Method for producing dendritic cells

The present invention relates to a method for producing and genetically engineering dendritic cells (DC) and uses therof.

During the last years the dendritic cell (DC) has been recognised as the central regulator of immunity. Human DCs are generated by in vitro differentiation from haematopoietic stem cells or peripheral blood monocytes in the presence of growth factors, typically interleukin (IL) 4 and granulocyte-macrophage colony-stimulating factor (GM-CSF). Recent evidence suggests that DCs have the capacity to flexibly respond to the encounter of microbial, traumatic, or metabolic stress. Thus, DCs do not only differentiate into one subtype that fulfils a particular function, e.g. activation or tolerance, type 1 or type 2 T-helper lymphocyte (Th1, Th2) polarisation, but assume distinct functional states in a time-kinetic fashion appropriate to the challenges encountered in a given environment (Fig. 1).

Monocytes leave the blood stream to enter various tissues and to become what is conventionally referred to as immature DCs (iDC). These iDCs are sentinels that sample their environment by taking up material from the extra cellular fluid as well as apoptotic bodies from physiologically dying cells, process, and present this material without co-stimulation in a tolerance-inducing form to T-lymphocytes. The tolerance-inducing iDC phenotype may be considered the default status of DCs. This state is maintained until the iDC encounters a danger signal that may be a pathogen associated molecular pattern (PAMP) transmitted by toll-like receptors (TLR), inflammatory cytokines, or T-lymphocyte derived signalling, most prominently mediated by CD40/CD40L interaction. This process is referred to as DC maturation, which coincides with a sequence of functional changes. These functional changes take place over a period of approximately 2 days, after which the DCs reach a status that is referred to as mature DCs (mDCs). Most prominently, the DC starts to up-regulate co-stimulatory molecules such as the B7 family members CD80 and CD86. This enables the DC to deliver an activating rather than a suppressive signal to T-lymphocytes

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that carry a T-cell receptor capable of interacting with an antigenic peptide in a complex with major histocompatibility complex (MHC) molecules on the DC membrane. At this stage also a stimulus-dependent polarisation takes place, with DCs secreting IL-12 as well as IL-12 family cytokines favouring a type 1 immune response that subsequently supports cellular immunity mediated by cytotoxic T-lymphocytes (CTL). IL-12 secretion is generally triggered by engagement of TLRs with their ligands, for example TLR4 engagement with LPS, but also by interaction of soluble or cell membrane bound CD40L molecules with CD40 on DCs. In contrast, the absence of IL-12 release triggers a type 2 polarisation that initiates a humoral immune response by supporting B-lymphocytes. Initiation of DC maturation without IL-12 secretion is accomplished by exposure of iDCs with cytokine cocktails that typically contain TNF-α and PG-E2 as well as various inflammatory cytokines including but not limited to type I and type II interferons, IL-1, or IL-6.

IL-12 release ceases after about 24 hours indicating that the encounter between DCs and T-lymphocytes needs to take place within that time window to allow efficient type 1 polarisation and CTL activation. In contrast, the expression of co-stimulatory molecules reaches its maximum after 2 days. Since per definition a mature DC is characterised only phenotypically by maximum expression of co-stimulatory molecules but not functionally, the IL-12 releasing type 1 polarising DC is referred to as semi-mature (sm) DC. After approximately 2 days the DC reaches the stage of so called maturity. During the second day of its differentiation the DCs loose their immune stimulatory capacity and acquire immune suppressive properties by up-regulation of molecules that mediate negative regulatory feedback loops (Fig. 1). The biological significance of this differentiation phase is the necessity of keeping immune responses under strict control. An activated immune cell, particularly a CTL that is enabled for the killing of other cells, poses a considerable threat to an organism. This is exemplified by the pathological consequences of immune responses that dodged their control: autoimmune disease such as type I diabetes or multiple sclerosis.

Therefore, the same DC that during day 1 after encountering a maturation signal primes immune responses will dampen this same immune response during day 2 of their differentiation process. Therefore, mature DCs are in fact not as originally thought immune stimulatory but rather immune suppressive cells and therefore inadequate for therapeutic interventions aimed at immune stimulation such as their use in cancer immune therapy or the treatment of microbial diseases.

