fact that the T cell-stimulating capacity of PGE2 overrides the IDO activity (Krause, Singer et al. 2007).

In summary, the exact biology of DC maturation is yet to be discovered and there is a need for more in-depth investigation of different maturation methods, with a more molecular focus. Here, we compared three different populations of mDCs generated by TNF-α, LPS, or a MC containing PGE2 with respect to their gene expression profile, phenotype, and functional properties. Our results show that the level of maturity differed depending on the treatment and that MC-generated mDCs (MC-mDCs) are phenotypically and functionally more mature than those treated by TNF-α or LPS. These results were confirmed at the transcriptional level, as MC appeared to have a more significant effect on transcripts of Th1-related genes than LPS or TNF-α.

4.3 Materials and methods

4.3.1 Generation of monocyte-derived immature and mature dendritic cells

Fast iDCs were generated as described previously (Landi, Babiuk et al. 2007). Briefly, human peripheral blood mononuclear cells were collected by density gradient separation, and monocytes were isolated using paramagnetic MicroBeads (Miltenyi Biotech, Auburn, CA, USA). The monocytes were then cultured in complete RPMI (CRPMI; RPMI 1640 [Invitrogen] supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 100 μM non-essential amino acids, 1 mM sodium pyruvate, 50 μM 2ME, 10 mM HEPES, and 50 μg/ml gentamycin), in the presence of recombinant human (rh)GM-CSF (100 ng/ml, Peprotech Inc., NJ, USA) and rhIL-4 (200 ng/ml, Peprotech). To generate mDCs, iDCs were collected on day 3, washed, and resuspended in CRPMI containing 50 ng/ml rhGM-CSF and 100 ng/ml rhIL-4. The cells were then dispensed in a 6-well plate and matured for 48 h using three different methods of maturation: rhTNF-α (Peprotech) at a concentration of 50 ng/ml, LPS (Sigma-Aldrich Canada

Ltd., Oakville, ON, Canada) at a concentration of 10 μg/ml, and a MC consisting of 20 ng/ml rhIL-1β (Peprotech), 10 ng/ml rhIL-6 (Peprotech), 50 ng/ml rhTNF-α, and 1 μg/ml PGE2 (Sigma-Aldrich). Immature DCs without addition of any maturation agent were used as control. To optimize the concentration of TNF-α and LPS, CD83 and CD86 marker expression was used as an indicator for the level of maturity. The concentrations resulting in the highest percentage of CD83 and CD86 expressing cells were chosen for further studies (data not shown).

4.3.2 Array analysis

After 48 h of incubation with different maturation factors, mDCs were washed in phosphate buffered saline pH 7.2 (PBS), and total RNA was extracted from the cells using a RNA/Protein Isolation NucleoSpin RNA/Protein Kit (MACHEREY-NAGEL**,** Bethlehem, PA, USA) according to the manufacturer's instructions. To make biotin-integrated cRNA, a SuperArray (SuperArray Biosciences, Frederick, MD, USA) reagent kit was used according to the instructions, and 3 μg of cRNA was hybridized to a specific Dendritic Cell and Antigen-presenting cell array with 288 spots of DC-specific genes (OHS-406; SuperArray). All steps were performed according to the protocol recommended by the supplier. However, for labeling and detection we optimized a one-step infrared labeling procedure using an infrared dye conjugated to streptavidin called IRDye CW800-SA (Perkin Elmer). Arrays were scanned using an Odyssey infrared scanner. The intensities were calculated using software Odyssey v.3, and raw integrated intensity values were loaded into GeneSpring software Version 7.3. Data were normalized based on GAPDH housekeeping genes on the array and median polishing was done as the second step for normalization.

4.3.3 Flow cytometry

DCs were washed in PBS and resuspended in FACS buffer (PBS, pH 7.2 with 0.2% gelatin and 0.03% sodium azide) in a round-bottom 96-well plate (Corning Incorporated, Corning, NY, USA). Subsequently, fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated monoclonal antibodies (Becton Dickinson Biosciences, Oakville, ON, Canada) were added to each well, and the plate was incubated on ice for 30 min. After incubation, cells were washed with FACS buffer, resuspended in cold PBS and analyzed by FACScan (BD Biosciences). The cells were gated electronically for Forward Scatter and Side Scatter properties to include the main population of the cells and exclude dead cells, and more than 10,000 events were analyzed. Cell Quest software (BD Biosciences) was used for analysis of flow cytometry data.

4.3.4 Phagocytosis assay

A phagocytosis assay was performed as described previously (Landi, Babiuk et al. 2007). Briefly, DCs were collected, washed in PBS, and resuspended in CRPMI. The cell suspension was dispensed in a 1.5 ml eppendorf tube, and FITC-conjugated dextran (DX) (Sigma-Aldrich) in PBS (1 mg/ml) was added to the DCs. The treatment tubes were incubated at 37ºC and the control ones were incubated on ice for 2 h in the dark. After incubation, the cells were collected, washed with cold PBS, and checked by flow cytometry for FITC-DX fluorescence as an indicator of phagocytosis.

4.3.5 Allostimulatory mixed leukocyte reaction (MLR)

Immature and mature DCs were collected, washed in PBS, and resuspended in CRPMI. The cell suspension was dispensed in the wells of a round-bottom 96-well plate. Subsequently, isolated $CD4^+$ cells ($CD4^+$ T cell isolation kit [Miltenyi]) with a purity of more than 95% from a different, HLA-mismatched healthy donor were added to each well, and the plate was incubated at 37ºC. After 3-5 days, ^{3}[H] thymidine was added to each well at 0.5 μCi, and the cells were incubated for an additional 16-18 hours. Subsequently, the plates were frozen at -20 ºC and at the appropriate time were thawed, harvested and read by a Packard microplate scintillation and luminescence counter (Packard BioScience Company, Meriden, CT, USA).

4.3.6 Chemotaxis assay

Chemotaxis assays were performed using a 24-well Transwell system (Corning Incorporated). After 2 days of incubation with maturation factors, mDCs were collected, washed and resuspended at 1×10^6 cells/ml in chemotaxis buffer (XRPMI; RPMI with 0.1% BSA, 10mM HEPES, 20 μM 2ME, 20 μg/ml gentamycin, 50 μg/ml GM-CSF, and 100 μg/ml IL-4). Chemokine CCL19 was diluted to different concentrations in XRPMI, and dispensed in a low-attachment 24-well plate (Corning Incorporated). Medium without any chemokine was used as a control. Transwell inserts were placed on each well, and 150 μl of cell suspensions from different treatments were added to the insert. Subsequently, the plate was incubated at 37 ºC for 2 h. Cells in each well were harvested, concentrated to 200 μl, and counted by FACScan for 1 min.

4.3.7 Statistical analysis

Data were analyzed using statistical software (GraphPad Prism Version 5.00). As variables were not normally distributed, the medians between pairs of groups were compared using the Mann-Whitney U test. Differences were considered significant if the two-tailed *P* value was lower than 0.05 with confidence intervals of 95%.

4.4 Results

4.4.1 Mature dendritic cells generated by different treatments vary significantly in their gross morphology

Immature DCs were treated with TNF-α, LPS or MC and 48 h later their morphology was examined under a light microscope. Mature DCs generated by TNF-α treatment (TNF-mDCs) were starting to develop thick dendrites with an irregular shape and were relatively non-adherent in comparison to iDCs. In contrast, DCs matured by LPS (LPS-mDCs) were elongated irregular large cells with a high tendency to attach to the culture plate; they resembled fibroblasts and were difficult to harvest. Finally, MC-mDCs were smaller and relatively round cells in comparison to TNF- and LPS-mDCs, and completely non-adherent with many small dendrites (Fig. 4.1).

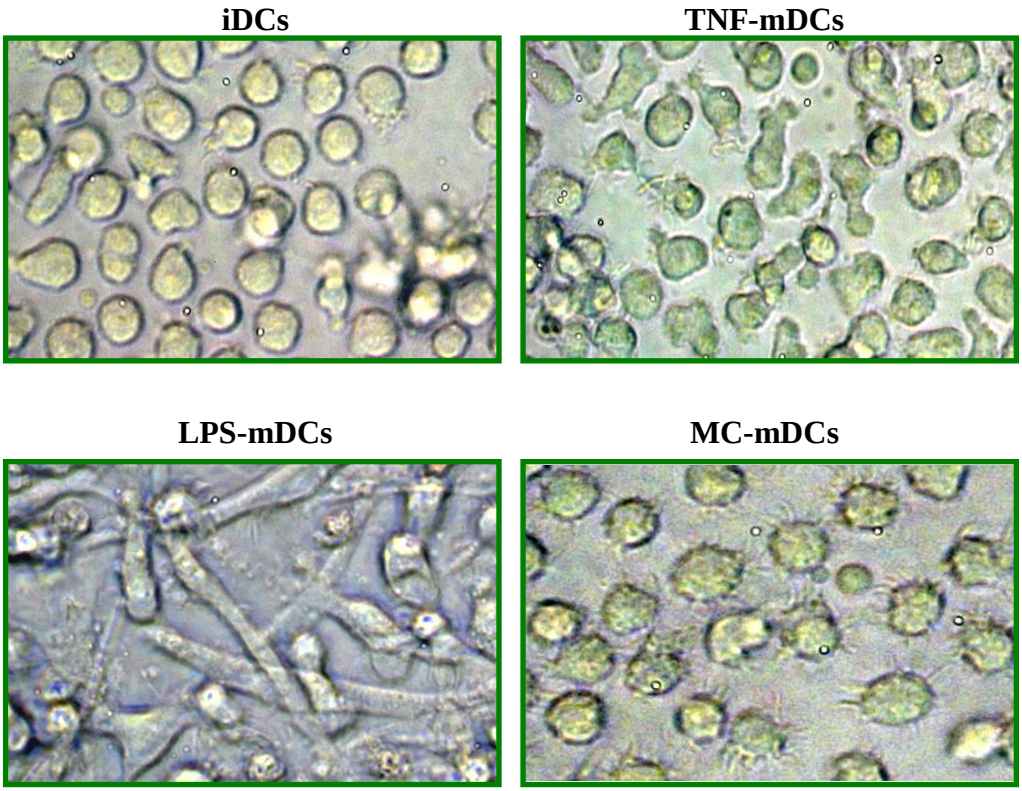


Figure 4.1. Immature DCs treated with different maturation agents or not treated; **iDCs:** immature DCs, **TNF-mDCs:** mDCs generated using TNF-α, **LPS-mDCs:** mDCs generated using LPS, and **MC-mDCs:** mDCs generated using maturation cocktail containing IL-1β, IL-6, TNF-α, and PGE2 (magnification of ×40).

4.4.2 Treatment of dendritic cells with maturation cocktail results in significant changes in gene expression

To further investigate the effects of different maturation methods, the transcriptional changes in iDCs incubated with TNF-α, LPS or MC were examined after 48 h, using non-treated iDCs as control. Although expression of many genes may be change during the first hours, the 48 h time-point was chosen to investigate more stable changes in gene expression in fully matured DCs, which persist even after degradation of cytokines and maturation stimuli. One and half-fold

changes in expression (based on normalized values) in comparison to control were used as cutoff. MC was found to change the expression of 86 genes, which corresponded to 95% of the changes (90 genes) observed for any treatment (Fig. 4.2a). In contrast, TNF-α induced a change in expression of only 29 genes (32%), which with exception of two genes were all in common with those changed by MC. Lipopolysacharide altered expression of 41 genes (45%), 90% of which were included in the MC list. Taking the additional 42 genes altered by MC into consideration, this indicates that MC treatment has more radical effects on the transcriptional profile of DCs in comparison to TNF-α and LPS.

To investigate the changes induced by each treatment in more detail, up- and down-regulated genes were compared separately. Of the 50 up-regulated genes, 40 genes were affected by MC, which was higher in number in comparison to the 30 genes changed by LPS and the 21 genes altered by TNF-α (Fig. 4.2b). Indeed, the MC-affected genes included 66% and 81% of the genes up regulated by LPS and TNF-α, respectively. Of the down-regulated genes, in addition to the 8 and 11 genes altered by LPS and TNF-α, respectively, MC was also found to distinctively reduce the expression of another 33 genes (Fig. 4.2c). Overall, the number of genes down-regulated by MC appeared to be significantly higher than the number of up-regulated ones.

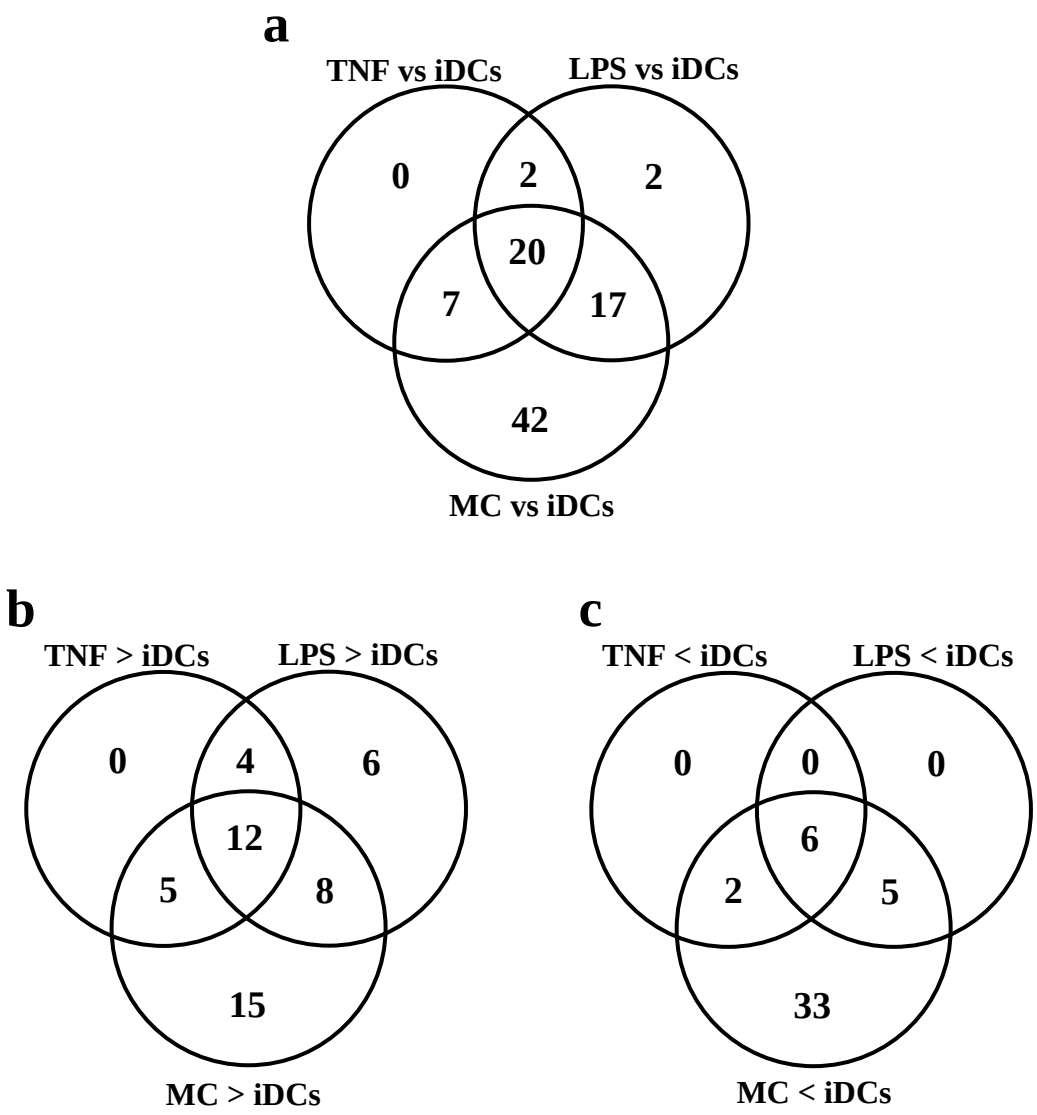


Figure 4.2. Gene expression changes in non-treated iDCs and TNF-, LPS-, or MC-mDCs. **(a)** Total number of genes changed after treatment. **(b)** Up-regulated genes. **(c)** Down-regulated genes. Only genes that changed in expression level by 1.5-fold in comparison to the iDCs are shown. **iDCs:** immature DCs, **TNF:** mDCs generated using TNF-α, **LPS:** mDCs generated using LPS, and **MC:** mDCs generated using maturation cocktail containing IL-1β, IL-6, TNF-α, and PGE2.

4.4.3 Maturation cocktail has more significant effects on dendritic cell maturation than TNF-α and LPS

Among the genes that were up-regulated by all treatments, CD83, CD86, CCR7, lymphocyte antigen 75 (LY75 or CD205), Epstein-Barr virus induced gene 3 (EBI3), IL-1β, and CCL22 are well-known maturation markers or mDC-associated cytokines/chemokines (Fig. 4.3a). The nuclear factor-kappa B1 (NFKB1), NFKB2, and proline-rich nuclear receptor coactivator 1(PNRC1) genes, which are components of the NFKB pathway were also up regulated by all treatments (Fig. 4.3a). The NFKB pathway has been reported to be involved in DC maturation (Lindstedt, Johansson-Lindbom et al. 2002; Ju, Hacker et al. 2003; Platzer, Jorgl et al. 2004; Valentinis, Capobianco et al. 2008). However, the increase in these transcripts was significantly higher in DCs treated by MC than DCs treated by TNF-α or LPS, with exception of IL-1β, which was higher in LPS-mDCs, and CCL22, which was almost equally increased by all treatments (Fig. 4.3a).

When compared in pairs, there were eight common genes between MC-mDCs and LPS-mDCs (IL-8, CXCL1, CXCL2, IL-6, CXCL5, CXCL16, CXCR4, and interferon stimulate gene 20 [ISG20 or CD25]), and five common genes between MC-mDCs and TNF-mDCs (reticuloendotheliosis viral oncogene homolog B [RELB], myristoylated alanin-rich protein kinase C substrate [MARCKS] L1, a disintegrin and metalloprotease domain [ADAM19], lysosomal-associated membrane protein 3 [LAMP3 or CD208] and B cell translocation gene 1 [BTG1]) (Fig. 4.3b). Although the gene expression levels for IL-8, IL-6, CXCL1, and CXCL2 was higher in LPS-mDCs than in MC-mDCs, the transcripts of most mDC-specific genes including CXCL16, CXCR4, ISG20, RELB, LAMP3, MARCKSL1, and BTG1 were found to be significantly higher after MC treatment than LPS or TNF-α treatments (Fig. 4.3b).

Among the genes up-regulated by TNF-α and/or LPS, but not by MC, a series of chemokines (CCL3L1, CCL4, CCL5, CCL7, and CCL8) involved in attraction of polymorphonuclear cells (PMN), T cells and iDCs, as well as ADAMDEC1, transmembrane 7 superfamily member 4 (TM7SF4 or DCSTAMP), low affinity immunoglobulin gamma Fc region receptor III-B (FCGR3B), and high affinity immunoglobulin E receptor, gamma chain (FCER1G) were observed (Fig. 4.3c). Surprisingly, the last two genes are involved in phagocytosis, which is normally reduced during maturation.

In addition to these genes, there was a list of 15 additional genes distinctively up-regulated by MC treatment, including several important ones such as IL-12β, IL-15, pleckstrin homology Sec7 and coiled-coil domains binding protein (PSCDBP), HLA-A, HLA-F, CD58, intercellular adhesion molecule 1 (ICAM1 or CD54), MARCKS, and PIM2 (Fig. 4.3d). A more detailed analysis of this gene list shows that most of these additional genes affected by MC are involved in DC maturation.

Of the down-regulated genes, all the common genes have been reported to be down-regulated in mDCs; however, the reduction in expression of CCL13, F13A1, and S100A4 was more significant in MC-mDCs than LPS-mDCs and TNF-mDCs (Fig. 4.3a). MC also more significantly reduced the transcript levels of ARHGDIB, CD1a, CD1c, CSF1R, FCER1A and VCL than LPS or TNF treatmens (Fig. 4.3b). In addition, the reduction in expression of 20 of the 33 MC-specific genes that are negatively regulated during maturation also favors DC maturation. This list includes CCL18, iDC-specific markers (CCR1 and CCR5), adhesion molecules (CLEC4A, ITGAX, and ITGB2), phagocytosis-related molecules (FCER1G, FCER2, FCGR2B, FCGR3B, and PDIA1), a series of the HLA class II molecules (HLA-DQA1, HLA-DRB5, HLA-

DRA, and HLA-DMA), HSP90AB1, machrophage migration inhibitory factor (MIF), TLR2, CD1b, and LIPA (Fig. 4.3d).