It is important to distinguish between immature (tolerance maintaining), semi-mature DCs (immune stimulatory), and mature (immune suppressive) DCs (Fig. 1). An iDC as outlined above maintains tolerance against autoantigens. An smDC has encountered one of the maturation stimuli described above and has irreversibly committed to differentiation into mDCs within approximately 2 days. Importantly, only during the first one of those 2 days it is enabled for IL-12 release, initiation of type I immune polarisation, and consequently support of a CTL mediated immune response. Once a maturing DC enters the second phase of differentiation after one day it acquires immune suppressive properties. It is a convention among immunologists to characterise an mDC by the expression of membrane molecules such as CD80, CD83, or CD86. However, in contrast to IL-12 that reaches maximum expression within a few hours and is lost after 24 hours, these membrane molecules reach their maximum expression only after 48 hours. In order to clearly distinguish the IL-12 secreting DCs that are described herein from what is conventionally understood by the name mature DC, the term semi-mature DCs was chosen. This, very importantly, shall not imply some kind of functional deficiency but only a certain differentiation stage at the time kinetic scale in Figure 1. The smDC is functionally different from an iDC as well as from an mDC.

WO 2007/117682 relates to mature dendritic cells which are transfected with mRNA molecules encoding for CD40L.

Koya R. C. et al. (J Immunoth. 26 (2003):451-460) describe the transfection of immature dendritic cells with viruses coding for CD40L. CD40L is required to mature these dendritic cells.

In Liu Y. et al. (Cancer Gene Therapy 9 (2002):202-208)

the transfection of immature dendritic cells with viruses encoding CD40L is disclosed.

It is an object of the present invention to provide a method for producing dendritic cells based on genetic engineering. These dendritic cells may be used to prepare pharmaceutical preparations.

The present invention relates to a pharmaceutical preparation comprising partially matured dendritic cells obtainable by a method comprising the steps of:

a) providing immature dendritic cells or precursor cells thereof or partially matured dendritic cells obtainable by contacting immature dendritic cells with at least one dendritic cell maturation agent to produce partially matured dendritic cells (semi-mature DCs, smDCs) as defined by their capacity to secrete IL-12,

b) manipulating the cells of step a), in particular the partially matured dendritic cells (semi-mature DCs, smDCs) releasing IL-12 of step a), to

(i) over-express at least one immune molecule capable of maintaining the T-lymphocyte stimulatory capacity of dendritic cells characterised by continued IL-12 secretion for at least 24 hours, preferably at least 48 hours, and selected from the group consisting of CD40L by introducing nucleic acid molecules encoding for said at least one molecule; and/or

(ii) inhibit or prevent the expression of at least one T-lymphocyte suppressive molecule acting within dendritic cells exposed to a primary maturation agent such as LPS/IFN-γ or being released from dendritic cells exposed to a primary maturation agent such as LPS/IFN-γ and being selected from the group consisting of interleukin 10 (IL-10), and indoleamine 2,3-dioxygenase (IDO), such as at least one of the genes given in the tables 3, 4 and/or 5, by knocking out the gene or a fragment thereof encoding said at least one T-lymphocyte suppressive molecule or by introducing nucleic acid molecules, preferably ribonucleic acid molecules, to inhibit or prevent the expression of the at least one T-lymphocyte suppressive molecule that is active within the dendritic cell or is delivered from the dendritic cells to T-cells and

c) optionally adding substances to transform precursor

cells of dendritic cells into dendritic cells.

The pharmaceutical preparation according to the present invention comprises dendritic cells obtainable by the methods disclosed herein. The dendritic cells that are subjected to genetic engineering aim at over-expression of molecules contributing to immune stimulation such as CD40L, or genetic engineering aimed at knocking down the expression of immune suppressive molecules such as IL-10 or IDO, and the newly identified molecules listed in tables 3, 4 and 5 below, which show an expression kinetic in DCs that is similar to IL-10 and IDO. The genetic engineering may be performed on any DCs or precursor cells, like hematopoietic stem cells, no matter whether these dendritic cells are exposed to a maturation agent such as a TLR ligand, a cocktail of inflammatory cytokines, or T-cell derived signals such as a CD40L mediated signal, or subjected to another procedure aimed at triggering the phenotypic switch from an immature to a mature DC, or they may be at an immature stage. The (genetic) manipulation may be performed before or after the exposure to a maturation stimulus. It is preferred to apply the maturation stimulus (or a combination of maturation stimuli) for a brief period of time only, e.g. for no longer than 24 hours, 12 hours, but especially preferred for 6 hours, but also for less than six hours. The favoured application of a brief (at least 2 hours) maturation stimulus to the DCs assures that a DC immune medicine after inoculation into a patient has retained the capacity for high efficiency initiation of T-cell stimulation. The genetic engineering of the DCs aims at improving that basic immune stimulatory capacity but is not intended to replace it.

Precursor cells of dendritic cells employed for the production of the dendritic cells of the pharmaceutical preparation of the present invention have to be transformed into dendritic cells. Means and methods to achieve this are known in the art.

"Precursor cells of dendritic cells" include monocytes, hematopoietic cells etc..

Another aspect of the present invention relates to a method for producing dendritic cells comprising the steps of:

a) providing immature dendritic cells,

b) contacting said immature dendritic cells with at least one dendritic cell maturation agent to produce partially matured dendritic cells (semi-mature DCs, smDCs) as defined by their capacity to secrete IL-12, and

c) manipulating the partially matured dendritic cells (semi-mature DCs, smDCs) releasing IL-12 of step b) to

(i) over-express at least one immune molecule capable of maintaining the T-lymphocyte stimulatory capacity of the dendritic cells characterised by continued IL-12 secretion for at least 24 hours, preferably at least 48 hours or longer, and the at least one immune molecule being selected from the group consisting of CD40L by introducing nucleic acid molecules encoding for said at least one molecule; and/or

(ii) inhibit or prevent the expression of at least one T-lymphocyte suppressive molecule acting within the dendritic cell exposed to a primary maturation agent such as LPS/IFN-γ or being released from dendritic cells exposed to a primary maturation agent such as LPS/IFN-γ and being selected from the group consisting of interleukin 10 (IL-10), and indoleamine 2,3-dioxygenase (IDO), such as at least one of the genes given in the tables 3 & 4, by knocking out the gene or a fragment thereof encoding said at least one T-lymphocyte suppressive molecule or by introducing nucleic acid molecules, preferably ribonucleic acid molecules, to inhibit or prevent the expression of the at least one T-lymphocyte suppressive molecule that is active within the dendritic cell or is delivered from the dendritic cells to T-cells.

Genetically engineered IL-12 releasing DCs of step b) of the methods described above over-express at least one molecule capable of extending the T-lymphocyte stimulatory time window and/or entirely preventing its closing after 24

hours characterised by maintained secretion of longer than 24 hours by introducing nucleic acid molecules encoding for said at least one immune stimulatory molecule; or show an inhibited or down-regulated expression of at least one molecule that is involved in the normal developmental progress of a DC after exposure to any effective maturation stimulus from the T-lymphocyte stimulatory into a T-lymphocyte suppressive time window that starts opening 24 hours after maturation; and/or inhibit or prevent the expression of at least one molecule that fulfils a function in mediating T-lymphocyte suppression by DCs that have developed to assume an immune suppressive phenotype. This is accomplished by knocking out the gene or a fragment thereof encoding said at least one molecule and/or by introducing nucleic acid molecules, preferably ribonucleic acid molecules, to inhibit or prevent the expression of at least one molecule that interferes with the normal development of DCs after exposure to a maturation stimulus from an immune stimulatory to an immune suppressive phenotype; and/or interfering with signals that are delivered from the DC to the T-cells causing suppressing the activity of this T-cell and thus suppressing an immune response. The dendritic cells of the present invention maximise T-lymphocyte stimulation, particularly CTL activation by using genetic engineering to broaden the stimulatory time window of approximately 24 hours or entirely prevent closing of this stimulatory time window after 24 hours. Alternatively to IL-10 or IDO, other molecules that are involved in the immune suppressive function of the DCs that starts approximately 24 hours after exposure to any maturation stimulus (table 3, 4, and 5) may be used. It will be these molecules that are preferably targeted in the manufacturing of genetically engineered immune stimulatory DCs. It is particularly preferred to use molecules that show a two-fold over expression in the presented DNA micro array data (table 3 and 4), more preferable an over-expression of at least six fold. The numbers given in tables 3 & 4 show the fold over-expression as indicated in the heading of the respective column. It is particularly preferred to knock down the expression in a DC immune medicine of the molecules that appear to be involved in immune suppression, as demonstrated

in the example depicted in Fig. 9.