Taken together, although there was a slight difference between the effects of TNF-α and LPS on the gene expression of DCs, it is clear that MC had a significant additional effect on the level of transcripts related to DC maturation. This suggests MC to be a superior cocktail for maturation of DCs.

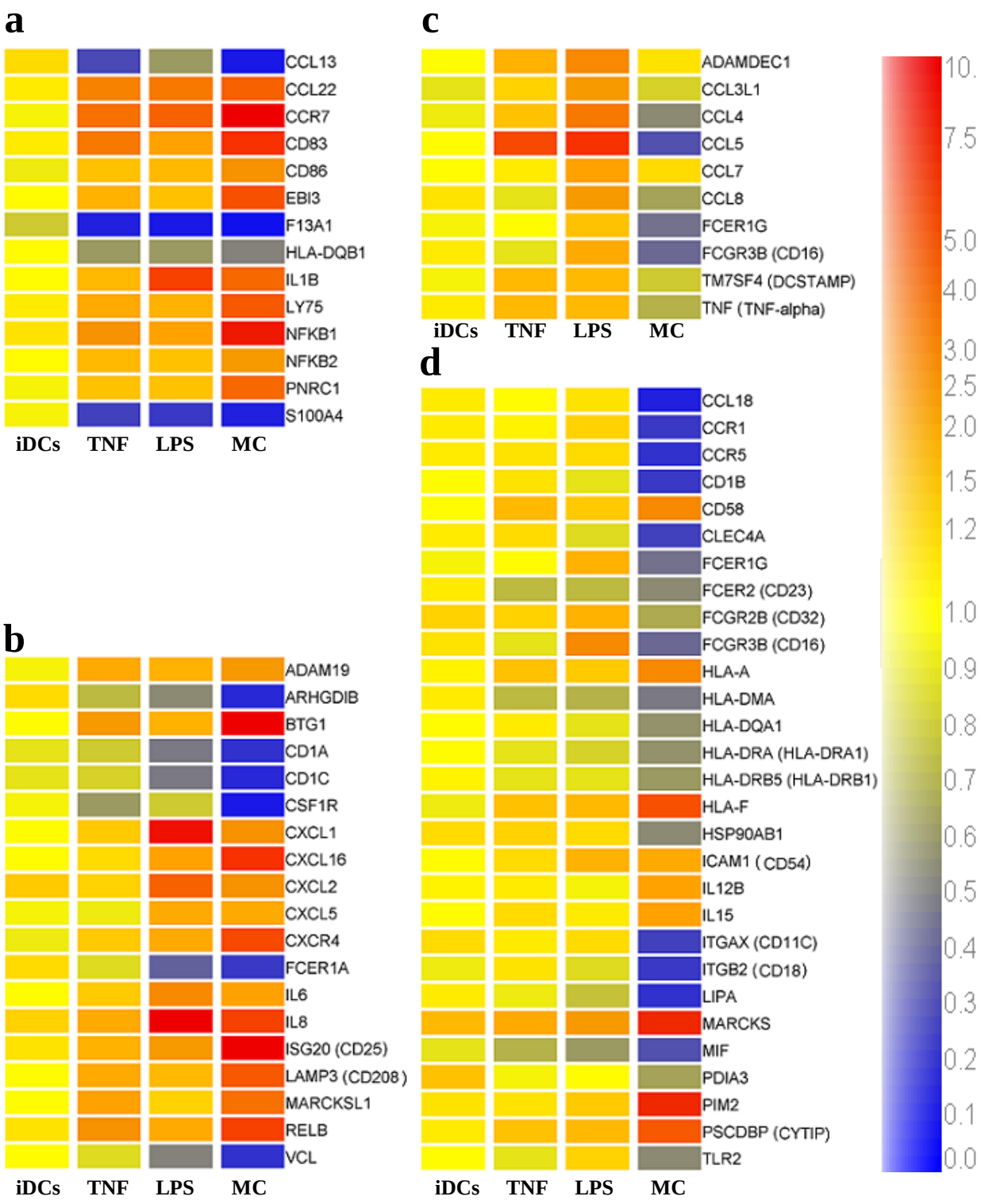

a
CCL13
CCL22
CCR7
CD83
CD86
EBI3
F13A1
HLA-DQB1
IL1B
LY75
NFKB1
NFKB2
PNRC1
S100A4
iDCs TNF LPS MC
b
ADAM19
ARHGDIB
BTG1
CD1A
CD1C
CSF1R
CXCL1
CXCL16
CXCL2
CXCL5
CXCR4
FCER1A
IL6
IL8
ISG20 (CD25)
LAMP3 (CD208)
MARCKSL1
RELB
VCL
iDCs TNF LPS MC
c
ADAMDEC1
CCL3L1
CCL4
CCL5
CCL7
CCL8
FCER1G
FCGR3B (CD16)
TM7SF4 (DCSTAMP)
TNF (TNF-alpha)
iDCs TNF LPS MC
d
CCL18
CCR1
CCR5
CD1B
CD58
CLEC4A
FCER1G
FCER2 (CD23)
FCGR2B (CD32)
FCGR3B (CD16)
HLA-A
HLA-DMA
HLA-DQA1
HLA-DRA (HLA-DRA1)
HLA-DRB5 (HLA-DRB1)
HLA-F
HSP90AB1
ICAM1 (CD54)
IL12B
IL15
ITGAX (CD11C)
ITGB2 (CD18)
LIPA
MARCKS
MIF
PDIA3
PIM2
PSCDBP (CYTIP)
TLR2
iDCs TNF LPS MC
10.
7.5
5.0
4.0
3.0
2.5
2.0
1.5
1.2
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0

Figure 4.3. Changes in gene expression levels of iDCs after treatment with TNF-α, LPS, or MC, shown by condition tree. **(a)** Maturation-related genes, which are commonly changed in all treatments. **(b)** Maturation-related genes, which are commonly changed between MC and LPS treatments, or MC and TNF-α treatments. **(c)** Genes that are changed by TNF-α and/or LPS, but not MC treatments. **(d)** Maturation-related genes, which are distinctively changed by MC treatment. **iDCs:** immature DCs, **TNF:** mDCs generated using TNF-α, **LPS:** mDCs generated using LPS, and **MC:** mDCs generated using maturation cocktail containing IL-1β, IL-6, TNF-α, and PGE2. The level of gene expression was normalized to non-treated iDCs. The color bar on the right shows the range of colors based on the level of gene expression. Yellow was set as normal (equal to 1), blue as low, and red as high level of gene expression, respectively. Results from three biological replicates are shown.

4.4.4 Maturation cocktail induces a Th1-favored transcriptional profile in mDCs

When the changes in the expression of Th1- and Th2-related genes were examined, MC induced a significantly higher increase in transcripts of Th1-related genes, with the exception of heat shock 90kDa protein 1 alpha class B (HSP90AB1) (Fig. 4.4). This included CXCL16, EBI3, and LAMP3, commonly up-regulated by all treatments, as well as the MHC class I molecules HLA-A and HLA-F, Th1-type cytokines IL-12β, IL-15, and the IL-12-induced gene PSCDBP. Conversely, the expression levels of Th2-related genes was lower in MC-mDCs than in LPS-mDCs or TNF-mDCs. Chemokine CCL13 was down-regulated by all treatments, and six additional genes were distinctively down-regulated by MC, including CCL18, TLR2, and the MHC class II molecules HLA-DQA1, HLA-DRB5, HLA-DRA, and HLA-DRM. The level of HLA-DQB1 transcript, a MHC class II molecule, was equally reduced by all treatments. Interestingly, expression of CCL22, an mDC-specific Th2-type chemokine was equally up-regulated by all treatments. Overall, MC appeared to be capable of generating Th1-promoting mDCs, by inducing Th1-biased changes in the transcripts of 16 out of 18 Th1/Th2-related genes.

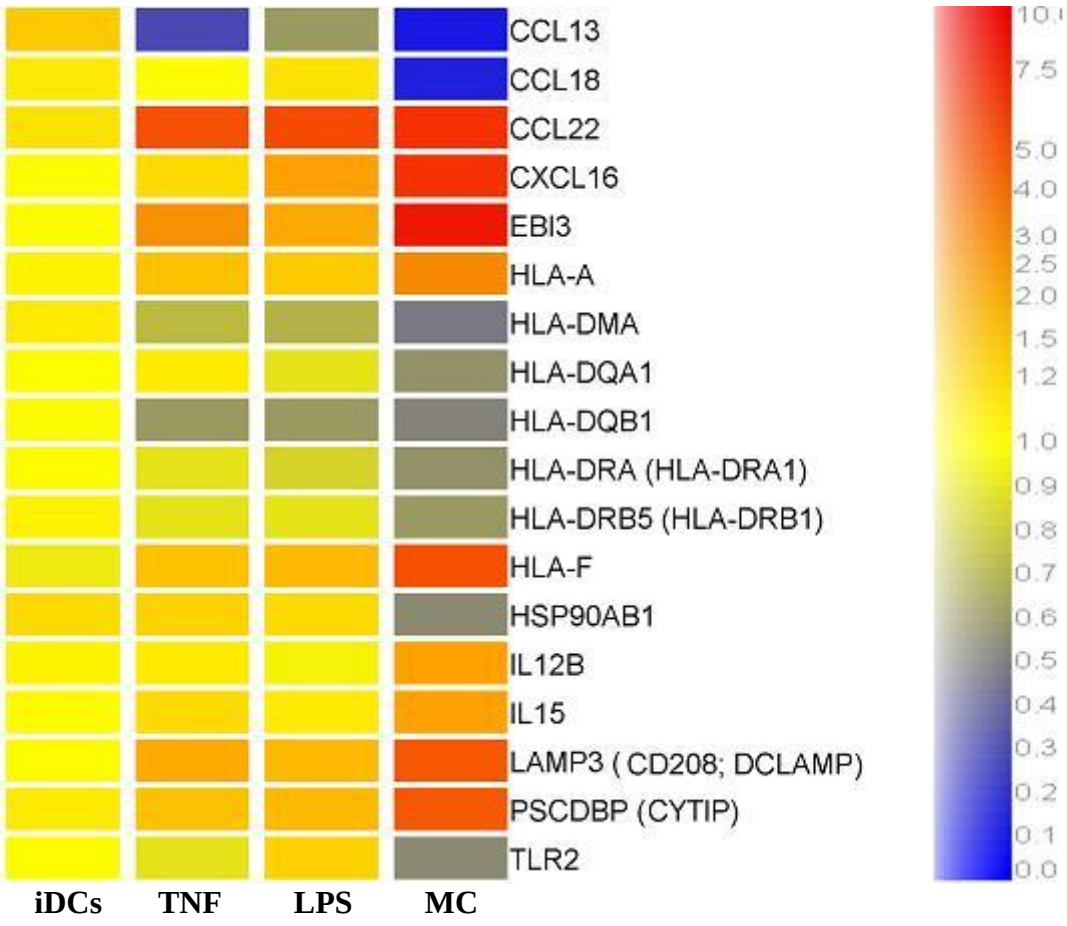


Figure 4.4. Changes in gene expression levels of 18 Th1- or Th2-related genes after treatment with TNF-α, LPS, and MC in comparison to non-treated iDCs. **iDCs:** immature DCs, **TNF:** mDCs generated using TNF-α, **LPS:** mDCs generated using LPS, and **MC:** mDCs generated using maturation cocktail containing IL-1β, IL-6, TNF-α, and PGE2. The level of gene expression was normalized to non-treated iDCs. The color bar on the right shows the range of colors based on the level of gene expression. Yellow was set as normal (equal to 1), blue as low, and red as high level of gene expression, respectively. Results from three biological replicates are shown.

4.4.5 Mature dendritic cells generated by maturation cocktail express higher levels of maturation markers

Since mDCs generated by different methods varied significantly in gross morphology and gene expression, we further characterized their phenotypes by evaluating the expression levels of known maturation markers (CD83, CD86, and HLA-DR). Flow cytometry data confirmed that MC-mDCs were most mature among all treated mDCs (Fig. 4.5). For instance, CD83, the characteristic functional marker for maturation was expressed on 75% of the MC-mDCs in comparison to an average of 36% of the LPS-mDCs and 22% of the TNF-mDCs (Fig. 4.5a, b). Cluster of differentiation 86 (CD86) expression, a co-stimulatory molecule that plays a role in antigen presentation, was also higher on MC-mDCs (Fig. 4.5c, d). For HLA-DR expression, the mean fluorescent intensity (MFI) was compared as the amount of this marker on each individual cell, but not the number of cells expressing HLA-DR, increases during maturation due to translocation from an intra-cytoplasmic location to the surface of the cell membrane. The MFI of HLA-DR in MC-mDCs was significantly higher than that of TNF- and LPS-mDCs (Fig. 4.5e, f). In summary, MC-mDCs showed significant surface marker changes by FACS analysis compatible with a mature phenotype.

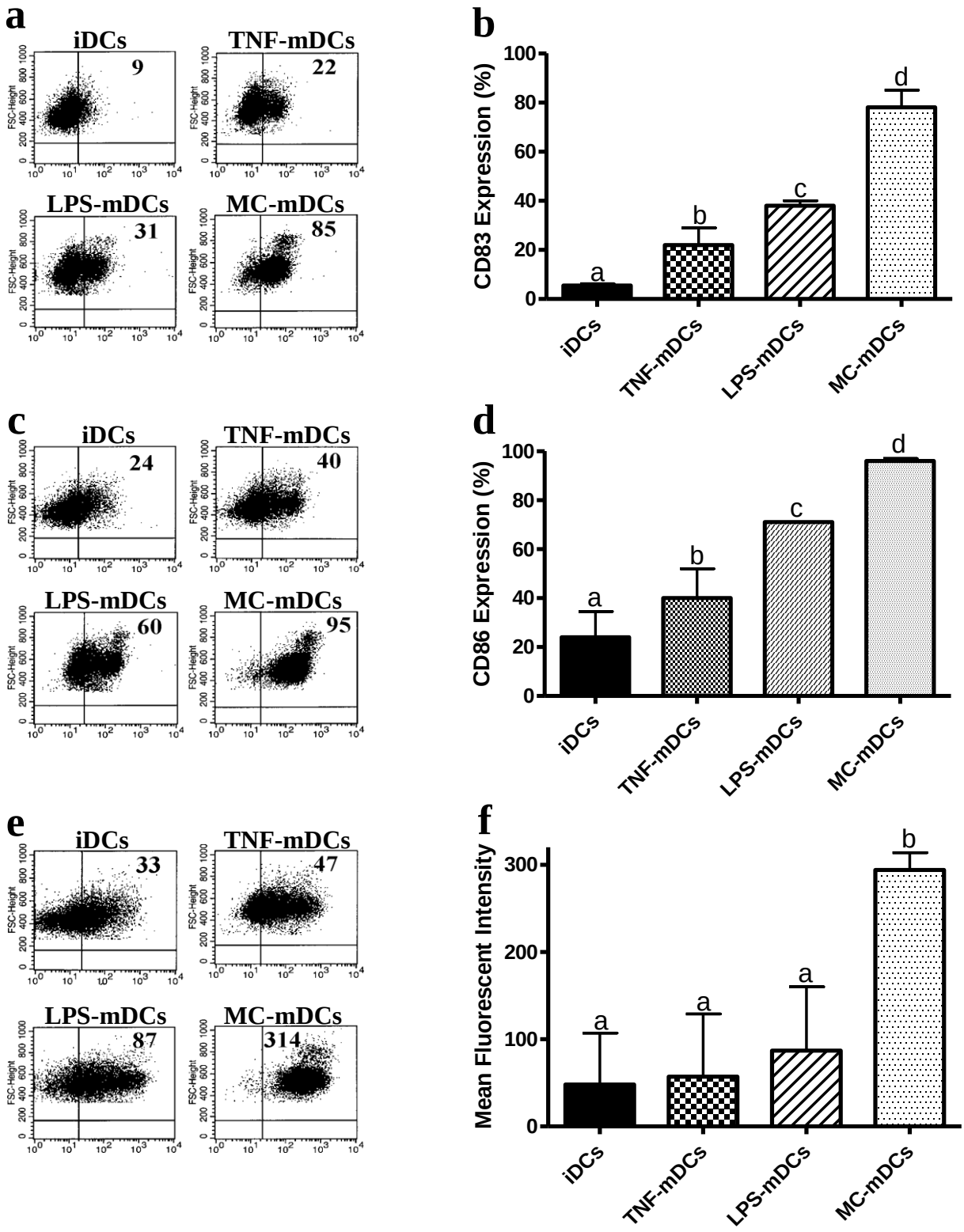

a
iDCs
9
TNF-mDCs
22
LPS-mDCs
31
MC-mDCs
85
FSC-Height
b
CD83 Expression (%)
a
b
c
d
iDCs
TNF-mDCs
LPS-mDCs
MC-mDCs
c
iDCs
24
TNF-mDCs
40
LPS-mDCs
60
MC-mDCs
95
d
CD86 Expression (%)
a
b
c
d
iDCs
TNF-mDCs
LPS-mDCs
MC-mDCs
e
iDCs
33
TNF-mDCs
47
LPS-mDCs
87
MC-mDCs
314
f
Mean Fluorescent Intensity
a
a
a
b
iDCs
TNF-mDCs
LPS-mDCs
MC-mDCs

Figure 4.5. Phenotypic marker expression on non-treated iDCs and DCs 48 h after treatment with TNF-α, LPS, or MC. **(a)** Representative FACS dot plot for each marker. Numbers in the upper right quadrants represent the percentage of positive cells for CD83 and CD86 and the mean fluorescent intensity for HLA-DR. **(b)** Percentage of cells expressing CD83, **(c)** Percentage of cells expressing CD86, **(d)** Mean fluorescent intensity (MFI) of HLA-DR. **iDCs:** immature DCs, **TNF-mDCs:** mDCs generated using TNF-α, **LPS-mDCs:** mDCs generated using LPS, and **MC-mDCs:** mDCs generated using maturation cocktail containing IL-1β, IL-6, TNF-α, and PGE2. Bars with different letters are statistically different from each other and those with the same letters are not statistically different from each other. Bars and error bars represent median with interquartile range.

4.4.6 Mature dendritic cells generated by maturation cocktail function more effectively than TNF- and LPS-mDCs

In order to check the functionality of the mDCs generated by the different treatments, they were checked in an *in vitro* phagocytosis assay. Tumor necrosis factor- and LPS-mDCs had similar phagocytic abilities, which however were significantly lower than that of iDCs; the MC-mDCs showed an even more significant reduction in their endocytic ability in comparison to the other treatments (Fig. 4.6a). Furthermore, TNF-, LPS-, and MC-mDCs were evaluated in an allostimulatory MLR assay, using $CD4^+$ T cells as responder cells. As shown in Fig. 4.6b, MC-mDCs induced enhanced T cell proliferation in comparison to all other DCs, which is compatible with improved antigen presentation, an important characteristic of mDCs. The difference between TNF- and LPS-mDCs was also significant suggesting that LPS likely has a stronger effect on the level of maturity with respect to antigen presentation. Finally, a chemotaxis assay using the chemokine CCL19, a ligand for CCR7, showed a very significant increase in the level of motility in MC-mDCs in comparison to TNF- and LPS-mDCs (Fig. 4.6c). In spite of their adherence in culture, LPS-mDCs showed a better motility than TNF-mDCs in the chemotaxis assay.

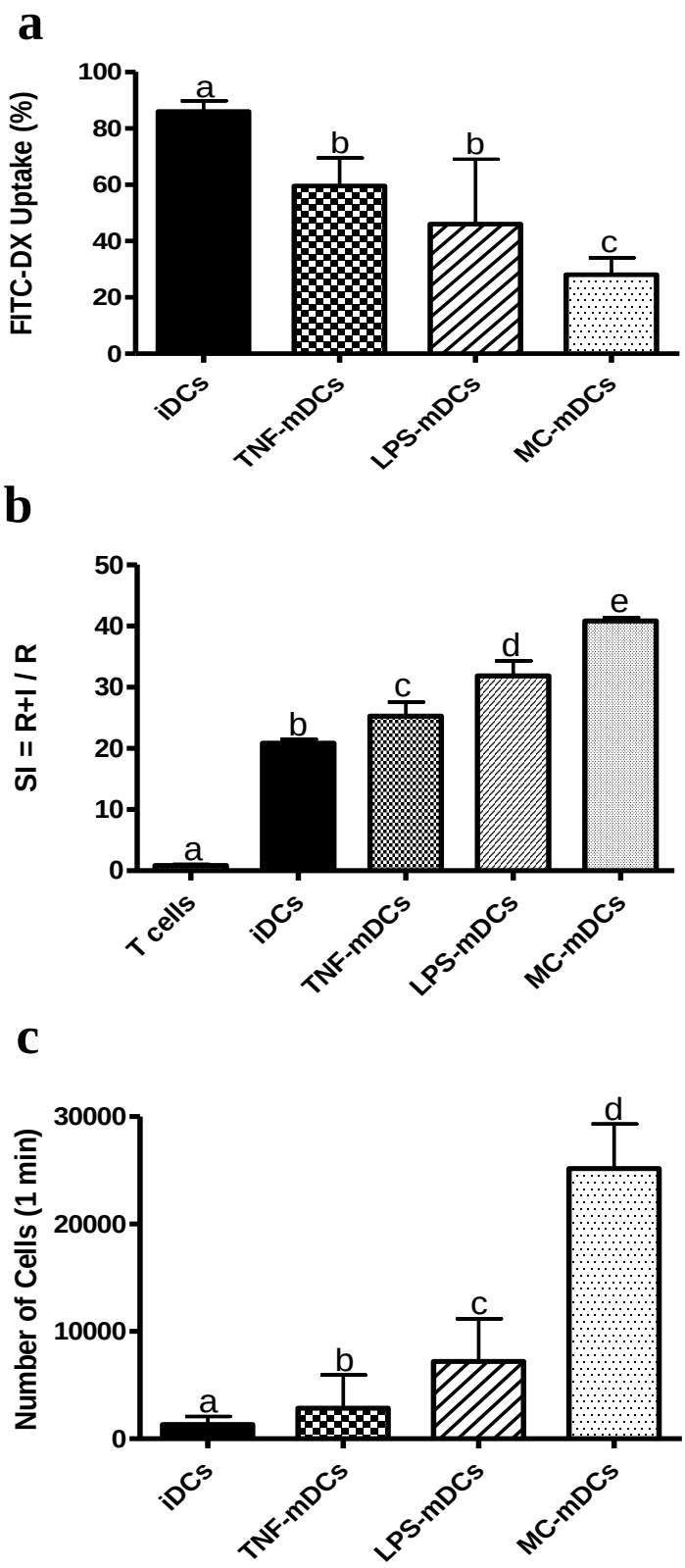

a
FITC-DX Uptake (%)
100
80
60
40
20
0
a
b
b
c
iDCs
TNF-mDCs
LPS-mDCs
MC-mDCs
b
SI = R+I / R
50
40
30
20
10
0
a
b
c
d
e
T cells
iDCs
TNF-mDCs
LPS-mDCs
MC-mDCs
c
Number of Cells (1 min)
30000
20000
10000
0
a
b
c
d
iDCs
TNF-mDCs
LPS-mDCs
MC-mDCs

Figure 4.6. Functional characterization of non-treated iDCs and DCs after treatment with TNF-α, LPS, or MC. **(a)** Phagocytosis assay. **(b)** MLR assay. Stimulation index (SI) was calculated with the formula: Radioactive count per minute (CPM) for culture of T cells (R) and DCs (I) : CPM in R, the control group (T cells culture alone). **(c)** Chemotaxis assay. CCL19 was used as chemoattractant in the bottom well of a Transwell system. **T cells**: T cells only, **iDCs:** immature DCs, **TNF-mDCs:** mDCs generated using TNF-α, **LPS-mDCs:** mDCs generated using LPS, and **MC-mDCs =** mDCs generated using maturation cocktail. The vertical axis (Y) shows the number of cells counted by flow cytometry in 1 min after concentration of the samples to 200 µl after harvesting. Bars with different letters are statistically different from each other and those with the same letters are not statistically different from each other. Bars and error bars represent median with interquartile range.

4.5 Discussion

Different methods of maturation have been used to generate mDCs for *in vitro* studies or clinical trials. A cocktail of IL-1β, IL-6, TNF-α and PGE2 has been widely used, but the presence of PGE2 in this cocktail was recently challenged because of the potential for induction of a Th2-biased immune response. In this study, we compared this cocktail with two common single agents, TNF-α and LPS, with respect to their effects on the maturation of DCs at the molecular and functional level. Our data showed that mDCs generated using these methods are very different in their morphology, gene expression profile, surface marker expression, and functional properties. In addition to the gene expression changes induced by TNF-α and LPS, MC modulated the transcription of a significant number of other genes, which are mostly related to maturation. Moreover, we did not observe any changes in gene expression profile corresponding to a Th2-bias in MC-mDC; conversely, MC induced Th1-promoting changes in the transcript levels of both Th1- and Th2-related genes in mDCs (Fig. 4.4).

Based on a comprehensive analysis of the functional properties of the genes affected by MC, the changes agreed with functions expected of mDCs. Several genes involved in antigen presentation (CD83, CD86, LY75, LAMP3, MARCKS, MARCKSL1, PSCDBP, ICAM1, CD58, HLA-A and HLA-F) were significantly or distinctively up-regulated by MC, while genes related to phagocytosis (CSF1R, FCER1A, FCER1G, FCER2, FCGR2B, FCGR3B, MIF, and PDIA3) were down-regulated. Genes favoring high directional motility (CCR7, CXCR4, ARHGDIB, MARCKS, MARCKSL1, ADAM19, BTG1, ICAM1, MIF), and lower adhesion properties (FCER1A, F13A1, S100A4, CLEC4A, ITGAX, ITGB2, VCL, and CSF1R) were up- or down-regulated as expected. These changes were mostly more significant after MC treatment than LPS or TNF-α treatments. The migration capacity is a crucial property of mDCs as *in vivo* injected DCs were reported to not leave the site of injection unless PGE2 had been added to the maturation cocktail (Morse, Coleman et al. 1999; Luft, Jefford et al. 2002; Scandella, Men et al. 2002; Scandella, Men et al. 2004; Legler, Krause et al. 2006). Consequently, the migration ability of mDCs is currently the focus of vaccine delivery techniques (Verdijk, Aarntzen et al. 2008). Maturation cocktail also significantly up-regulated several genes related to the NFKB pathway (NFKB1, NFKB2, PNRC1, RELB, and PIM2), which has been reported to be involved in DC maturation (Lindstedt, Johansson-Lindbom et al. 2002; Ju, Hacker et al. 2003; Platzer, Jorgl et al. 2004; Valentinis, Capobianco et al. 2008).

Finally, the gene expression profile of MC-mDCs was evaluated for a possible shift toward transcripts of Th1- or Th2-related genes. Interestingly, the transcript levels of MHC class I (HLA-A and HLA-F) and MHC class II (HLA-DQA1, HLA-DRB5, HLA-DRA, and HLA-DMA) molecules were distinctively up- and down-regulated, respectively, by MC. In contrast, no changes were detected for these transcripts in LPS-mDCs and TNF-mDCs. In addition, the

transcript levels of three Th1-type cytokines, IL-12β, IL-27β, and IL-15, were significantly or distinctively up-regulated by MC. As a subunit of the Th1-type cytokine IL-27, EBI3 is an early product of activated antigen-presenting cells and drives rapid clonal expansion of naive $CD4^+$ T cells. Moreover, the expression of IL-27 mRNA precedes that of IL-12 and strongly synergizes with IL-12 to trigger IFN-γ production of naive $CD4^+$ T cells (Pflanz, Timans et al. 2002). This agrees with a report indicating the induction of a rapid *in vivo* Th1 response by MC-mDCs (Schuler-Thurner, Schultz et al. 2002).

The secretion of IL-12β (IL-12p40) is tightly regulated and restricted to the cells producing bioactive IL-12p70, whereas IL-12α (IL-12p35) is constitutively expressed in many cell types (D'Andrea, Rengaraju et al. 1992; Trinchieri 2003). The detection of IL-12p40 homodimer in DCs treated by PGE2 suggested an antagonist role against IL-12p70 (Kalinski, Vieira et al. 2001). However, since the production of IL-12p40 homodimer has never been observed by non-transfected human cells (Carra, Gerosa et al. 2000), even under conditions in which a large amount of free IL-12p40 chain was produced, an extensive review on IL-12 suggested that the IL-12 p40 homodimer is not a physiological antagonist of bioactive IL-12p70 in humans (Trinchieri 2003). Conclusively, the production of IL-12p40 by MC is likely an early event in the process of maturation providing the ability of subsequent production of bioactive IL-12p70 by mDCs.

As a Th1-type cytokine expressed by maturing DCs (Ohteki, Tada et al. 2006; Lucas, Schachterle et al. 2007), IL-15 increases the secretion of IFN-γ by NK and activated T cells (Borger, Kauffman et al. 1999), as well as generation of CTLs (Kuniyoshi, Kuniyoshi et al. 1999). In addition, IL-15 is required for type I IFN-mediated expression of MHC class I molecules on DCs, mediating effective induction of NKs (Jinushi, Takehara et al. 2003).

Impaired production of IL-15 is suggested to be an immune evasion mechanism in HCV infection, a well-known chronic infection combined with a weakened Th1-type immune response. Furthermore, the MC-mDCs showed a significantly higher level of expression of LAMP3, PSCDBP, and CXCL16, in comparison to LPS-mDCs and TNF-mDCs. As an important mDC marker, accumulation of $LAMP3^{+}$ mDCs was reported to be involved in induction of proliferation of memory effector CTLs and regression of metastatic melanoma (Elliott, Scolyer et al. 2007), a Th1-type immune response. The PSCDBP is an IL-12-induced gene in activated DCs and was reported to regulate DC-T cell adhesion by mediating DC-T cell de-attachment after antigen presentation (Hofer, Pfeil et al. 2006) indicating a programmed change compatible with mDC characteristics. The Th1-type chemokine CXCL16, a TNF-α- and IFN-γ-induced chemokine (Abel, Hundhausen et al. 2004), is produced by mDCs found in the T cell zones of lymphoid organs, where mDCs are supposed to interact with T cells (Matloubian, David et al. 2000). Expression of CXCL16 in LNs attracts several subsets of T cells and NKTs favoring better antigen presentation.

In contrast, MC dramatically reduced the transcripts of CCL13 and CCL18, two well-known Th2-type chemokines, supporting the notion of driving the immune response away from a Th2-type. Chemokine CCL13 is a well-known biomarker of asthma, a well-known Th2-driven immune-related disease (Kalayci, Sonna et al. 2004). The secretion of CCL18 is reported to be induced by IL-10 (a Th2-biased cytokine) in myeloid DCs (Vulcano, Struyf et al. 2003) resulting in generation of Tregs (Adema, Hartgers et al. 1997; Vulcano, Struyf et al. 2003). Moreover, it is well known that CCL18 is one of the most abundant chemokines produced by iDCs and selectively down-regulated during the maturation process. Finally, the reduction in TLR2 transcript might be supportive of a Th1-type shift in the transcriptional profile of MC-mDCs as

TLR2 appears to promote Th2/Th17 responses by human DCs through TLR4 and TLR8 by abrogating the type-1 IFN amplification loop (Wenink M. 2009). This agrees with previous studies demonstrating the production of IL-10 by monocytes in response to *in vitro* stimulation of TLR2 (Dolganiuc, Chang et al. 2006), and the impairment of DCs in cross-presentation due to the systemic activation of TLR2 (Wilson, Behrens et al. 2006). The only detected potential Th2-biased change in the transcriptional profile of MC-mDCs was a 2-fold reduction in the expression of HSP90AB1; however, considering the series of Th1-biased transcriptional changes, this does not independently support the notion of Th2-biased mDCs generated by PGE2-containing MC.

Finally, the level of CCL22 transcript, an mDC-specific chemokine and ligand for CCR4 expressed on activated Th2 T cells, was similarly up regulated by all treatments. This observation is important as the increase in CCL22 production by PGE2 was suggested as further evidence for a Th2-biased immune response (McIlroy, Caron et al. 2006) . Our results, however, contradict this by showing comparable up-regulation of CCL22 transcript by TNF-α, a well-known inducer of CCL22 secretion (Soumelis, Reche et al. 2002), LPS, and MC, and agrees with other reports (Godiska, Chantry et al. 1997; Vulcano, Albanesi et al. 2001; Penna, Vulcano et al. 2002; Bonecchi, Locati et al. 2004). There are several additional issues disputing the conclusion made by this report (McIlroy, Caron et al. 2006). First, the effect of PGE2 used individually, and not in a MC combination, on CCL22 production was evaluated. Secondly, the report showed that CCL22 prodution was reduced in iDCs by IL-10. As IL-10 is a well-known Th2-type cytokine, this observation supports the notion that the production of CCL22 in DCs is not necessarily associated with Th2-type immune responses. Third, the increase in CCL22 transcript in our study was combined with higher transcript levels of Th1-type cytokines/chemokines IL-12β, IL-27β,

IL-15, CXCL16, and a reduction in transcript levels of two Th2-type chemokines, CCL13 and CCL18.

Taken together, considering the changes in transcripts of the cytokines IL-12β, IL-27β, IL-15, the chemokines CCL13, CCL18, CCL22, CXCL16, as well as PSCDBP, LAMP3, and TLR2, combined with Th1-promoting changes in expression of the MHC class I and II genes, it appears that MC-mDCs tend to skew the immune response less toward a Th2 type than TNF-α, and LPS, a known inducer of IL-12. Conversely, it may even promote a shift toward a Th1-type transcriptional profile. Of the other genes dramatically altered by MC, ISG20 (CD25) is a specific marker for mDC, while CCR1 and CCR5 are considered iDC markers. For most of the additional genes that were solely up-regulated by MC, there is clear evidence that they are important and involved in DC maturation.

In conclusion, there is no strong evidence at the gene expression level that PGE2-containing MC generates Th2-promoting mDCs, or permanently blocks the capability of the generated mDCs to secrete IL-12. In fact, they may preserve this capacity for the next maturation step process called licensing, which is happening in the LNs (Gilboa 2007), where they encounter the CD40L signal on T cells in a proper microenvironment. This environment may include specific TLR ligands as it has been recently shown that the primary inhibitory effect of PGE2 on IL-12 secretion can be overcome with TLR ligands (Boullart, Aarntzen et al. 2008). In other words, *in vitro* generated mDCs may benefit from lack of IL-12 secretion at the time of maturation as long as they preserve this potential, as it should happen in the licensing phase of maturation in the LNs (Reis e Sousa 2001). In addition, IL-12 secretion during maturation may even result in exhaustion of mDCs and failure of further IL-12 secretion (Langenkamp, Messi et al. 2000). This contention is in agreement with a comprehensive review (Gilboa 2007)

suggesting that DCs need to be generated *in vitro* such that they undergo optimal maturation but not licensing, because full activation of DCs *ex vivo* might be counterproductive. Moreover, although early exposure of mDCs to signals such as CD40L or TLR ligands may increase production of IL-12, it reduces the migratory capacity of mDCs supporting the necessity of a late trigger for IL-12 secretion (Luft, Maraskovsky et al. 2004; Shi, Harrison et al. 2004; Boullart, Aarntzen et al. 2008). This agrees with the reports suggesting IL-12 to be a late-phase cytokine in DC maturation which is secreted under tight regulation based on a combination of multiple TLR ligands and CD40L (Schulz, Edwards et al. 2000), while a single stimulus is insufficient to induce secretion of IL-12 (Snijders, Kalinski et al. 1998; Schulz, Edwards et al. 2000). Moreover, the Th1-promoting transcriptional profile observed in MC-mDCs indicates that an IL-12 based assessment of the abilities of MC-mDCs to generate a Th1- or Th2-type immune response might not be necessarily conclusive.

To our knowledge, this is the first study that compares the effect of several maturation agents on the gene expression profiles and functional abilities of the resulting mDCs showing a Th1-promoting effect of PGE2-containing cocktail on mDCs combined with advanced mDC-specific characteristics. In conclusion, we suggest retaining this agent in the cocktail, because of its significant effects on all functional aspects of maturation combined with the induction of a Th1-biased transcriptional profile in mDCs, while re-evaluating the effects of PGE2 on DC maturation with respect to IL-12 production.

5 Human dendritic cells expressing hepatitis C virus core protein display transcriptional and functional changes consistent with maturation

Submitted for publication.

5.1 Abstract

Hepatitis C virus (HCV) causes a chronic liver infection, which may result in cirrhosis and hepatocellular carcinoma. Impairment of the maturation process in dendritic cells (DCs) may be one of the mechanisms responsible for immune evasion of HCV. The core and NS3 proteins are among the most conserved proteins of HCV and play a key role in viral clearance. To evaluate the effects of these proteins on DCs, monocyte-derived immature DCs (iDCs) were transfected with *in vitro* transcribed (IVT) HCV core or NS3 RNA. While these HCV genes had no inhibitory effect on DC maturation, transfection of DCs with IVT core RNA appeared to result in changes compatible with maturation. To investigate this in more detail, the transcriptional profiles of DCs transfected with IVT core, NS3 or green fluorescent protein (GFP) RNA were examined using a DC-specific membrane array. Of the 288 genes on the array, 46 genes were distinctively up- or down-regulated by transfection with IVT core RNA in comparison to NS3 or GFP RNA treatments. Forty-two of these genes are involved in DC maturation. The effects of core on maturation of DCs were further confirmed by a significant increase in surface expression of CD83 and HLA-DR, a reduction of phagocytosis, as well as an increase in proliferation and

IFN-γ secretion by T cells in a mixed leukocyte reaction assay. These results show that HCV core does not have an inhibitory effect on human DC maturation; but could be a target for the immune system.

5.2 Introduction

Hepatitis C Virus (HCV), a member of the Flaviviridae family, is the causative agent of a blood-borne hepatitis becoming persistent in more than 70% of cases, and continuing as a chronic-active hepatitis, which then results in liver cirrhosis and/or hepatocellular carcinoma (HCC) (Alter 1997; Alter and Seeff 2000). After the development of a hepatitis B vaccine, HCV is becoming the leading cause of liver transplantation and HCC, as one of the confirmed oncogenic viruses (El-Serag and Mason 2000; Hassan, Hwang et al. 2002; Rosen 2002; Koike 2005). According to a World Health Organization (WHO) report in 1999 (http://www.who.int/en/), at least 3.1% of the world population is chronically infected with this virus. It is estimated that each year 3 to 4 million people are newly infected (WHO, 2000 report), who play important roles as carriers and reservoirs of the disease (Major and Feinstone 1997; Moradpour, Brass et al. 2002; Meylan, Curran et al. 2005). There is no vaccine to prevent HCV infection, and while the existing therapy is expensive, it is not effective against all subtypes of HCV, and causes serious side effects. Overall, with a five times higher prevalence (3.1%) in comparison to HIV infection (0.6%; UNAIDS 2008 Report; http://www.unaids.org/en/), a further increased rate in mortality from HCC associated with chronic hepatitis C can be expected during the next 20-25 years.

Viruses use multiple mechanisms to evade the host's immune defence and become persistent. One of the strategies non-cytopathic viruses like HCV use to establish a chronic

disease is to infect immune cells. Dendritic cells (DCs), the most potent antigen presenting cells, have been suggested to be targets for HCV and impaired in chronic hepatitis C patients. This has been attributed to either reduced numbers of circulating DCs or functional impairment of circulating or monocyte-derived DCs (Mo-DCs) (Kanto, Hayashi et al. 1999; Auffermann-Gretzinger, Keeffe et al. 2001; Bain, Fatmi et al. 2001; Kanto, Inoue et al. 2004; Tsubouchi, Akbar et al. 2004; Szabo and Dolganiuc 2005; Averill, Lee et al. 2007; Della Bella, Crosignani et al. 2007; MacDonald, Semper et al. 2007; Miyatake, Kanto et al. 2007; Miyazaki, Kanto et al. 2008). Furthermore, inhibition of maturation or allostimulatory ability of *in vitro* generated DCs from healthy donors transfected with HCV genes or exposed to HCV proteins has been reported (Sarobe, Lasarte et al. 2002; Dolganiuc, Kodys et al. 2003; Waggoner, Hall et al. 2007; Saito, Ait-Goughoulte et al. 2008; Zimmermann, Flechsig et al. 2008). These observations have been confirmed in murine models (Hiasa, Horiike et al. 1998; Large, Kittlesen et al. 1999; Kim, Lee et al. 2002; Sarobe, Lasarte et al. 2003).