By reversing the strategy outlined above it is possible to design a genetically engineered T-lymphocyte suppressive DC immune medicine for the treatment of pathological over-activity of the immune system, e.g. in allergies or autoimmune diseases, as well as in stem cell and organ transplantation. Immune suppression is physiologically mediated by a DC that has differentiated for more than 24 hours after exposure to any maturation stimulus. The immune suppressive capacity of such a DC is enhanced by interfering with the expression of T-lymphocyte stimulatory molecules during the first 24 hours of DC differentiation; and/or by over-expressing molecules that confer T-lymphocyte suppression by genetically engineering the DC according to the strategies outlined above.

The use of smDCs as a target for genetic manipulation is a central and critical part of the present invention. The immune stimulatory effects of mDCs that were published in the past are mainly due to the highly artificial experimental setting in which many of these experiments were performed, for example the use of synthetic peptides, which do not exist in nature, instead of the real targets of DCs: native protein antigen molecules or even whole cells, both of which require completely different mechanisms of uptake and processing by DCs. Many other investigators used the murine system for their research and there are critical differences between humans and mice that cause much confusion. However, it is now generally accepted that mDCs have immune suppressive properties.It surprisingly turned out that dendritic cells obtained with the method according to the present invention exhibit a broader stimulatory window (i.e. increased and prolonged expression of IL-12). It was found that genetic engineering of a semi-mature (sm) DC - a DC in which the physiologic differentiation process is initiated by exposure to any maturation stimulus capable of triggering IL-12 secretion from DCs, but which, however, is removed preferably after two to twelve hours, more preferably after six hours - to over-express the CD40L molecule has the capacity to maintain its T-lymphocyte stimulatory capacity for at least 24 hours, preferably 48 hours, and even up to five or even ten days. It is furthermore

preferred to culture such genetically engineered DCs in medium containing IFN-γ. Such DCs render smDCs by a typically six hour-exposure to a Toll-like receptor (TLR) ligand, preferably but not exclusively lipopolysaccharide (LPS), again preferably in the presence of IFN-γ, - see Table 1 - and genetically engineered to over-express CD40L, assume a phenotype that is characterised by continued secretion of IL-12 for at least one, preferably three, and even up to five days and the maintenance of the immune stimulatory capacity in an allogeneic mixed leukocyte reaction (alloMLR) for at least 24 hours, preferably 48 hours, but up to five days. Applied to the design of a DC immune medicine, this confirms the existence of an early immune stimulatory and a later immune suppressive window of DC differentiation and associated function. The general principle in the development of stimulatory DC immune medicines may therefore be to broaden the early immune stimulatory window in order to more effectively trigger immune activation and reduce or close the later immune suppressive window, or vice versa for designing a suppressive DC immune medicine (Fig. 1).

In general, for producing of an immune stimulatory DC medicine ("DC immune medicine"; "immune medicine") for the treatment of, e.g., cancer or infectious diseases, an initial maturation stimulus such as LPS/IFN-γ needs to be applied to the DC in order to initiate the physiologic differentiation from iDCs into smDCs. Other TLR ligands (table 1) may serve the same purpose as LPS; combinations of TLR ligands may give a stronger but not a qualitatively different signal. If the stimulatory potential of a T-lymphocyte stimulatory DC immune medicine is based only on the artificial manipulation of the gene transfer without the initial exposure to a TLR ligand mediated maturation stimulus (e.g. by direct genetic engineering of immature DCs), important contributions to the DC function will be lost and the T-lymphocyte stimulatory DC immune medicine may not reach its full potential. A critical difference of the genetically engineered DC immune medicine according to the present invention to a DC immune medicine that is manufactured by only an exposure to a maturation agent or combinations thereof, e.g. LPS/IFN-γ (smDC), is that for