In contrast, other studies produced contradictory results showing normal functional properties of DCs in chronic HCV patients or DCs expressing HCV genes (Sun, Bodola et al. 2001; Longman, Talal et al. 2004; Longman, Talal et al. 2005; Piccioli, Tavarini et al. 2005; Li, Krishnadas et al. 2006; Li, Li et al. 2006; Zhou, Lukes et al. 2007; Barnes, Salio et al. 2008; Thumann, Schvoerer et al. 2008). Furthermore, DCs transfected with HCV genes were shown to be good candidate vaccines because of their *in vitro* and *in vivo* stimulatory effects (Li, Krishnadas et al. 2006; Li, Li et al. 2006; Yu, Huang et al. 2006; Yu, Babiuk et al. 2007; Yu, Babiuk et al. 2008). However, the use of a viral adenovirus as vectors in some of these studies is questionable as adenovirus itself may be able to mature DCs (Morelli, Larregina et al. 2000; Philpott, Nociari et al. 2004; Basner-Tschakarjan, Gaffal et al. 2006). Even if the empty vector is

used as a control, there is a concern about a potential synergic effect of the combination of HCV and vector proteins in inducing maturation, which may not occur when HCV or vector genes are expressed individually.

In this study, we used an efficient non-viral method (Landi, Babiuk et al. 2007) to transfect human DCs with *in vitro* transcribed (IVT) core or NS3 RNA, and then analyzed their functional properties. Overall, our data suggest that these HCV genes do not inhibit DC maturation. In contrast, according to transcriptional profiles and functional assays, core appeared to induce maturation of immature DCs (iDCs). These IVT core RNA-transfected DCs secreted IL-12p70 in response to LPS or poly I:C treatment and induced proliferation and IFN-γ secretion by T cells in a mixed leukocyte reaction (MLR) assay. In conclusion, by reporting induction of DC maturation by HCV core protein, we propose core as a strong immunogenic component of HCV with the potential to induce Th1-type immune responses, which suggests core to be a target antigen for the immune defence.

5.3 Materials and methods

5.3.1 Generation of monocyte-derived immature dendritic cells

Immature DCs were generated as described previously (Landi, Babiuk et al. 2007). Briefly, peripheral blood mononuclear cells were collected from human venous blood by density gradient separation, and subsequently monocytes were isolated by positive selection on LS columns (Miltenyi Biotech, Auburn, CA, USA) using a CD14-specific antibody conjugated to paramagnetic MicroBeads (Miltenyi). The cells were resuspended in complete RPMI (CRPMI; RPMI 1640 [Invitrogen] supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 100

μM non-essential amino acids, 1 mM sodium pyruvate, 50 μM 2ME, 10 mM HEPES, and 50 μg/ml gentamycin) prepared with phenol red-free RPMI 1640 (Invitrogen Canada Inc., Burlington, ON, Canada) as described previously (Landi, Babiuk et al. 2007). Recombinant human (rh) GM-CSF (100 ng/ml, Peprotech Inc., NJ, USA) and rhIL-4 (200 ng/ml, Peprotech) were added, and then the $CD14^+$ monocytes were dispensed into the wells of a 6-well plate (Corning Incorporated, Corning, NY, USA). On day 3, iDCs were harvested, washed, counted, and used in subsequent experiments.

5.3.2 Construction of plasmids containing HCV genes

The pGEM4Z-5'UT-eGFP-3'UT-64A vector (pGEM) was kindly provided by Dr. E. Gilboa (Duke University Medical Centre, Durham, NC, USA). This plasmid contains the GFP gene flanked by the 5' and 3' un-translated regions and a poly A tail. To construct pGEM plasmids containing HCV genes, a core DNA fragment was generated by polymerase chain reaction (PCR) using genomic DNA isolated from HCV BK strain and a NS3 DNA fragment was amplified from pHCVrep1bl. Designed primers, plasmids pGEM4Z-5'UT-core-3'UT-64A and pGEM4Z-5'UT-NS3-3'UT-64A, and gene sequences of core and NS3 are displayed in Fig. 5.7. The PCR products were purified and cloned into the pGEM vector, from which the GFP gene was excised by appropriate restriction enzyme digestion. The insertion of the subcloned HCV genes was confirmed by sequencing.

5.3.3 Nucleofection of immature dendritic cells with HCV genes

The plasmids containing one of the HCV genes or green fluorescent protein (GFP) were used to make IVT RNA using the mMESSAGE mMACHINE Kit (Ambion, Austin, TX, USA) according to the manufacturer's instructions. Nucleofection of iDCs was performed according to the manufacturer's instructions with some modifications (Amaxa Company, Köln, Germany). Briefly, the iDCs were collected on day 3, washed twice, and resuspended in 100 µl of Human Dendritic Cell Nucleofection Solution (Amaxa). Five µg of IVT RNAs were added per 1×10^6 cells, and the samples were nucleofected using program K2. The iDCs were then collected and dispensed in the wells of a 24-well plate (Corning Incorporated) containing pre-warmed CRPMI supplemented with 50 ng/ml rhGM-CSF and 200 ng/ml rhIL-4. The plate was incubated at 37ºC, and DCs and supernatants were collected at appropriate time points for further analysis or assays. To induce maturation, the iDCs were treated for 48 h with rhTNF-α (Peprotech) at 50 ng/ml, LPS (Sigma-Aldrich Canada Ltd., Oakville, ON, Canada) at 10 µg/ml, or a maturation cocktail (MC) consisting of 20 ng/ml rhIL-1β (Peprotech), 10 ng/ml rhIL-6 (Peprotech), 50 ng/ml rhTNF-α, and 1 µg/ml PGE2 (Sigma). The iDCs and supernatants were collected 48 h after nucleofection for further analysis.

5.3.4 Detection of core, NS3 and GFP protein expression in dendritic cells by western blotting

After nucleofection of iDCs and transfection of Cos7 cells with IVT core, NS3 or GFP RNA, the cells were centrifuged at 300 x g for 5 min, and resuspended in 200 µl of sodium dodecyl sulfate (SDS) loading buffer containing 2-mercapto-ethanol. Subsequently, the cell lysates were boiled for 5 min, and loaded on a 12% reducing SDS-polyacrylamide gel. The

proteins were transferred to a nitrocellulose membrane, which was then incubated with core-, NS3- or GFP-specific monoclonal antibody (Anogen, YES Biotech Laboratories Ltd., Mississauga, ON, Canada), followed by secondary antibody conjugated to an infrared dye (IRDye CW800, LI-COR Biosciences, Lincoln, Nebraska USA), and scanned on an infrared scanner (Odyssey, LI-COR).

5.3.5 Array analysis

After nucleofection, the iDCs were washed twice in PBS (pH 7.2, Invitrogen), and total RNA was extracted from the cells using a RNA/Protein Isolation NucleoSpin RNA/Protein Kit (MACHEREY-NAGEL**,** Bethlehem, PA, USA) according to the manufacturer's instructions. This kit was used in order to simultanously isolate proteins from the same sample for proteomic study. Total RNA samples were checked on a bioanalyzer for quality assurance and kept at -80ºC until use. Biotin-integrated complementary RNA (cRNA) was generated using a reagent kit from SuperArray Biosciences (SuperArray Biosciences, Frederick, MD, USA) according to the manufacturer's instructions. Three biological replicates were used, and all cRNA samples were made simultaneously and hybridized under the same conditions to reduce the technical variability. A final amount of 3 μg of cRNA was used to hybridize on a specific Dendritic Cell and Antigen-presenting Cell Array (OHS-406) membrane (SuperArray). All hybridization steps were performed according to the protocol provided by the supplier with exception of the labeling and detection steps. To be able to scan the arrays with an infrared scanner, we optimized a one-step method to label streptavidin with IRDye CW800-SA (Perkin Elmer, Waltham, Massachusetts, USA). After incubation with CW800-SA-streptavidin, the arrays were washed, and scanned using the Odyssey scanner (LI-COR). The settings were optimized to get the best

images with a broad range of intensities within the limits of the equipment. A customized grid with circle-shape features was designed, and before calculation of the intensities, every spot on the array was visually checked, and noises and artifacts were excluded using Odyssey software V.3 (LI-COR). The intensities were calculated and raw integrated intensity values were loaded into GeneSpring software Version 7.3 (Agilent Technologies, Santa Clara, California, USA). Data normalization was performed based on GAPDH housekeeping genes on the array, and only median polishing was added as the second step for normalization. Changes of 1.5-fold (based on normalized values) in comparison to non-transfected or IVT NS3- or GFP-transfected iDCs were used as cutoff.

5.3.6 Flow cytometry

DCs were washed in PBS, and resuspended in FACS buffer (PBS, pH 7.2 with 0.2% gelatin and 0.03% sodium azide) in a round-bottom 96-well plate (Corning Incorporated). Subsequently, FITC- or PE-conjugated monoclonal antibodies specific for CD83 or HLA-DR, as well as appropriate isotype controls (Becton Dickinson [BD] Biosciences, Oakville, ON, Canada) were added to each well, and the plate was incubated on ice for 30 min. After incubation, the cells were washed three times with FACS buffer, resuspended in cold PBS and analyzed by FACScan (BD Biosciences). The cells were gated electronically for Forward Scatter (FSC) and Side Scatter (SSC) properties and more than 10,000 events were analyzed by FACScan using Cell Quest software (BD Biosciences).

5.3.7 Phagocytosis assay

A phagocytosis assay was performed as described previously (Landi, Babiuk et al. 2007). Briefly, DCs were collected, washed once in PBS, resuspended in CRPMI at 1×10^6 cells/ml, and then incubated at 37 ºC or on ice with FITC-conjugated dextran (FITC-DX, Sigma, 1 mg/ml of PBS) for 2 h in the dark. After incubation, the cells were collected, washed three times with cold PBS, and checked by flow cytometry for FITC-DX uptake.

5.3.8 Allostimulatory mixed leukocyte reaction (MLR)

Carboxyfluorescein diacetate succininidyl ester (CFSE) (CellTrace™ CFSE Cell Proliferation Kit, Molecular Probes, Invitrogen) was used to label responder $CD4^+$ T cells as described previously (Lyons and Parish 1994). Using a $CD4^+$ T cell isolation kit (Miltenyi), $CD4^+$ T cells were isolated from a different, HLA-mismatched healthy donor with a purity of more than 95% based on CD3 and CD4 markers. After incubation with CFSE and washes according to the manufacturer's instructions, $CD4^+$ T cells were resuspend in CRPMI, and added to each well of a round-bottom 96-well plate (Corning Incorporated) at 3×10^5 cells/well. Dendritic cells were harvested, washed in PBS, resuspended in CRPMI, and dispensed in the wells at 3×10^4 cells/well. The plate was incubated at 37 ºC. On days 3, 5, and 8, the supernatants were collected for cytokine analysis and the cells were collected and analyzed by FACScan. The T cell populations were gated electronically for further analysis on the FL1 channel. Based on the fluorescent intensities for CFSE, dividing T cells were gated separately from non-dividing T cells and their ratio used to compare the magnitude of T cell proliferation.

5.3.9 Cytokine secretion analysis

Transfected iDCs were collected 24 h after nucleofection and stimulated by LPS (Sigma) or poly I:C (Sigma) to check their ability to secrete IL-12p70 and/or IL-10. After incubation for 24 h, the plates were centrifuged at 400 x g, and the supernatants were collected, and analyzed for production of IL-12p70 and IL-10 using ELISA Sets (BD biosciences). The supernatants from the MLR assays were also collected on days 3, 5, and 8, and checked for IFN-γ and IL-10 secretion by ELISA (BD biosciences).

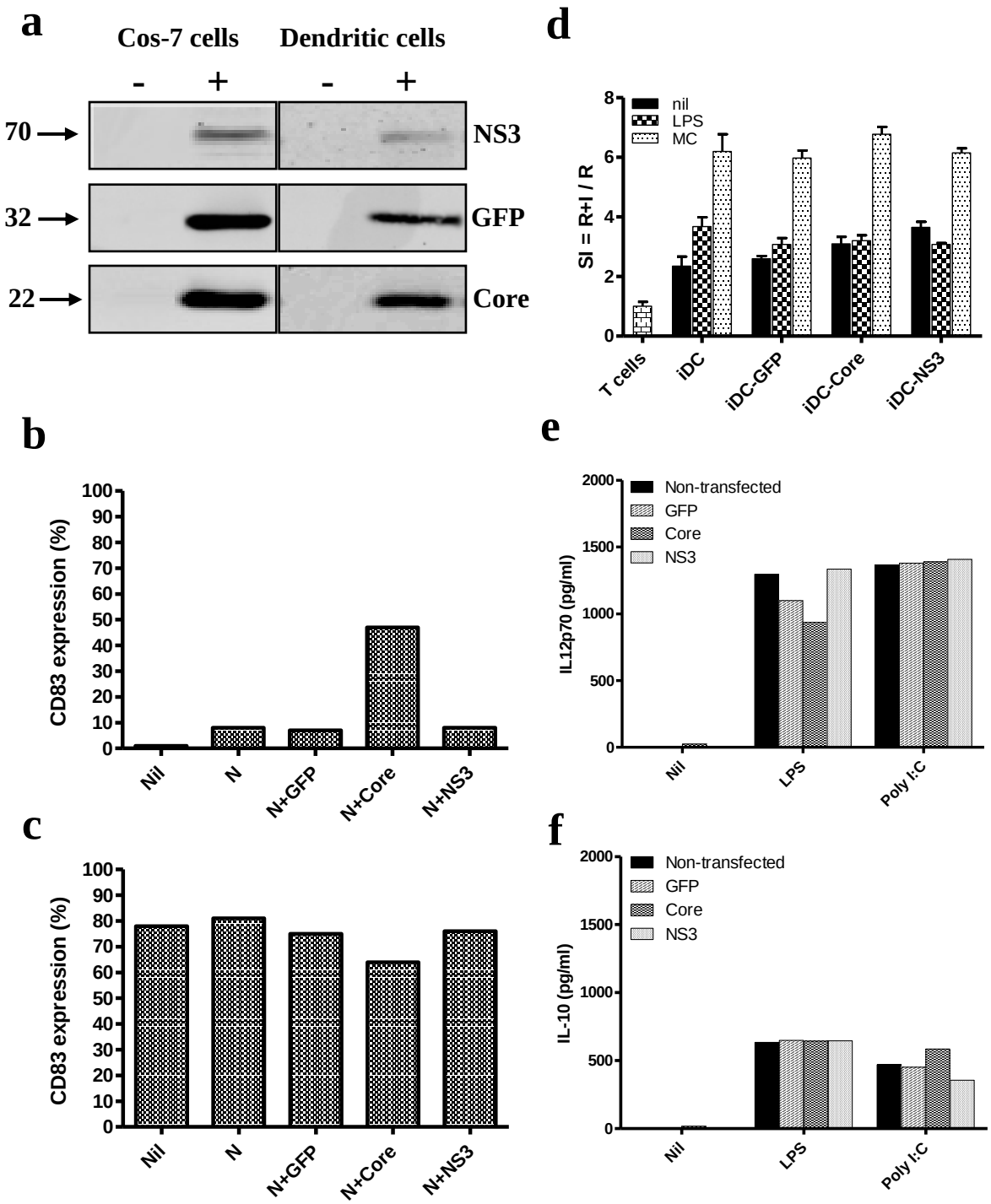
5.3.10 Statistical analysis

Data were analyzed using statistical software (GraphPad Prism Version 5.00, Prism Software Corporation, Irvine, CA, USA). Variables were assumed to not be normally distributed; thus, the median values between pairs of groups were compared using the Mann-Whitney U test. Differences were considered significant if the two-tailed *P* value was lower than 0.05 with confidence intervals of 95%. For the array data only genes up- or down-regulated in the same direction in all three replicates in core-nucleofected DCs were considered to be relevant. After normalization of the fold-change values of these genes, only those genes that showed statistical significance between the core and GFP or NS3 treatments with a p value of <0.05 or lower based on a parametric student *t*-Test were considered significant and discussed as genes distinctively affected by core.

5.4 Results

5.4.1 Transfection of immature dendritic cells with IVT core or NS3 RNA does not impair dendritic cell maturation

To evaluate the effects of core or NS3 expression on human DCs, monocyte-derived iDCs (Mo-iDCs) were transfected with IVT core, NS3 or GFP RNA, treated with LPS, TNF-α or maturation cocktail, and then phenotypically and functionally characterized. As shown in Fig. 5.1a, iDCs nucleofected with IVT core, NS3 or GFP RNA expressed the respective proteins at levels comparable to those in transfected Cos-7 cells, which confirms the intracellular expression of the target proteins, and supports the efficiency of nucleofection of Mo-DCs (90-98%) (Landi, Babiuk et al. 2007). Based on expression of CD83 and MLR, core and NS3 did not show an inhibitory effect on DC maturation (Fig. 5.1b, c, and d). Furthermore, IVT core and NS3 RNA-transfected DCs retained their ability to secrete high levels of IL-12p70 in response to LPS or poly I:C stimulation (Fig. 5.1e), while similar levels of IL-10 secretion were induced, regardless of the DC treatment (Fig. 5.1f). Interestingly, in the absence of maturation factors, transfection with IVT core RNA appeared to result in induction of maturation (Fig. 5.2b).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CD83 expression (%)
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N+Core
N+NS3
e
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GFP
Core
NS3
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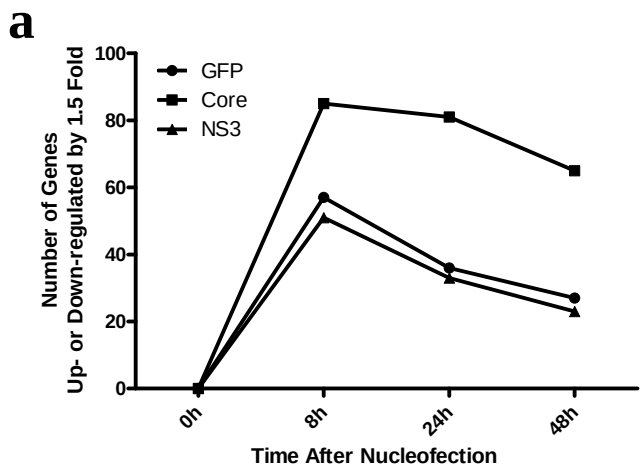
Core
NS3
IL-10 (pg/ml)
Nil
LPS
Poly I:C

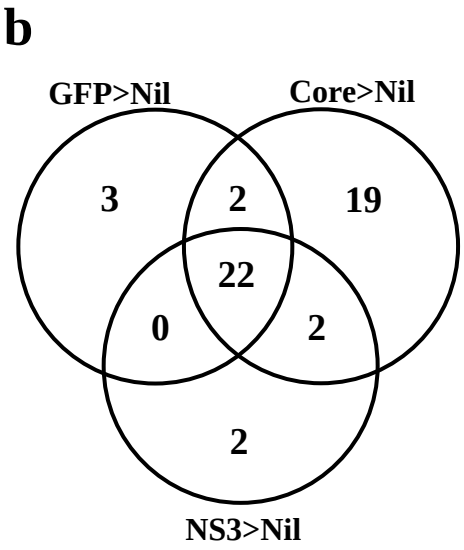
Figure 5.1. Effects of transfection with IVT core, NS3 or GFP RNA on iDCs treated with maturation factors. **(a)** Expression of HCV core, NS3 and GFP proteins in iDCs after nucleofection with IVT core, NS3, or GFP RNA and in Cos-7 cells after electroporation with IVT core, NS3, or GFP RNA. Molecular weight markers are shown in the left margin. **(b)** CD83 expression without treatment with maturation factors in iDCs transfected with IVT core (N+core), NS3 (N+NS3) or GFP (N+GFP) RNA on iDCs after 48 h. N: nucleofected only. Nil: No treatment. **(c)** CD83 expression after treatment with maturation cocktail (MC) in iDCs transfected with IVT core (N+core), NS3 (N+NS3) or GFP (N+GFP) RNA on iDCs after 48 h. N: nucleofected only. Nil: No treatment. **(d)** MLR of iDCs transfected with IVT core, NS3, or GFP RNA, and either treated with LPS or MC, or left untreated (nil). Stimulation index was calculated with the formula: Radioactive count per minute (CPM) for culture of T cells (R) and DCs (I) : CPM in R, the control group (T cells culture alone). **(e)** IL-12 p70 and **(f)** IL-10 production by DCs transfected with IVT core, NS3 or GFP RNA and non-transfected DCs, 24 h after induction with LPS or poly I:C. Nil on the X-axis refers to untreated DCs. Bars represent median values (n = 5).

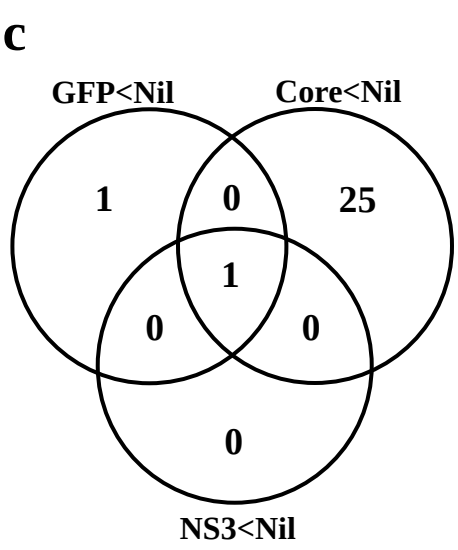
5.4.2 Transfection of immature dendritic cells with IVT core RNA results in significant changes in gene expression

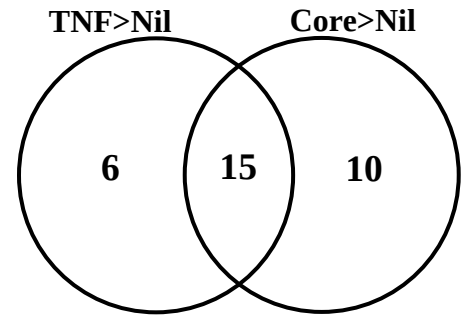
To examine the effects of core at the transcriptional level, iDCs were nucleofected with IVT core RNA, without addition of any maturation factors, and collected 8, 24 or 48 h later. Total RNA was extracted, and cRNA was made and analyzed using DC-specific arrays. IVT NS3 or GFP RNA-transfected, and non-transfected were included as controls. Furthermore, non-transfected iDCs were treated with TNF-α to compare the effect of core to a commonly used maturation factor. Since the changes in gene expression at 48 h were highly representative of changes at 8 and 24 h (Fig. 5.2a) and likely representative of specific and stable effects due to foreign protein expression within DCs and not from immediate effects of the nucleofection itself, the 48 h time-point was selected for further studies. This time point was also most relevant, because mDCs are usually characterized 48 h after incubation with maturation agents.

When a 1.5-fold change in gene expression over non-transfected iDCs was used as cutoff to evaluate the effects of IVT core, NS3, or GFP RNA on iDCs after 48 h, core was found to induce changes in expression of 71 genes, which corresponds to 89% of the changes observed after any of the treatments (Table 5.1). In contrast, NS3 induced changes in expression of 27 genes (34%), while GFP changed expression of 29 genes (36%) and TNF-α altered expression of 26 genes (33%). A more detailed analysis of the gene list suggested that a significant number of the genes affected by core are involved in DC maturation.


a
Number of Genes
Up- or Down-regulated by 1.5 Fold
100
80
60
40
20
0
GFP
Core
NS3
0h
8h
24h
48h
Time After Nucleofection


b
GFP>Nil
Core>Nil
3
2
19
22
0
2
2
NS3>Nil


c
GFP<Nil
Core<Nil
1
0
25
1
0
0
0
NS3<Nil


d
TNF>Nil
Core>Nil
6
15
10

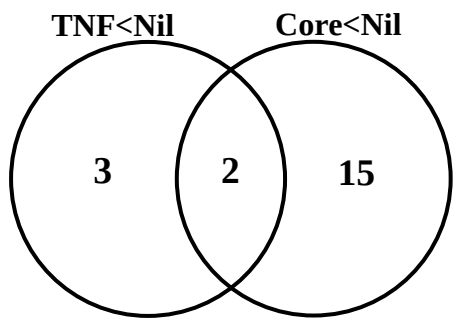

e
TNF<Nil
Core<Nil
3
2
15

Figure 5.2. Gene expression changes in IVT core, NS3, or GFP RNA-transfected iDCs. **(a)** Number of up- and down-regulated genes in IVT core, NS3, or GFP RNA-transfected iDCs in comparison to non-nucleofected iDC at different time points after transfection. **(b,c)** Comparison of gene expression changes after nucleofection with IVT core, NS3 and GFP RNA. **(b)** Up-regulated genes. **(c)** Down-regulated genes. Only genes that changed in expression level by 1.5-fold in comparison to the non-nucleofected iDCs (Nil) treatment are shown. **(d,e)** Comparison of changes in expression of maturation-related genes after nucleofection with IVT core RNA and treatment with TNF-α. **(d)** Up-regulated genes. **(e)** Down-regulated genes. For core-affected genes, only genes that were significantly up- or down-regulated in comparison to IVT NS3 or GFP RNA-transfected iDCs are used for comparison. Only genes that changed in expression level by 1.5-fold in comparison to the non-nucleofected iDCs (Nil) treatment are shown.

Table 5.1. List of 80 genes up- or down-regulated by ≥1.5-fold in IVT core-, NS3- and GFP RNA-transfected and TNF-α-treated iDCs, in comparison to non-transfected iDCs.

Gene	*GFP*	*Core*	*NS3*	*TNF*	*Gene*	*GFP*	*Core*	*NS3*	*TNF*
ADAMDEC1	2.2	3.4	2.0	1.5	FCER1G	1.7	1.2	1.4	**-1.1**
ADAR	1.7	1.7	1.5	1.1	FCER2	1.3	**-2.1**	1.0	**-1.4**
ARHGDIB	**-1.4**	**-5.2**	**-1.3**	**-1.0**	GBP3	1.1	1.6	1.3	**-1.0**
B2M-1	1.7	2.2	1.8	1.3	GPX4	**-1.0**	**-1.0**	1.1	1.8
BASP1	**-1.3**	1.3	**-1.3**	1.8	HLA-A	2.0	4.0	2.3	1.5
BTG1	1.0	2.5	1.1	1.6	HLA-DPB1	**-1.0**	**-1.5**	**-1.0**	**-1.0**
CCL1	1.0	1.5	**-1.0**	1.0	HLA-DQA1	1.1	**-1.6**	**-1.1**	**-1.1**
CCL13	**-1.1**	1.2	**-1.2**	**-2.4**	HLA-DQB1	1.4	**-1.6**	1.2	**-1.1**
CCL2	2.1	4.8	1.9	**-1.6**	HLA-F	2.1	3.5	1.8	1.5
CCL22	**-1.0**	1.2	1.1	1.6	HLA-G	1.4	1.7	1.6	1.4
CCL3L1	1.9	5.3	2.3	1.3	HSP90B1	1.4	1.1	1.5	1.1
CCL4	1.6	4.0	2.7	1.5	IFI16	1.8	2.0	1.7	**-1.1**
CCL5	2.7	8.3	2.4	3.5	IFI27	4.1	12.2	2.2	**-1.1**
CCL7	1.1	3.1	**-1.1**	**-1.3**	IFI35	3.3	5.7	2.4	1.0
CCL8	2.6	14.7	1.9	**-1.6**	IFI44	2.3	3.5	2.3	**-1.1**
CCR1	1.8	1.6	1.5	**-1.3**	IFI6	4.1	4.3	2.9	**-1.2**
CCR5	1.6	1.2	1.4	1.1	IFIT1	8.0	30.0	4.7	**-1.3**
CCR7	1.3	9.1	1.3	3.7	IL8	1.2	3.7	1.9	1.5
CD1A	**-1.2**	**-3.7**	**-1.0**	**-1.2**	INHBA	1.3	2.3	1.1	1.4
CD1B	1.3	**-1.5**	1.6	1.2	ISG15	14.0	20.0	7.0	1.0
CD1C	1.2	**-2.6**	1.2	**-1.0**	ISG20	5.1	33.6	2.5	1.7
CD209	**-1.2**	**-1.6**	**-1.1**	**-1.1**	ITGAX	1.2	**-1.7**	**-1.1**	1.1
CD33	**-1.0**	**-1.6**	1.1	**-1.3**	ITGB2	1.6	**-1.7**	1.2	1.2
CD4	**-1.5**	**-2.3**	**-1.4**	**-1.1**	LAMP3	**-1.1**	1.5	**-1.0**	1.6
CD52	**-1.0**	**-2.0**	**-1.1**	**-1.3**	LIPA	**-1.1**	**-2.2**	1.0	1.0
CD74	**-1.1**	**-1.6**	**-1.2**	**-1.0**	LY75	**-1.3**	1.6	1.0	2.5
CD80	1.2	1.9	1.2	**-1.1**	MARCKSL1	**-1.2**	2.0	1.0	1.6
CD83	1.1	2.7	1.2	1.9	MIF	**-1.5**	**-2.6**	**-1.2**	**-1.3**
CD86	2.0	3.4	1.8	1.3	NFKB1	1.2	2.3	**-1.0**	1.9
CD8A	**-1.3**	**-1.6**	**-1.2**	**-1.1**	NFKB2	**-1.0**	1.4	**-1.1**	1.5
CLEC4A	**-1.4**	**-2.1**	**-1.0**	1.2	PDIA3	1.5	1.7	2.1	1.2
CSF1R	**-1.0**	**-2.1**	**-1.1**	**-1.4**	PLAUR	1.1	1.7	1.3	1.3
CST3	**-1.4**	**-2.9**	**-1.4**	**-1.4**	PNRC1	**-1.6**	2.1	**-1.1**	2.0
CXCL10	**-1.0**	3.1	1.1	**-1.2**	PRG1	1.9	2.1	2.2	1.3
CXCL16	1.3	1.7	1.1	**-1.0**	PSCDBP	1.2	2.4	1.3	1.6
CXCR4	**-1.2**	1.8	**-1.3**	1.3	RELB	1.2	1.8	1.0	1.7
EBI3	**-1.0**	1.8	**-1.1**	2.5	S100A4	**-1.1**	**-4.8**	1.1	**-2.2**
ECGF1	1.9	1.7	1.4	**-1.0**	SOD2	1.5	3.7	1.4	1.4
F13A1	**-3.6**	**-8.5**	**-2.2**	**-4.1**	TRAP1	1.1	**-1.8**	**-1.1**	1.0
FCER1A	**-1.5**	**-3.6**	**-1.1**	**-1.2**	VCL	**-1.4**	**-3.1**	**-1.4**	**-1.0**

5.4.3 Transfection of immature dendritic cells with IVT core RNA has distinctive effects on the expression of maturation-related genes in comparison to dendritic cells transfected with GFP or NS3

When analyzed in more detail, of the 50 up-regulated genes in transfected DCs (Fig. 5.2b), 22 genes were common for core, NS3 and GFP. However, the increase in expression of 10 of these genes was consistently higher in IVT core RNA-transfected DCs than in DCs transfected with IVT NS3 or GFP RNA. This increase was observed in all replicates and was statistically significant (student *t*-Test) (Fig. 5.3a). With the exception of CCL3L1, all of these genes have been reported as up-regulated during DC maturation, including genes encoding chemokines involved in T cell attraction (CCL5 and CCL8), the co-stimulatory molecule CD86, MHC-I molecules HLA-A and HLA-F, and IFN-stimulated genes (ISGs), such as IFI27, IFIT1 and ISG20. Detailed information regarding these genes and their role in DC maturation is provided in Table 5.2. Furthermore, the expression of mitochondrial superoxide dismutase 2 (SOD2), one of two genes up-regulated by both core and GFP, but not NS3 treatment, was significantly higher in IVT core RNA-transfected DCs than in IVT GFP RNA-transfected DCs, whereas the expression levels of IL-8, a gene up-regulated by both core and NS3, but not GFP treatment, was significantly higher in IVT core RNA-transfected DCs than in IVT NS3 RNA-transfected DCs (Fig. 5.3a). These two genes have also been frequently reported as up-regulated during DC maturation (Dietz, Bulur et al. 2000; Le Naour, Hohenkirk et al. 2001; Moschella, Maffei et al. 2001; Lindstedt, Johansson-Lindbom et al. 2002; Messmer, Messmer et al. 2003; Bles, Horckmans et al. 2007). Since only two respectively three genes were specifically up-regulated by IVT NS3- and GFP RNA treatment (Fig. 5.2b), expression of NS3 did not appear to have any significant effect on DCs when compared to GFP. Interestingly, three of these genes (FCER1,

ITGB2, and CD1b) are members of the families down- regulated during maturation, while CCR5 is a known iDC marker.

Of the 19 genes up-regulated in IVT core RNA-transfected DCs (Fig. 5.2b), 16 genes were significantly increased in expression when compared to non-transfected DCs (Fig. 5.3b). Fourteen of these 16 genes are clearly up-regulated during DC maturation (Table 5.2). In summary, of the 28 genes significantly up-regulated after transfection with IVT core RNA in comparison to transfection with IVT NS3 and GFP RNA, 25 genes (89%) have been reported as up-regulated in mDCs. This suggests that induction of maturation is one of the *in vitro* effects of core on human DCs. Figure 5.4a shows the actual images for several of these up-regulated genes supporting the reproducibility and quality of these arrays.

Of the 27 down-regulated genes in transfected DCs, 25 genes were specific for core (Fig. 5.2c). Seventeen of these genes were significantly down-regulated when compared to non-transfected, as well as IVT NS3 and GFP RNA-transfected DCs. In contrast, only one gene (PNRC1) was down-regulated by GFP, while F13A1 was down-regulated by core, NS3 and GFP. However, the level of F13A1 gene expression was significantly lower in IVT core RNA-nucleofected DCs for all replicates in comparison to other treatments, so we consider F13A1 a gene distinctively down-regulated by core treatment. Interestingly, PNRC1, which is up-regulated during maturation, was down-regulated by GFP. Overall, of the 18 genes significantly down-regulated by transfection with IVT core RNA in comparison with IVT NS3 or GFP RNA transfected DCs or non-nucleofected DCs (Fig. 5.3c), 17 are down-regulated during DC maturation (Table 5.2). The actual images for several of the down-regulated genes are shown in Fig. 5.4b.

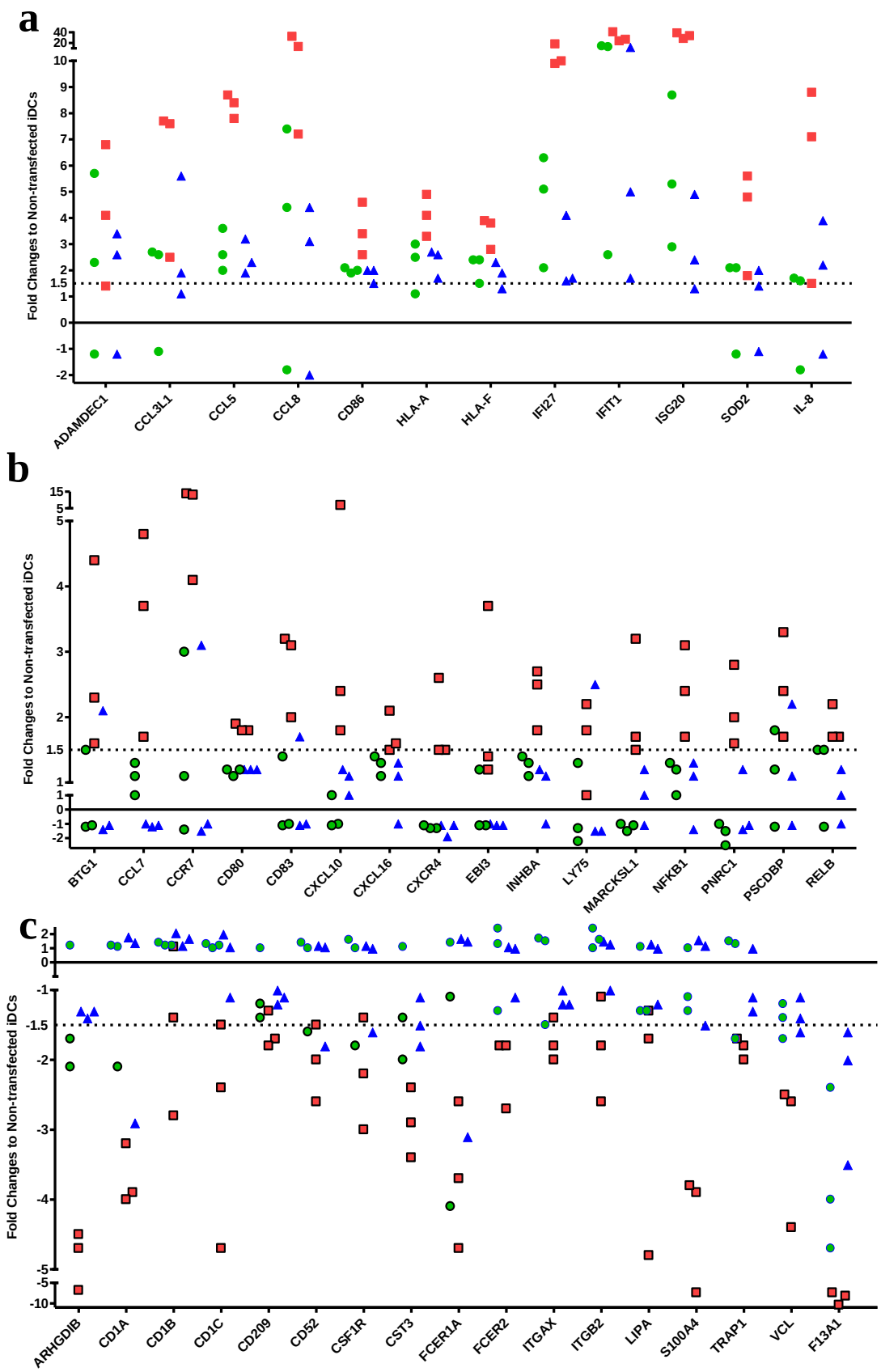

a
Fold Changes to Non-transfected iDCs
ADAMDEC1
CCL3L1
CCL5
CCL8
CD86
HLA-A
HLA-F
IFI27
IFIT1
ISG20
SOD2
IL-8
b
Fold Changes to Non-transfected iDCs
BTG1
CCL7
CCR7
CD80
CD83
CXCL10
CXCL16
CXCR4
EBI3
INHBA
LY75
MARCKSL1
NFKB1
PNRC1
PSCDBP
RELB
c
Fold Changes to Non-transfected iDCs
ARHGDIB
CD1A
CD1B
CD1C
CD209
CD52
CSF1R
CST3
FCER1A
FCER2
ITGAX
ITGB2
LIPA
S100A4
TRAP1
VCL
F13A1

Figure 5.3. Changes in gene expression levels after transfection with IVT core, NS3 or GFP RNA. **(a)** Genes up-regulated in iDCs after transfection with IVT core, NS3 or GFP RNA in comparison to non-nucleofected iDCs. SOD2 was common between core and GFP, and IL-8 was common between core and NS3 only. The changes induced were significantly higher in IVT core RNA-treated iDCs when compared to IVT NS3 or GFP RNA-treated iDCs. **(b)** Genes significantly up-regulated in iDCs transfected with IVT core RNA in comparison to IVT NS3 and GFP RNA-treated iDCs, and non-nucleofected iDCs. **(c)** Genes significantly down-regulated in iDCs transfected with IVT core RNA in comparison with IVT NS3 and GFP RNA-treated iDCs and non-nucleofected iDCs. F13A1 gene expression was down-regulated in all transfected groups. Squares: IVT core RNA-transfected iDCs. Triangles: IVT NS3 RNA-transfected iDCs. Circles: IVT GFP RNA-transfected iDCs. Each data point is representative of one biological replicate. The dotted line at 1.5 fold change shows the cutoff point for analysis.

Table 5.2. Functional categorization and description of genes significantly changed in expression levels by IVT core RNA in the direction expected for DC maturation.