the latter it is critical that the smDC immune medicine is applied during the corresponding brief window of DC differentiation. Such a stimulatory DC immune medicine has therefore to be applied early after exposure to the maturation stimulus, whereas the genetic engineering e.g. by over-expression of CD40L aims at broadening the immune stimulatory time window of DC differentiation allowing for a less time critical application but most importantly prevent the development of DCs from an immune stimulatory to an immune suppressive phenotype (Fig. 1). A comparable improvement of the immune stimulatory capacity of DCs may be accomplished by knocking down molecules suspected to be critically involved in immune suppression as indicated by an expression profile that is similar to the expression of the known immune suppressive molecules IL-10 or IDO (listed in Fig. 3, 4 and 5); or molecules that have already shown to be involved in immune suppression, as knocking them down in DCs resulted in improved T-cell stimulatory capacity of engineered DCs (Fig. 9). Also, the immune stimulatory time window of the older smDC immune medicine closes after 24 hours, whereas the novel genetically engineered DC immune medicine will maintain its T-lymphocyte stimulatory potential for at least one, preferably three, but up to five days longer. A comparable concept holds true for a T-lymphocyte suppressive DC immune medicine. The immature DCs first need to be exposed to a conventional maturation stimulus, such as LPS/IFN-γ, in order to initiate differentiation towards an mDC phenotype corresponding to the T-lymphocyte suppressive window of DC differentiation. The genetic engineering to over-express T-lymphocyte suppressive molecules from the DC immune medicine may be done before maturation of immature DCs by a maturation stimulus such as LPS/IFN-γ, but also when targeting precursor cells of DCs such as monocytes from the peripheral blood, or haematopoietic stem and precursor cells, especially but not exclusively when gene transfer methods are used that result in stable integration into the genome such as retroviral gene transfer. In addition to genetic engineering before exposure to the maturation stimulus, the genetic engineering may be done six hours and up to 48 hours after initiation of maturation by e.g. LPS/IFN-γ.

When immature DCs are genetically engineered to over-express immune-suppressive molecules, important contributions by the physiologic T-lymphocyte suppressive activity of DCs longer than 24 hours after exposure to a maturation stimulus would be lost, for which reason we prefer the genetic engineering of DCs only in combination with the exposure of these DCs before (even at a precursor cell level) or after the genetic engineering to a maturation stimulus such as LPS/IFN-γ. Without genetic engineering of a T-lymphocyte suppressive DC immune medicine, the application of such a suppressive DC immune medicine has to be done during that suppressive window of DC differentiation whereas a T-lymphocyte suppressive DC immune medicine genetically engineered to over-express molecules that mediate suppression of T-lymphocyte activity allows for a much more flexible administration to the patient.

Table 1: TLR ligands

Receptor	**Naturally occurring**	**Synthetic analogues**	**Fully synthetic small molecules**
Exogenous ligands			
TLR1	Not determined	Triacyl lipopeptides	-
TLR2	Lipoproteins/lipopeptides Peptidoglycan Lipoteichoic acid Lipoarrabinomannan Atypical lipopolysaccharide	Di- and triacyl lipopeptides	-
TLR3	Double-stranded RNA	PolyI:C	-
TLR4	Lipopolysaccharide HSP60 (Chlamydia pneumonie)	LPS/lipid A mimetics, such as MLP	Synthetic lipid A, E5564
TLR5	Bacterial flaggelin	Discontinuous 13-aminoacid peptide	-
TLR6	Not determined	Diacyl lipopeptides	-
TLR7	(G+U) rich single-stranded RNA (mouse only)	Oligonucleotides	Imidazole quinolines (imiquimod, resiquimod), guanosine nucleotides (loxoribine)
TLR8	(G+U) rich single-	-	Imidazole

Receptor	Naturally occurring	Synthetic analogues	Fully synthetic small molecules
	stranded RNA (human only)		quinolines (imiquimod)
TLR9	Bacterial DNA Viral DNA Other DNA with non-methylated CpG sequences	CpG oligonucleotides	-
Endogenous ligands			
TLR2	HSP70	-	-
TLR4	HSP60 Oligosaccharides of hyaluronic acid	-	-

By genetically engineering DCs which have also received an LPS/IFN-γ or similar maturation stimulus before or after genetic engineering, to over-express T-lymphocyte stimulatory molecules and molecules that prevent closing of the immune stimulatory window, a DC differentiation will be possible to broaden the immune stimulatory time window of DC differentiation. It was elected to demonstrate the feasibility of the present invention by using CD40L gene transfer, as the interaction of CD40 expressed from DCs and CD40L expressed from activated T-lymphocytes delivers a potent activation and maturation signal to DCs. Such experiments are preferably performed in the presence of IFN-γ, which is a critical co-factor in the maturation of DCs, and all experiments with CD40L transgenic cells reported in the examples were done in the presence of IFN-γ. The same principle as CD40L gene transfer may be applied to other molecules that confer improved stimulatory capacity to DCs. Alternatively, a T-lymphocyte suppressive DC immune medicine may be designed by knocking out the expression of molecules, such as CD40 or IL-12 or similar molecules, or by over-expressing molecules that confer T-lymphocyte suppression from the DC immune medicine.