Gene Name and Description	Fold change
Phagocytosis:	
CD209: A cell surface receptor involved in the primary interaction between host and pathogens, which acts as cell-adhesion and pathogen-recognition receptor (Barreiro, Patin et al. 2005).	**-1.6**
CSF1R: A receptor for which down-regulation is associated with loss of phagocytic and adhesive properties of DCs (Takashima and Kitajima 1998).	**-2.1**
FCER1A: A high affinity receptor for IgE which plays a role in uptake of antigens bound to IgE by LCs (Mudde, Hansel et al. 1990).	**-3.6**
FCER2: A low-affinity receptor for IgE, which is down-regulated in mDCs (Hashimoto, Suzuki et al. 2000; Lindstedt, Johansson-Lindbom et al. 2002), and considered an iDC marker (Shin, Jin et al. 2008).	**-2.1**
Antigen Processing and Presentation:	
ADAMDEC1: A secreted protein in the disintegrin metalloproteinase family, which is involved in cell migration, cell adhesion, cell-cell and cell-matrix interactions, and signal transduction. It is predominantly expressed in DCs located in germinal centers of the lymphatic tissue (where mDCs are supposed to interact with T cells) and absent in iDCs. High levels of gene expression upon DC maturation were observed (Mueller, Rissoan et al. 1997; Fritsche, Muller et al. 2003).	**3.4**
HLA-A: and	**4**
HLA-F: Members of the MHC-I family involved in antigen presentation, and up-regulated during DC maturation (Hashimoto, Suzuki et al. 2000; Bleharski, Niazi et al. 2001; Messmer, Messmer et al. 2003).	**3.5**
CD80: A co-stimulatory molecule involved in antigen presentation.	**1.9**
CD83: A functional maturation marker involved in antigen presentation	**2.7**
CD86: A co-stimulatory molecule involved in antigen presentation.	**3.4**
CD205 A member of the MMR family mediating the capture, endocytosis and subsequent processing and presentation of antigens by DCs. It can deliver antigens into both the MHC- I and II presentation pathways, indicating that CD205-mediated endocytosis might be coupled to a mechanism for cross-presentation (Bonifaz, Bonnyay et al. 2002). It is up-regulated upon DC maturation suggesting two distinct functions for this molecule in iDCs and mDCs (Messmer, Messmer et al. 2003; Butler, Morel et al. 2007).	**1.6**
MARCKSL1: Myristoylated alanine-rich C-kinase substrate-like 1 playing important roles in cell shape, cell motility, cell secretion, transmembrane transport, and regulation of the cell cycle. It is consistently up-regulated during DC maturation (Dietz, Bulur et al. 2000; Hashimoto, Suzuki et al. 2000; Le Naour, Hohenkirk et al. 2001; Lindstedt, Johansson-Lindbom et al. 2002; Tureci, Bian et al. 2003).	**2**
PSCDBP: An IL-12-induced gene, regulating DC-T cell adhesion by mediating DC-T cell de-attachment after antigen presentation (Hofer, Pfeil et al. 2006). It is up-regulated during DC maturation (Bleharski, Niazi et al. 2001; Messmer, Messmer et al. 2003; Tureci, Bian et al. 2003), which is indicative of a programmed change compatible with the characteristics of mDCs.	**2.4**
CST3: Encodes human Cystatin C, which is an endogenous cysteine proteinase inhibitor in the Golgi system (Mussap and Plebani 2004). A reduction in expression level during DC maturation (Zavasnik-Bergant, Repnik et al. 2005) has suggested an increase in the activity of antigen processing in the cytoplasm compatible with an mDC state.	**-2.9**
CD1a: and	**-3.7**
CD1b: and	**-1.5**
CD1c: Members of the CD1 family, mediating the presentation of lipid antigens to T cells. They are down-regulated during DC maturation (Palucka, Taquet et al. 1998; Yanagihara, Komura et al. 1998; Hashimoto, Suzuki et al. 2000; Le Naour, Hohenkirk et al. 2001; Messmer, Messmer et al. 2003).	**-2.6**
LIPA: Lipase A, a crucial enzyme for the intracellular hydrolysis of taken-up lipoprotein particles. It is expressed at high levels in iDCs (Dietz, Bulur et al. 2000; Hashimoto, Suzuki et al. 2000; Ahn, Lee et al. 2002) and decreased in mDCs matured by LPS (Messmer, Messmer et al. 2003). This is suggestive of an enzyme activity focused at the early stages of maturation.	**-2.2**
Cell Motility:	
ADAMDEC1: Described above.	**3.4**
MARCKSL1: Described above.	**2**
CCR7: A specific receptor for CCL19 and CCL21 involved in directional migration of mDCs and T cells toward lymph nodes, selectively expressed on mDCs and T cells.	**9.1**
CXCR4: A specific receptor for CXCL12 involved in directional migration of mDCs and LCs toward lymph nodes, selectively expressed on mDCs and migrating LCs.	**1.8**
ARHGDIB: A well-known anti-metastatic gene (Harding and Theodorescu 2007) (Schunke, Span et al. 2007), which	**-5.2**

may also have an inhibitory effects on DC motility (Messmer, Messmer et al. 2003).	
Cell Adherence: **CSF1R:** Described above. **ITGAX (CD11c):** and **ITGB2 (CD18):** Leukocyte adhesion receptors, involved in cellular attachment. Indeed, podosomes are linked to the extracellular matrix through these receptors, a critical process for cell adhesion (Gaidano, Bergui et al. 1990; Burns, Hardy et al. 2004). **VCL:** A membrane cytoskeletal protein, which is required for strong cell adhesion (Demali 2004). **F13A1**: An important coagulation factor, which cross-links between fibrin chains and stabilizes the fibrin clot. With its role in coagulation cascades and consistent and extensive reduction in its expression during DC maturation (Hashimoto, Suzuki et al. 2000; Messmer, Messmer et al. 2003), it could be a molecule needed to be down regulated during DC maturation.	 **-2.1** **-1.7** **-1.7** **-3.1** **-8.5**
T cell Attraction: **CCL5:** Ligand for CCR1, CCR3, and CCR5, and **CCL8**: Ligand for CCR1, CCR2, CCR3, and CCR5 (Lindstedt, Johansson-Lindbom et al. 2002; Messmer, Messmer et al. 2003; Skelton, Cooper et al. 2003; Tureci, Bian et al. 2003). They play an important role in attracting naïve $CD8^+$ through the CCR5 receptor to the site where DCs are interacting with $CD4^+$ cells (Castellino, Huang et al. 2006). These would favour better DC-T cell contact resulting in stronger antigen presentation. **CXCL10:** A specific ligand for CXCR3 (expressed on Th1-type T cells and NK cells). It attracts $CD8^+$ T cells and is secreted during induction of DC maturation by IFN-α2a (Padovan, Spagnoli et al. 2002). **CXCL16:** A specific ligand for CXCR6. It is produced by DCs found in the T cell zones of lymphoid organs, where mDCs are supposed to be located and interact with T cells (Matloubian, David et al. 2000). Expression of CXCL16, which attracts several subsets of T cells and natural killer T (NKT) cells, is induced by TNF-α and IFN-γ (Abel, Hundhausen et al. 2004). **EBI3:** Epstein-barr virus induced gene 3, an early product of activated antigen-presenting cells, driving rapid clonal expansion of naive $CD4^+$ T cells (Pflanz, Timans et al. 2002). It is up-regulated during DC maturation (Ngo, Tang et al. 1998; Yanagihara, Komura et al. 1998; Tureci, Bian et al. 2003).	 **8.3** **14.8** **3.1** **1.7** **1.8**
Other: **IFI27:** and **IFIT1:** and **ISG20:** An important part of the IFN defense mechanism against viruses. Their expression is up-regulated during DC activation and maturation (Hashimoto, Suzuki et al. 2000; Liang, Ristich et al. 2008) (Hashimoto, Suzuki et al. 2000; Messmer, Messmer et al. 2003; Velten, Rambow et al. 2007). ISG20 is even proposed as a maturation marker for DCs. **SOD2:** A mitochondrial antioxidant enzyme, which is suggested to have a role in the immune response as it is up-regulated in cells phagocytosing virus in other species (Zhang, Li et al. 2007). **IL-8:** A ligand for CXCR1 and CXCR2 attracting PMN to the site of infection. **BTG1:** B-cell translocation gene 1, which positively alters the migration capacity of DCs. **NFKB1:** Nuclear factor κB (NFKB) is a transcriptional regulator, which was recently suggested to be involved in partial maturation of DCs (Valentinis, Capobianco et al. 2008). **PNRC1:** Proline-rich nuclear receptor coactivator 1, a transcriptional regulator playing a role in signal transduction. It was also up-regulated as a result of NFKB signaling in response to LPS stimulation in a monocyte cell line (Carayol, Chen et al. 2006). **RELB:** V-reticulo-endotheliosis viral oncogene homolog B, a member of the NFKB family. It is suggested to have a critical role in maturation of DCs (Valentinis, Capobianco et al. 2008). Its up-regulation and nuclear translocation during DC maturation is considered a hallmark for maturation (Lindstedt, Johansson-Lindbom et al. 2002; Ju, Hacker et al. 2003; Platzer, Jorgl et al. 2004). **CD52:** An MHC-II-related gene, down-regulated in mDCs (Hashimoto, Suzuki et al. 2000). **S100A4:** S100 calcium binding protein A4, down-regulated during DC maturation. **TRAP1:** TNF receptor-associated protein 1, down regulated during DC maturation (Bles, Horckmans et al. 2007).	 **12.2** **30** **32.6** **3.7** **3.7** **3.7** **2.3** **2.1** **1.8** **-2** **-4.8** **-1.8**

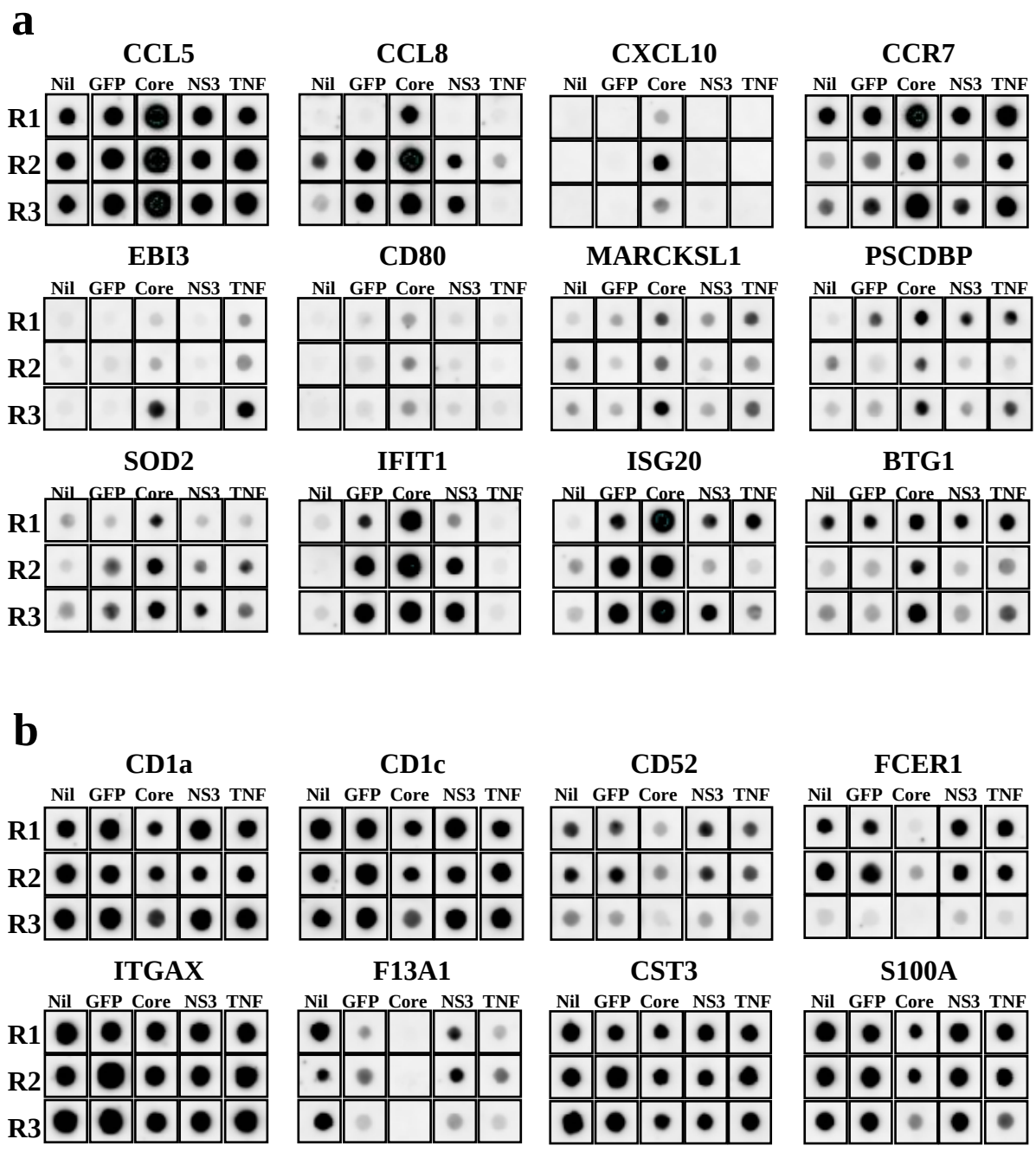


Figure 5.4. Dendritic Cell and Antigen-presenting Cell Arrays (OHS-406) (SuperArray) incubated with 3 μg of cRNA from iDCs after transfection with IVT core, NS3 or GFP RNA or treatment with TNF-α, or from untreated iDCs (Nil); R1, R2 and R3 represent three biological replicates. **(a)** Up-regulated genes. **(b)** Down-regulated genes.

Collectively, 91% (42 out of 46 genes) of all genes distinctively up- or down-regulated by core behaved according to changes expected during DC maturation, which suggests a function for core in DC maturation. To further confirm the differences in the effects of core, NS3 and GFP on iDCs, the genes 1.5-fold up- or down-regulated by core in comparison to GFP or NS3 treatment were identified. This approach demonstrated that of the 42 genes described above only two (CD209, and CXCL16) were not included; however, even though not changed by 1.5-fold, the expression levels of these genes were still significantly different in IVT core RNA-transfected DCs in comparison with IVT NS3 or GFP RNA-transfected DCs.

5.4.4 The effects of core on the gene expression profile of dendritic cells are similar to those of TNF-α as one of the most common maturation agents

To compare the effects of core to those induced by TNF-α, a routinely used maturation agent, the maturation-related genes up-regulated after IVT core RNA treatment were pooled with the genes altered during TNF-α treatment. Of 25 mDC-specific genes up-regulated by core (Fig. 5.2d), 15 were also up-regulated by TNF-α. This accounts for 71% of all genes up-regulated during TNF-α treatment and 60% of all genes up-regulated by core. Indeed, this strongly suggests that there is a significant effect of core on DC maturation with a similar pattern to that of TNF-α. When the 17 mDC-specific genes down-regulated by core were pooled with the genes down-regulated by TNF-α in a Venn diagram (Fig. 5.2e), 2 common genes (F13A1 and S100A4) were identified, while 15 genes were down-regulated during nucleofection with IVT core RNA, but not by TNF-α.

In summary, the effects of core were similar to the changes occurring during maturation of DCs by TNF-α, specifically with respect to the up-regulated genes. However, core promoted

the maturation process of DCs more extensively by shutting down a significant number of additional genes, mostly involved in endocytosis and cell adhesion, thus facilitating cell motility. These data strongly suggest that transfection of DCs with IVT core RNA results in stimulation of maturation, which could result in more efficient antigen presentation by mDCs followed by a more effective immune response.

5.4.5 Transfection of immature dendritic cells with core induces phenotypic changes suggestive of maturation

Expression of CD83, a functional maturation marker involved in antigen presentation, and HLA-DR, which translocates from the cytoplasm to the cell surface during maturation was examined. The number of IVT core RNA-transfected DCs expressing CD83 was equivalent to the number of TNF-α-treated DCs expressing CD83 (Fig. 5.5a), but significantly higher than the number of IVT NS3 or GFP RNA-transfected or non-nucleofected iDCs expressing CD83. Interestingly, the effect of NS3 was similar to that of the control treatments and significantly different from that of core. In addition, the cellular expression level of HLA-DR (shown by mean fluorescent intensity = MFI) was significantly increased after transfection with IVT core RNA, but not by IVT NS3 or GFP RNA (Fig. 5.5b). Although a significant increase was observed in MFI of HLA-DR in TNF-α-treated iDCs in comparison to untreated iDCs, the MFI of HLA-DR was significantly lower in TNF-α-treated iDC when compared to IVT core RNA-transfected iDCs, while there was no difference in comparison to the IVT NS3 or GFP RNA-transfected iDCs.

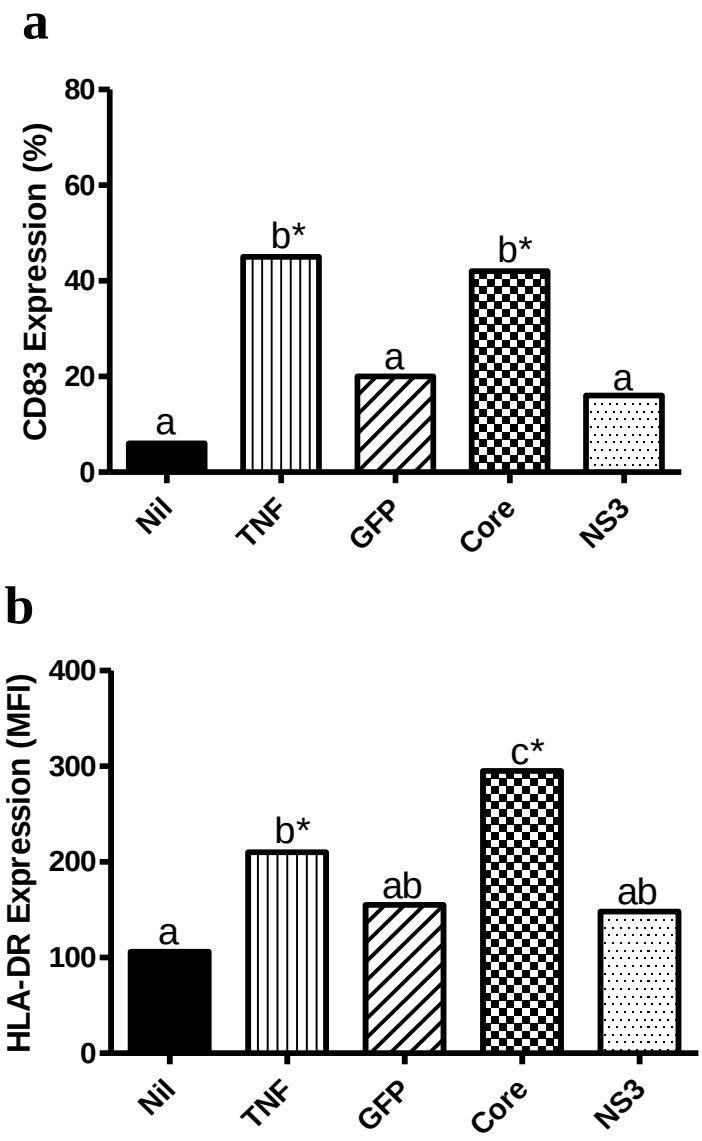


Figure 5.5. Phenotypic marker expression of iDCs 48 h after transfection with IVT core, NS3 or GFP RNA or treatment with TNF-α, or from untreated iDCs (Nil). The percentage of cells expressing CD83 **(a)** and the mean fluorescent intensity (MFI) of HLA-DR **(b)** are shown, respectively. Bars represent median values (n = 5); bars with different letters are statistically different from each other and those with the same letters are not statistically different from each other. The star (*) is representative of a *p* value of < 0.05.

5.4.6 Transfection with core induces functional changes in immature dendritic cells compatible with maturation

A reduction in phagocytic ability is a characteristic of mDCs, so the transfected DCs were examined in an *in vitro* phagocytosis assay. Transfection with IVT core RNA induced a significant reduction in the endocytic ability of the iDCs in comparison to all other treatments including TNF-α. In contrast, transfection with IVT NS3 RNA did not show any effect on the endocytic ability of the iDCs (Fig. 5.6a) suggesting that NS3 does not influence DC maturation. Furthermore, IVT core, NS3 and GFP RNA-transfected DCs were evaluated in an allostimulatory MLR assay, using CFSE-incubated T cells as responder cells. As shown in Fig. 5.6b, iDCs nucleofected with IVT core RNA induced enhanced T cell proliferation in comparison to all other iDCs, which is compatible with improved antigen presentation, an important characteristic of mDCs. The difference between IVT core RNA-transfected DCs and IVT GFP RNA-transfected or untreated DCs was statistically significant on days 3, 5 and 8, whereas the IVT core RNA-transfected DCs were different from IVT NS3 RNA-transfected DCs on day 8. Representative FACS plots of this assay on day 8 are shown in Fig. 5.6c.

In addition to their ability to promote proliferation of T cells, IVT core RNA-transfected DCs induced T cells to secrete IFN-γ at significantly higher levels when compared to iDCs transfected with IVT NS3 or GFP RNA, or non-transfected iDCs (Fig. 5.6d), while there were no differences in IL-10 production between the treatments (Fig. 5.6e). These data show that HCV core might promote the induction of a Th1-type immune response.

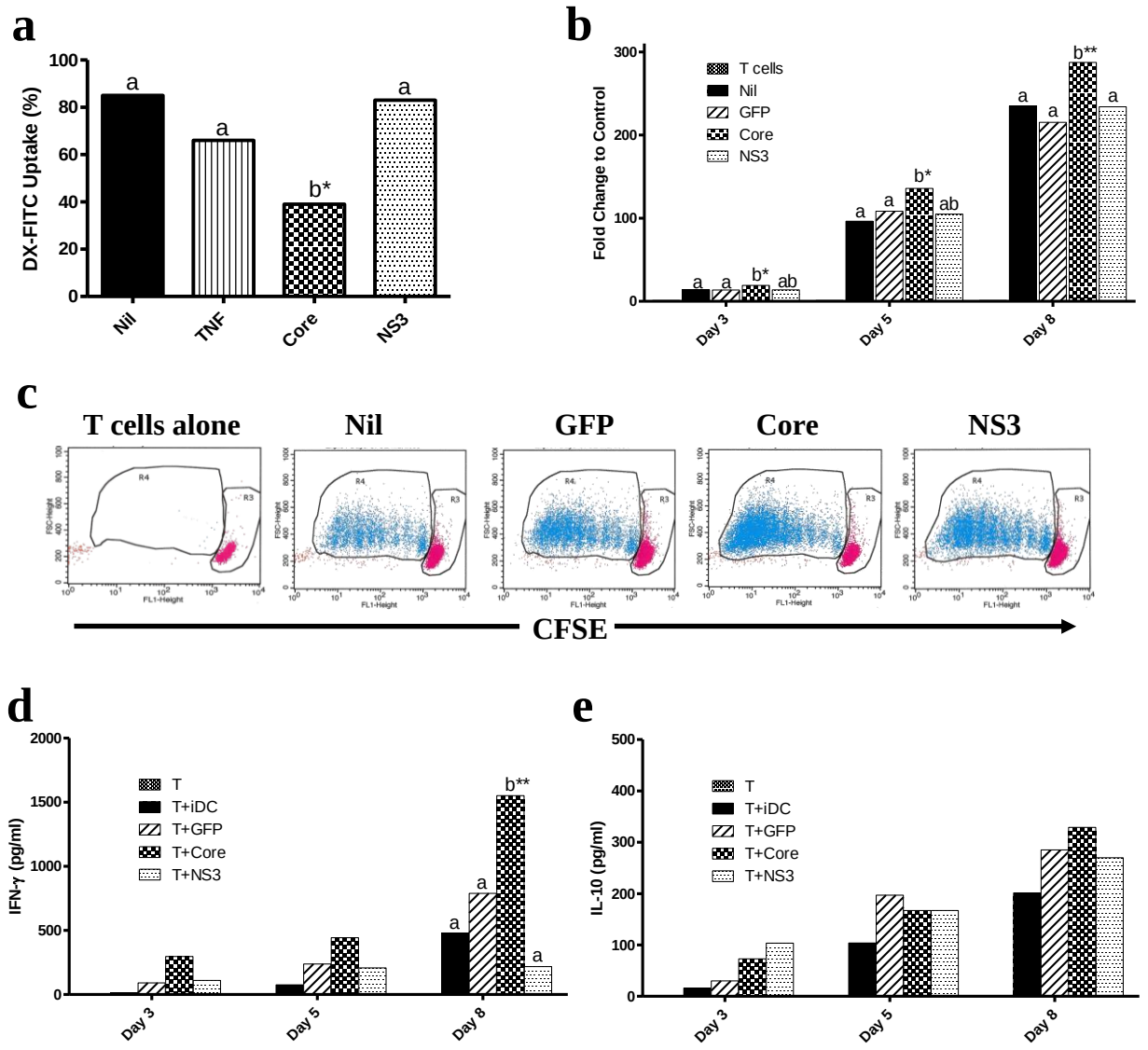


Figure 5.6. Functional characterization of DCs nucleofected with IVT core or NS3 RNA or incubated with TNF-α. Non-transfected iDCs (Nil) or GFP-transfected iDCs were also included. **(a)** Phagocytosis assay. **(b)** MLR assay. Stimulation index was calculated with the formula: Dividing T cells : Non-dividing T cells in each treatment group / Dividing T cells : Non-dividing T cells in the control group (T cells culture). **(c)** FACS dot plot representative of the day 8 reading of the MLR assay. R3 and R4 show the population of non-divided and divided T cells, respectively. **(d)** IFN-γ secretion and **(e)** IL-10 secretion by T cells induced by different treatments of iDCs. T: T cells only; T+iDC: T cells incubated with iDCs; T+ GFP, core or NS3: T cells incubated with transfected iDCs. Bars represent median values (n = 5); bars with different letters are statistically different from each other and those with the same letters are not statistically different from each other. The stars (*) and (**) represent a *p* value of < 0.05 and < 0.01, respectively.

Core primers :

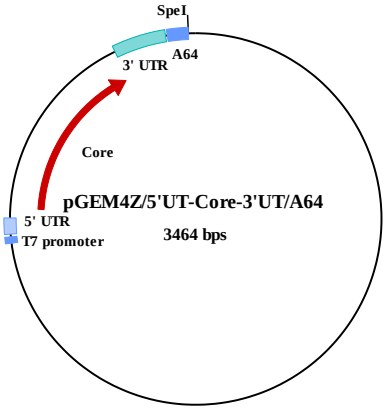
5’ primer 5’ GAATACGGATCCACCATGAGCACGAATCCTAAACC 3’

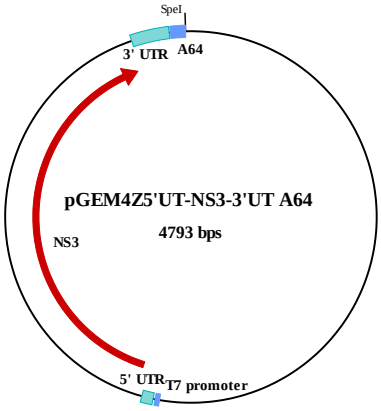
3’ primer: 5’ TAGCATCGAATTCTCTTAAGCGGAAGCTGGGGTGGT 3’

NS3 primers:

5’ primer 5’ ACGATGGATCCACCATGGCGCCTATTACGGCCTA 3’

3’ primer 5’ TAGCATCGAATTCACTCAGGTGCTCGTGACGACCTC 3’





Core gene sequences including T7 promoter and A64 regions:

TAATACGACTCACTATAGGGAGACAAGCTTGCTTGTTCTTTTTGCAGAAGCTCAGAA
TAAACGCTCAACTTTGGCAGATCTGCAGGTCGACTCTAGAGGATCCACCATGAGCAC
GAATCCTAAACCTCAAAGAAAAACCAAACGTAACACCAACCGCCGCCCACAGGACG
TCAAGTTCCCGGGCGGTGGTCAGATCGTTGGTGGAGTTTACCTGTTGCCGCGCAGGG
GCCCCAGGTTGGGTGTGCGCGCGCCCAGGAAGACTTCCGAGCGGTCGCAACCTCGT
GGAAGGCGACAACCTATCCCCAAGGCTCGCCGGCCCGAGGGCAGGACCTGGGCTCA
GCCCGGGTACCCTTGGCCTCTCTATGGCAATGAGGGCTTAGGGTGGGCAGGATGGCT
CCTGTCACCCCGCGGCTCCCGGCCTAGTTGGGGCCCCACGGACCCCCGGCGTAGGTC
GCGTAATTTGGGTAAGGTCATCGATACCCTCACATGCGGCTTCGCCGATCTCATGGG
GTACATTCCGCTCGTCGGCGCCCCCCTGGGGGGCGCTGCCAGGGCCCTGGCACATGG
TGTCCGGGTTCTGGAGGACGGCGTGAACTATGCAACAGGGAATCTGCCCGGTTGCTC
TTTCTCTATCTTCCTCTTGGCTCTGCTGTCCTGCCTGACCACCCCAGCTTCCGCTTAA
GAGAATTCTAGTGACTGACTAGGATCTGGTTACCACTAAACCAGCCTCAAGAACACC
CGAATGGAGTCTCTAAGCTACATAATACCAACTTACACTTACAAAATGTTGTCCCCC
AAAATGTAGCCATCGTATCTGCTCCTAATAAAAAGAAAGTTTCTTCACATTCTTTAA
TTAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
AAAAAAAAAA

NS3 gene sequence including T7 promoter and A64 regions:

TAATACGACTCACTATAGGGAGACAAGCTTGCTTGTTCTTTTTGCAGAAGCTCAGAA
TAAACGCTCAACTTTGGCAGATCTGCAGGTCGACTCTAGAGGATCCACCATGGCGCC
TATTACGGCCTACTCCCAACAGACGCGAGGCCTACTTGGCTGCATCATCACTAGCCT
CACAGGCCGGGACAGGAACCAGGTCGAGGGGGAGGTCCAAGTGGTCTCCACCGCA
ACACAATCTTTCCTGGCGACCTGCGTCAATGGCGTGTGTTGGACTGTCTATCATGGT
GCCGGCTCAAAGACCCTTGCCGGCCCAAAGGGCCCAATCACCCAAATGTACACCAA
TGTGGACCAGGACCTCGTCGGCTGGCAAGCGCCCCCCGGGGCGCGTTCCTTGACACC
ATGCACCTGCGGCAGCTCGGACCTTTACTTGGTCACGAGGCATGCCGATGTCATTCC
GGTGCGCCGGCGGGGCGACAGCAGGGGGAGCCTACTCTCCCCCAGGCCCGTCTCCT
ACTTGAAGGGCTCTTCGGGCGGTCCACTGCTCTGCCCCTCGGGGCACGCTGTGGGCA
TCTTTCGGGCTGCCGTGTGCACCCGAGGGGTTGCGAAGGCGGTGGACTTTGTACCCG
TCGAGTCTATGGAAACCACTATGCGGTCCCCGGTCTTCACGGACAACTCGTCCCCTC
CGGCCGTACCGCAGACATTCCAGGTGGCCCATCTACACGCCCCTACTGGTAGCGGCA
AGAGCACTAAGGTGCCGGCTGCGTATGCAGCCCAAGGGTATAAGGTGCTTGTCCTG
AACCCGTCCGTCGCCGCCACCCTAGGTTTCGGGGCGTATATGTCTAAGGCACATGGT
ATCGACCCTAACATCAGAACCGGGGTAAGGACCATCACCACGGGTGCCCCCATCAC
GTACTCCACCTATGGCAAGTTTCTTGCCGACGGTGGTTGCTCTGGGGGCGCCTATGA
CATCATAATATGTGATGAGTGCCACTCAACTGACTCGACCACTATCCTGGGCATCGG
CACAGTCCTGGACCAAGCGGAGACGGCTGGAGCGCGACTCGTCGTGCTCGCCACCG
CTACGCCTCCGGGATCGGTCACCGTGCCACATCCAAACATCGAGGAGGTGGCTCTGT
CCAGCACTGGAGAAATCCCCTTTTATGGCAAAGCCATCCCCATCGAGACCATCAAG
GGGGGGAGGCACCTCATTTTCTGCCATTCCAAGAAGAAATGTGATGAGCTCGCCGC
GAAGCTGTCCGGCCTCGGACTCAATGCTGTAGCATATTACCGGGGCCTTGATGTATC
CGTCATACCAACTAGCGGAGACGTCATTGTCGTAGCAACGGACGCTCTAATGACGG
GCTTTACCGGCGATTTCGACTCAGTGATCGACTGCAATACATGTGTCACCCAGACAG
TCGACTTCAGCCTGGACCCGACCTTCACCATTGAGACGACGACCGTGCCACAAGAC
GCGGTGTCACGCTCGCAGCGGCGAGGCAGGACTGGTAGGGGCAGGATGGGCATTTA
CAGGTTTGTGACTCCAGGAGAACGGCCCTCGGGCATGTTCGATTCCTCGGTTCTGTG
CGAGTGCTATGACGCGGGCTGTGCTTGGTACGAGCTCACGCCCGCCGAGACCTCAGT
TAGGTTGCGGGCTTACCTAAACACACCAGGGTTGCCCGTCTGCCAGGACCATCTGGA
GTTCTGGGAGAGCGTCTTTACAGGCCTCACCCACATAGACGCCCATTTCTTGTCCCA
GACTAAGCAGGCAGGAGACAACTTCCCCTACCTGGTAGCATACCAGGCTACGGTGT
GCGCCAGGGCTCAGGCTCCACCTCCATCGTGGGACCAAATGTGGAAGTGTCTCATAC
GGCTAAAGCCTACGCTGCACGGGCCAACGCCCCTGCTGTATAGGCTGGGAGCCGTT
AAAACGAGGTTACTACCACACACCCCATAACCAAATACATCATGGCATGCATGTCG
GCTGACCTGGAGGTCGTCACGAGCACCTGAGTGAATTCTAGTGACTGACTAGGATCT
GGTTACCACTAAACCAGCCTCAAGAACACCCGAATGGAGTCTCTAAGCTACATAAT
ACCAACTTACACTTACAAAATGTTGTCCCCCAAAATGTAGCCATCGTATCTGCTCCT
AATAAAAAGAAAGTTTCTTCACATTCTTTAATTAAAAAAAAAAAAAAAAAAAAAA
AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA

Figure 5.7. Designed primers, pGEM-core and pGEM-NS3 plasmids and gene sequences.

5.5 Discussion

The effect of HCV infection on DC maturation has been the subject of controversy in the past few years. To test the effects of HCV proteins on human DCs, iDC generated from healthy volunteers were transfected with IVT core or NS3 RNA and evaluated for their capacity to mature in comparison to non-transfected iDCs or IVT GFP RNA-transfected iDCs. The core and NS3 proteins are among the most conserved proteins of HCV and are thought to play a key role in viral clearance. Transfection of DCs by these HCV genes did not show an inhibitory effect on DC maturation. In contrast, we obtained evidence for induction of DC maturation by transfection with IVT core RNA. Therefore, the effects of core on DC maturation were further investigated at the transcriptional and functional level.

Core appeared to induce radical changes in gene transcripts. When the 71 genes affected by core were compared to the number affected by other treatments, 46 were significant and distinctive to core in all three replicates. Forty-two of these genes (91%) were changed at the transcriptional level in agreement with maturation of DCs. By categorizing these 42 genes according to functional properties (Table 2), the changes agreed with the functions expected of mDCs. Several known phagocytic genes (CD209, CSF1R, FCER1A, and FCER2) were down-regulated and at the same time the transcript levels of genes involved in antigen processing and presentation (ADAMDEC1, HLA-A, HLA-F, CD80, CD83, CD86, and CD205) were increased. A reduction in gene expression of CST3, an inhibitor of proteinase activity in the Golgi system, suggested an increase in the level of antigen processing in the cytoplasm compatible with an mDC state. An increase in expression of an IL-12-induced gene, PSCDBP, which plays a role in de-attachment of DCs from T cells, is indicative of a programmed change compatible with the characteristics of mDCs. Although CD1 receptors (CD1a, CD1b, and CD1c) are involved in

presentation of lipid antigens, the slight reduction in gene expression during DC maturation is in agreement with other studies (Palucka, Taquet et al. 1998; Yanagihara, Komura et al. 1998; Hashimoto, Suzuki et al. 2000; Le Naour, Hohenkirk et al. 2001; Messmer, Messmer et al. 2003). A reduction was also consistently observed in cell surface expression of CD1a on mDCs (data not shown) and might have occurred in compensation of the up-regulation of the other important receptors involved in presentation of peptides, especially when this occurs with reduced levels of Lipase A (LIPA) enzyme in order to adopt higher levels of processing and presentation of peptide antigens. In fact, the expression of LIPA, which is a crucial enzyme for the intracellular hydrolysis of lipoprotein particles after uptake, was shown to be high in iDCs (Dietz, Bulur et al. 2000; Hashimoto, Suzuki et al. 2000; Ahn, Lee et al. 2002) and decreased in response to LPS (Messmer, Messmer et al. 2003). This might suggest that LIPA enzyme activity is highest in the early stages of maturation.

As high motility of mDCs is required, the increased gene expression of the ADAMDEC1 and MARCKSL1 genes and decreased expression of ARHGDIB, CSF1R, ITGAX, ITGB2, VCL and F13A1 genes, which are involved in cell motility and cell adherence, respectively, are interesting, while the increased gene expression of the chemokine receptors CCR7 and CXCR4 favours motility toward the lymph nodes. A consistent increase in the level of EBI3 gene expression, an IL-12 related gene involved in clonal expansion of naïve T cells, as well as an increase in the expression of a number of chemokine genes (CCL5, CCL8, CXCL10, and CXCL16) involved in attraction of different subtypes of T cells and NK cells facilitate a potential interaction between mDCs and T cells, which supports the induction of immune responses. The high CCL5 and CCL8 gene expression may play a role in attraction of iDCs, which would also boost the immune response. In addition to these changes, the involvement of

components of the NFKB pathway, suggested to be related to DC maturation by adenoviruses, could indicate a role of this important pathway in DC maturation by core.

Although there are insufficient data on the functions of the other genes affected by transfection with IVT core RNA, the expression levels of these genes changed in the same direction as reported for maturation elsewhere. Some of these genes are well known antiviral factors (IFI27, IFIT1, ISG20), which, as an early event in the immune defense, are not expected to impede the maturation process. SOD2, an important mitochondrial antioxidant enzyme, is also suggested to play a role in the immune response as it is up-regulated in cells phagocytosing virus in other species (Zhang, Li et al. 2007).

Transfection with core changed the expression of most of the genes affected by TNF-α in a similar manner, which supports the contention of core inducing maturation of human iDCs. In addition, the spotted complementary oligonucleotides on the arrays are 60 nucleotides in length (considered long oligonucleotides), which recognize 3'-biased sequences of each gene with enough distance from 3' end of transcript. This makes the results of this 288-spot array very reliable and thus conclusive, especially with consistency in the three biological replicates (Fig. 5.4). Most importantly, these maturation-compatible changes in the transcriptional profiles of the DCs were confirmed with phenotypic and functional studies, which showed that the effects of core were stronger than those of TNF-α, resulting in higher levels of CD83 and HLA-DR expression. Furthermore, IVT core RNA-transfected iDCs showed a reduction in endocytosis, while the induction of T cell proliferation was enhanced, suggesting an augmented antigen presentation capacity, as expected of mDCs. Finally, IVT core RNA-transfected DCs retained the capacity to secrete IL12-p70, and induced IFN-γ production by T cells in a MLR assay, in agreement with the increased gene expression of the IL-12-related proteins PSCDBP and EIB3.

Collectively, HCV core did not inhibit the maturation of DCs, but appeared to have the ability to mature DCs with a type-1 cytokine profile.

Although this seems surprising in view of the reports suggesting inhibition of maturation by HCV genes, induction of DC maturation by core is indeed in agreement with the physiological process expected from an intact and competent immune system when encountering a viral antigen. This effect on maturation agrees with the contention that core might decrease the viral load and maintain a low steady state dose of HCV in infected cells in order to escape from antigen presentation (Kato 2001). Transfection with IVT RNA has the advantage of being very efficient without the presence of other viral genes. Although signalling through TLR7/8 may contribute to maturation of iDCs transfected with ssRNA, this did not appear to be a major contributing factor, as very few gene expression changes were observed in IVT NS3 or GFP RNA-transfected iDCs. The secondary structure in core RNA (Tuplin, Wood et al. 2002) might have led to activation of TLR3 and type I IFNs; however, this is not necessary due to induction of TLR3 by dsRNA, as core protein could have effects on the downstream steps of the TLR3 signalling pathways as reported for other HCV proteins. Moreover, TLR3 is an intracytoplasmic receptor and usually induced by constitutive replication of viral RNA while the IVT core RNA has a very short half-life, as it is rapidly degraded in the cytoplasm. Finally, in spite of similarities in the changes in gene profile (Avril, de Tayrac et al. 2009), several genes changed differently in the presence of core in comparison to TLR3. For example, adhesion molecules that are down-regulated due to core favouring increased DC motility are up regulated by TLR3 signalling (Lee, Hooper et al. 2007; Liu, Kimura et al. 2008), while CXCR4, which is up-regulated by core is down regulated by TLR3 agonists (Zhang, Sun et al. 2009). As a result, HCV core protein might not only be a factor in immune evasion, but could also be an excellent

immunogenic antigen to induce a strong immune response through maturing DCs. However, since the inhibitory effects of HCV proteins may only occur when the viral genome is replicated during the natural course of infection, HCV may still evade the immune system by interfering with the maturation process *in vivo*. This hypothesis is supported by two recent studies, showing that infection of *in vitro* generated DCs with the full HCV genome results in some degree of DC impairment in culture (Saito, Ait-Goughoulte et al. 2008; Shiina and Rehermann 2008), although other reports are not in agreement (Ebihara, Shingai et al. 2008).

To our knowledge, this is the first study of the effects of HCV core and NS3 on gene expression profiles of DCs. The results, which suggest that core induces DC maturation, were confirmed by phenotypic and functional assays. Core is one among the most conserved HCV proteins, and thus is expected to elicit immune responses to a broad range of virus variants (Lechner, Wong et al. 2000). This proposes core as a target for the immune system, and as such, a good candidate to induce a Th1-type immune response.

6 General discussions and conclusions

This work was based on evidence suggesting inhibition of DC maturation by HCV. Our initial goal was to evaluate the effect of HCV on DCs from patients at different stages of chronic infection; however, limited access to patients led us to evaluate the effects of HCV genes on *in vitro* generated DCs from healthy donors. Since the maturation process was the focus of our study, iDCs were generated, transfected by HCV genes, matured, and evaluated for changes in their functional charcteristics and gene expression profiles indicating inhibition of maturation due to the presence of HCV proteins.

Our first objective was to optimize the generation of DCs from monocytes. We had to work with human primary DCs since there is no appropriate DC cell line available to be used as a prototype and stimulation of available monocyte cell lines such as THP-1 is not generating fully qualified DCs for our study. Since there is a limited number of human DCs in the peripheral circulation, we generated DCs *in vitro* through the isolation and stimulation of monocytes. Particular attention was paid to this process as different culture conditions may result in different DC subtypes, which potentially behave differently in response to expression of HCV proteins. After optimization of DC generation, we mainly focused on transfection of DCs. In this regard, two parameters were crucial, the efficiency of transfection and the viability of the DCs after transfection. As DCs have short life span, we hypothesized that if we generate DCs more rapidly, this may enhance their viability after transfection. To achieve this goal, attention was paid to avoid stimulation of monocytes during the isolation process, as it could drive them toward MΦs, which in turn could delay the process of differentiation to DCs. Several key variables were optimized. First, as the PBMC suspension was to be incubated with $CD14^+$ MicroBeads at 4 ºC, the temperature was gradually reduced to avoid stimulation of monocytes. Secondly, very gentle

pipetting techniques with widebore pipette tips were practiced. Thirdly, the culture media was modified by reducing and replacing FBS with L-glutamine favoring less stimulation of monocytes. Finally, the concentration of IL-4 was increased as it has been reported to be the crucial cytokine in differentiation of monocytes to DCs, and to be responsible for better survival of hematopoietic cells. Indeed, higher levels of IL-4 in combination with the changes mentioned above resulted in faster conversion of monocytes to iDCs. The role of IL-4 in differentiation of monocytes to iDCs was confirmed as we observed a dramatic appearance of DC markers in differentiating monocytes when IL-4 was used in isolation. This eventually led to the development of a new method allowing fast generation of iDCs (Chapter 3). Although a report had been published on the generation of mDCs within 48 h, our protocol was the first one on fast generation of iDCs. When these iDCs were compared to conventionally generated iDCs, they shared all phenotypic and functional characteristics, and were equally capable of converting to functional mDCs.

Although this method of iDC generation is a significant advancement in *in vitro* generation of DCs, future studies could be designed to compare different cytokine cocktails as an approach to better understand DC biology. In addition, different subtypes of DCs may be generated and their characteristics compared, as it is not known whether pre-programmed subtypes of DCs determine the type of immune response based on the expression of different PRRs on their surface such as TLRs, or whether each DC population has the ability to generate both types of Th1 versus Th2 immune responses based on the nature of the pathogen they encounter or the presence of cytokines secreted by other immune cells involved in this process (Maldonado-Lopez, De Smedt et al. 1999; Kapsenberg 2003).

Since most of the previously reported non-viral approaches to transfect DCs have failed or ended in significant damage and death of the DCs, our next objective was to develope an efficient method to transfect iDCs while retaining high viability. The previous failures are likely due to the fact that DCs are professional APCs and strictly control the trafficking of molecules across their cell membranes making it difficult to artificially transfer genes into their nucleus. Although viral vectors were successfully used for this purpose (Hiasa, Horiike et al. 1998; Jooss, Yang et al. 1998; Ponnazhagan, Mahendra et al. 2001; Dullaers, Breckpot et al. 2004; Siavoshian, Abraham et al. 2005), potential effects on DC function and maturation due to the presence of vector genes have been reported for several viral vectors (Engelmayer, Larsson et al. 1999; Salio, Cella et al. 1999; Drillien, Spehner et al. 2000; Jenne, Hauser et al. 2000; Kruse, Rosorius et al. 2000; Morelli, Larregina et al. 2000; Philpott, Nociari et al. 2004; Basner-Tschakarjan, Gaffal et al. 2006). Therefore, we sought non-viral approaches to transfect DCs. Furthermore, for clinical use, there is a need for DC-based immunotherapy without the risk of exposing individuals to other viral genes or integration of the viral genome. Non-viral approaches of gene transfer have been used in recent years; however, inefficient gene transfer to DCs, low levels of gene expression or low cell viability by non-viral transfection methods have limited studies on DCs (Chamarthy, Jia et al. 2004; Dullaers, Breckpot et al. 2004; Erhardt, Gorschluter et al. 2005; Tan, Beutelspacher et al. 2005).

By investigating different methods of transfection, we successfully established a new method of nucleofection for iDCs with high efficiency and viability (Chapter 3). First, we evaluated reagents that seemed to be less harmful to DC survival. Although acceptable viability was achieved, the efficiency and more importantly the level of protein expression was generally too low. Conventional electroporation of DNA plasmids on the other hand resulted in much

lower viability, while it did not also show acceptable transfection efficiency. By using IVT RNAs with conventional electroporation combined with optimized parameters (such as voltage, capacitance, and resistance), we observed up to 80% transfection efficiency and acceptable viability for DCs; however, the level of protein expression was still insufficient for functional studies and transient. Nevertheless, these optimized methods might be valuable when temporary and low expression levels of the target protein are preferred. The use of TTR with IVT RNA resulted in high efficiency and viability with an interesting stimulatory effect on DC maturation, which is an advantage if the generation of mDCs is desired. Although it is not clear but the effect on maturation could be a result of lipid-based formulation of TTR. Finally, we were able to optimize a new method of nucleofection, which resulted in the highest efficiency reported for DCs, as well as long-term viability and high levels of protein expression in DCs. This method is based on the X1 program of an Amaxa nucleofection device as optimal setting in combination with IVT RNA. An important feature of this approach was that DCs keep their immature status, making them suitable for studying the effects of HCV genes on the maturation process. In addition, they showed normal potential to secrete IL-12 in response to LPS stimulation. In conclusion, this newly established transfection method could be considered as a milestone in DC research since transfection of DCs has been a significant barrier for a long time.

To extend this work, we propose to investigate potentially gentler methods of gene delivery into DCs, as the nucleofection method still uses electrical shock, which may affect DC functionality. One approach could be the use of cationic or anionic micro particles for transfer of genes or proteins into the DCs. A relatively stable combination of candidate genetic material (DNA or RNA) or proteins with micro particles of different sizes could be evaluated for the potential to passively enter DCs via phagocytosis. The current problem with this approach is low

efficiency, but if the efficiency improves, this method may also induce DC maturation, which is an additional advantage for *in vivo* application of DCs when mDCs are needed. Another interesting approach could be delivery of target antigens via specific receptors on the DC surface, which are normally taken up after interacting with their ligands (Boscardin, Hafalla et al. 2006). Members of the MMR receptor family such as CD205 are good candidates for this approach. Genes of microbial proteins can be genetically engineered into anti-receptor antibodies plasmids, transcribed *in vitro* while attached to given antibodies, which could easily interact with their receptor on the cell surface and deliver the proteins into the cell through the natural mechanism of phagocytosis. This method is indeed the most natural way of antigen delivery for extracellular proteins and could increase the antigen presentation efficiency by more than 100-fold relative to non-targeted antigens (Bozzacco, Trumpfheller et al. 2007). Other candidates for this kind of approach include MMR/CD206 and DC-SIGN/CD209 receptors. A disadvantage of phagocytic receptor-associated antigen transfer into DCs is the type of the immune response induced as exogenously-obtained antigens are generally presented in MHC class II pathways; however, this possibility could be investigated and addressed in light of cross-presentation capabilities of DCs.

After establishing successful transfection of iDCs, we intended to evaluate the effects of HCV genes on the process of DC maturation. By reviewing studies on the generation of mDCs for *in vitro* assays or *in vivo* purposes, we noticed that insufficient attention was paid to the method used for maturation and thought that the controversial reports on the effects of HCV on DC maturation could be due to this issue. Thus, prior to evaluating the effects of HCV genes on DC maturation, we performed an extensive study on the characteristics of mDCs generated by different methods of maturation (Chapter 4). Our data showed that mDCs generated with

different methods varied significantly in their morphology and level of maturity leading to different functional abilities as mDCs. A cytokine cocktail containing IL-1β, IL-6, TNF-α, and PGE2 proved to be a superior maturation agent in comparison to TNF-α and LPS, based on the induction of phenotypic and functional characteristics compatible with mDCs. The PGE2-containing cocktail also induced transcriptional changes in a significant number of additional genes in comparison to the other two methods. These genes were mostly involved in DC maturation and corresponded to changes expected of mDCs. Although the quality of the immune response induced by the PGE2-containing cocktail has been questioned, based on the transcriptional profile of MC-mDCs, there was no evidence for a Th2-type immune response. In contrast, we observed an increase in transcript levels of IL-12β, IL-27β, which normally precedes the secretion of IL-12 during maturation, IL-15, and an IL-12-induced gene, PSCDBP, as well as Th1-promoting changes in the transcripts of chemokines CXCL16, CCL13, and CCL18. IL-12 and IL-15 are Th1-type cytokines, and IL-27β is a Th1-biased cytokine, which is usually an early-secreted cytokine in the process of maturation, preceding secretion of IL-12. This sequential order of cytokine secretion resembles the licensing phase in LNs *in vivo* and provides support for retaining PGE2 in the MC cocktail, especially for DCs matured for clinical use, as under physiological conditions the secretion of IL-12 at later stages in the LNs is crucial. Moreover, the *in vitro* secretion of IL-12 during maturation is not beneficial as it may result in exhaustion or paralysis of DCs after a transient production of IL-12 during maturation (Reis e Sousa, Yap et al. 1999; Langenkamp, Messi et al. 2000). More interestingly, these changes were accompanied by increases in the transcript levels of MHC class I molecules HLA-A and HLA-F, combined with reduction in the expression of MHC class II molecules such as HLA-DQA1, HLA-DRB5, HLA-DRA, and HLA-DMA. Finally, the transcript levels of TLR2, which was

recently suggested to be involved in Th2-type immune responses, was reduced. Thus, we suggest retaining of PGE2 in the cocktail for maturation of DCs.

As an extension of the maturation study, a more extensive analysis of different combinations of maturation factors, in particular TLR ligands, is worth performing. A full panel of DC markers could be applied, and some of the interesting genes should be studied using quantitative real time PCR. By using larger gene arrays, there is a chance of finding new genes and proteins involved in maturation, which could be considered new markers for mDCs.

Although achieving effective maturation of DCs *ex vivo* has not been a major problem, *in vivo* migration of DCs, which is a critical parameter for successful immunotherapy has been a challenge (Verdijk, Aarntzen et al. 2008). This aspect of mDCs was initially underestimated, as in preliminary *in vivo* trials, DCs loaded with target antigens did not leave the site of injection and were not able to trigger an immune response. If DCs do not migrate toward LNs, where they can interact with T cells, they cannot deliver the antigen for presentation and the immune response will be aborted. In addition, as *ex vivo* generated DCs may not be as healthy as *in vivo* ones, they might be rapidly cleared from the body before getting to the point of antigen presentation. Thus, faster and more effective motility of DCs bearing target antigens is required. In this regard, *in vitro* maturation stimuli should result in a high level of CCR7 expression, the selective receptor for homeostatic chemokines CCL19 and CCL21 constitutively produced in secondary lymphatic tissues (Willimann, Legler et al. 1998; MartIn-Fontecha, Sebastiani et al. 2003). In complementary studies, transfected mDCs can be labeled with radioactive compounds, fluorochrome-conjugated antibodies, or paramagnetic beads and be traced. Carboxyfluorescein diacetate succininidyl ester (CFSE), which passively diffuses into the cells and becomes impermeant and stable for a long time, could be a potential dye to label and follow DCs *in vivo*.

In summary, maturation is not a simple one-step event; conversely, it is a progressive well-programmed process with a high level of complexity, the details of which are mostly yet to be discovered. To simplify and quantify this concept, and to compare results from different laboratories, we suggest a definition of a maturity index for the level of maturity based on a mathematical model. Based on a maturity index, mDCs generated in different studies could be properly compared and the results could potentially be predicted in experiments or trials. This may also indentify the level of DC maturity as the reason for the controversial reports on the effects of HCV on DCs in different studies. Defining a maturity index is part of an ongoing interdisciplinary study in collaboration with mathematicians. Although to mathematically define a biological concept is in its initial phase, modeling of maturation could be a starting point to engage mathematical modeling in conceptual aspects of immunology.

As our ultimate objective, we proceeded to evaluate the effects of HCV genes on DCs. Fast iDCs were transfected with different HCV genes individually or in various combinations and checked for their characteristic in the presence or absence of different maturation agents. Interestingly, we did not observe any inhibitory effect on DC maturation by individual or combinations of HCV genes transferred to DCs generated from healthy donors. In contrast, we observed a considerable increase in CD83 expression due to core expression in DCs, without addition of any maturation agent. Thus, we designed experiments to compare the effects of core protein on iDCs with those induced by a common maturation agent, TNF-α. Non-structural protein 3 was included as a HCV gene that had no effects on DC maturation in our preliminary studies. Our data showed that not only did core not impair the maturation of DCs, it actually induced maturation of DCs (Chapter 5). According to transcriptional profiles, core altered the expression levels of a significant number of genes related to the process of DC maturation. The

core-transfected DCs also developed a mature phenotype based on the expression of CD83 and HLA-DR, as well as characteristics of mDCs in phagocytosis and MLR assays. These effects were similar to, but much stronger than those of TNF-α. Furthermore, contrary to other reports core-transfected DCs still retained their ability to secrete significant amounts of IL-12 in response to LPS or poly I:C stimulation and induced proliferation and IFN-γ secretion by T cells in a MLR assay. The fact that IVT GFP and NS3 RNAs did not induced DC maturation confirmed that this effect is not due to TLR7/8 receptor stimulation by IVT ssRNAs. In addition, several genes involved in adhesion and motility of DCs has changed differently in core transfected DCs in comparison to TLR3 signalling (Lee, Hooper et al. 2007; Liu, Kimura et al. 2008; Avril, de Tayrac et al. 2009; Zhang, Sun et al. 2009). This shows that the double-stranded secondary structure of IVT core RNA (Tuplin, Wood et al. 2002) is not likely responsible for maturation induction. To further support this notion, an experiment with point mutations in core RNA could be designed to rule out a possible TLR3 stimulation as a contributing factor in maturation instead of the expression of core protein.

Overall, by reporting induction of DC maturation by HCV core protein, we proposed core as a strong immunogenic component of HCV with the potential to induce Th1-type immune responses, which suggests core to be a target antigen for the immune defense. This conclusion agrees with previous reports on the induction of humoral and cellular immune responses by core (Iwata, Wakita et al. 1995; Lasarte, Garcia-Granero et al. 1998; Alisi, Giambartolomei et al. 2003). Based on these results, core could possibly be used as a target antigen to formulate vaccines with appropriate adjuants in *in vivo* systems. An alternative would be the evaluation of DCs loaded with core protein or genes expressing core in initiating an appropriate immune response in *in vivo*.

To our knowledge, this is the first study evaluating the effects of HCV genes on DC maturation based on gene expression in addition to phenotypic and functional characteristics. The results strongly suggest that core protein can induce DC maturation, while NS3 had no effect on DCs. This may not necessarily contradict results obtained with DCs generated from CHC patients, as inhibitory effects may only occur when the whole viral genome is replicated, resulting in evasion of HCV via interference with the maturation process. Indeed, there are several reasons, which may explain the contradictory observations.

To address the discrepancies between studies with patients' DCs and those with *in vitro* tranfected DCs from healthy individuals, the first potential reason might be the introduction of the virus or viral genes in two different manners (Kanto, Hayashi et al. 1999; Kanto, Inoue et al. 2004; Li, Li et al. 2006). Typically, DCs in patients are exposed to the virus such that the whole genome is replicated during natural infection, which may be optimal condition for the expression of viral proteins leading to successful evasion from immune system. In contrast, the presence of a complete set of defense strategies may effectively limit viral replication *in vivo*, while *in vitro* infected or transfected cells may fail to overcome the viral effects. In conclusion, the results from studies on DCs generated from patients cannot be compared to the results from studies on DCs that are infected or transfected *in vitro*.

To perform complementary studies, infecting DCs from healthy volunteers with a recently characterized clinical isolate of HCV (Kato, Furusaka et al. 2001), Japanese fulminant hepatitis virus 1 (JFH-1) would be interesting, resembling natural infection *in vivo*. This approach is not straightforward as the virus hardly proliferates in primary DC cultures, except under very specific conditions. However, since the results of an *in vitro* study on the effects of

JFH-1 virus on DC maturation might better model a true HCV infection, it would be worth improving the replication of JFH-1 in primary DCs.

Contradictory results obtained in experiments with DCs generated from chronic patients, could be due to a difference in the methods of DC generation and maturation. Thus, the results of even two similar experiments using patient DCs might still not be in agreement if the same method for DC generation and maturation was not used. For similarly *in vitro* generated and matured DCs from healthy volunteers, the use of different methods of loading DCs with viral antigens (Dolganiuc, Kodys et al. 2003; Zhou, Lukes et al. 2007; Thumann, Schvoerer et al. 2008; Zimmermann, Flechsig et al. 2008) might be an additional reason for discrepancies. The method of transfection needs to be carefully examined, especially when the use of viral vectors is considered, because two separate viral genes could act synergistically with respect to induction or inhibition of DC maturation.

Thus, non-viral methods seem more reasonable to avoid such a effect. However, a common disadvantage of electroporation-based non-viral transfection methods is their negative effect on the functionality of DCs, which could be due to general damage to the cellular function and not necessarily to inhibition of specific pathways of maturation. This may also explain the differences in results between a viral method versus a non-viral method or direct exposure of the DCs to viral proteins. The solution to this problem was the development of non-viral non-invasive protocols for gene transfer into DCs. In comparison to other *in vitro* studies, our study had the advantage of not using a viral vector for transfection of DCs supporting that the observed effect on maturation is primarily related to HCV core protein.

The other issue is whether a combination of two or more genes might have a stronger effect on DCs. Although we investigated this possibility and did not observe any inhibitory

effects of multiple HCV genes, studies with the complete genome of the virus, VLPs or the use of JFH-1 virus could further clarify this matter. The level of expression of each protein is also important, as during HCV infection low antigenicity could be one of the evasion mechanisms. By using different amounts of IVT RNAs expressing HCV proteins, this effect could be investigated. Ideally, transfection of DCs with the whole genome of a virus may result in proportionally appropriate amounts of viral proteins, which resembles a natural course of infection even if the whole viral replication cycle is not completed. However, these approaches should be compared to determine which combination or level of protein expression might have effects on DC maturation.

The genetic material used for transfection might also influence the DCs. For example, transfection of DCs using RNA may result in a low level of maturation induction via interacting with TLR receptors. Therefore, if RNA is used for transfection and no inhibitory effect is observed, weak effects of the viral proteins might have been compromised by the effect of introduced RNA in the cells. In our study however, proper controls and complementary work such as gene expression profiling ruled out this possibility, as the transcriptional profiles of mDCs differed those expected from a TLR-specific pathway (Chapter 5). In summary, although there are several ways to explain discrepancies between observed effects of HCV on DC maturation, at this stage a definite explanation is not readily available. More studies need to be performed, specifically with DCs from hepatitis C patients.

Therefore, to further extend this study, we have planned to evaluate the response of DCs from hepatitis C patients to different maturation stimuli. Chronic, treated versus untreated, and spontaneously resolved versus chronic cases will be chosen for this investigation, and transcriptional studies will be designed as complementary approaches to functional evaluation of

the DCs. Finally, a collective review of all reported studies may lead to a possible conclusion on the effects of HCV on DC maturation.

In conclusion, the most significant results included i) Development of a new method for rapid generation of iDCs with minimal handling and high reproducibility in biological replicates. ii) Establishment of a new nucleofection method for DC transfection with excellent efficiency and viability as well as high level of protein expression. iii) The first extensive and comparative study on DC maturation using gene profiling suggesting a different level of maturity of mDCs as a possible reason for the controversy of HCV effects on DC maturation. And iv) Induction, but not inhibition of maturation of DCs by HCV core protein but not NS3 protein, supported by a gene expression study that proposes core as a candidate for vaccine design against HCV.

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