By interfering with the expression and/or function of T-lymphocyte suppressive molecules, the immune suppressive window of DC differentiation may be closed or made narrower or moved to a later time point. The feasibility of this approach is demonstrated by knocking down the expression of molecules that interfere with T-lymphocyte activation by DCs. The

improvement of T-lymphocyte function by knocking down DC-derived T-lymphocyte suppressive signals, as e.g. the enzyme IDO that metabolises tryptophan, on which activated T-lymphocytes heavily depend, into kynurenines that have pro-apoptotic effects on activated T-lymphocytes, is shown in the example section. As a second example the expression of IL-10 was targeted, which is considered the prototypic immune suppressive molecule and which is expressed by DCs during the immune suppressive differentiation time window. In order to knock down the expression of target molecules, RNA interference is preferably used, but other technologies, such as the intracellular expression of single chain monoclonal antibodies or anti-sense RNA, may serve the same purpose. Alternatively, over-expression of said molecules (e.g. IDO or IL-10) or similar molecules may serve to design a T-lymphocyte suppressive DC immune medicine on the basis of pre-matured smDCs. The results in the example section (Fig. 9) show that knocking down the expression of molecules that have an expression kinetic comparable to IL-10 and/or IDO also results in an improved T-cell stimulatory capacity of genetically engineered DCs.

The structure and properties of a DC need to be described in a dynamic fashion that takes into consideration the developmental stages of a DC. Each of these stages may be characterised by the absence or presence of certain marker molecules. This also indicates that the molecular features of a DC depend on the specific stage of differentiation of this DC and the conditions that caused a DC to assume a certain differentiation pathway. The developmental plasticity of a DC also explains why it is advantageous to use what is called a semi-mature type 1 DC (smDC1) ("T cell activating dendritic cells characterised by the release of interleukin 12"). To initiate the switch from the tolerance maintenance function to the immune stimulatory stage, the DC needs to be exposed to a maturation stimulus (dendritic cell maturation agent). This opens the immune stimulatory time window during which the DC most importantly releases IL-12 as a response to the combination of LPS and IFN-γ or similar reagents that are added to the DC manufacturing culture. IL-12 acts via a

specific receptor on helper T-lymphocytes and causes them to assume a Th1 phenotype, resulting in the support of cytolytic immunity. In order to allow this DC/T-lymphocyte interaction and the development of cytolytic immunity the DCs are preferably inoculated into the organism (e.g. human) at an early time point during the immune stimulatory window. It is particularly preferred that said DCs are injected 6 hours after the maturation stimulus. Obviously, inoculation is associated with removal of the DC culture medium that contains the dendritic cell maturation agent (e.g. stimulatory molecules LPS and IFN-γ). A sufficiently sustainable signal is transmitted into the DC by a 2, preferably 4, more preferably 6-hour exposure to said maturation agents (e.g. LPS and IFN-γ) so that after said exposure the DC is irreversibly committed to complete the process of maturation and no longer depends on the presence of the ligands, i.e. DC maturation agents. Formally, however, at the time of application the DCs have not yet completed their maturation process, which takes 1-2 days. The smDC1 design takes optimal advantage of the immune stimulatory time window during the first 24 hours after initiation of maturation and before the immune suppressive time window opens and starts to down-modulate the immune response.

At an early phase after exposure to a maturation stimulus, such as LPS/IFN-γ, DCs possess strong immune activating properties (the activating window, figure 1), whereas at later stages of their development they enter an immune suppressive phase (the suppressive window, figure 1). Molecular mechanisms of T-cell activation are well studied and understood. The molecular nature of and the events initiating negative regulatory feedback loops are much less studied. Thus, the design of the DC immune medicine according to the present invention aims at broadening the immune stimulatory window for enhancement of the immune activation and downscaling or closing the immune suppressive window, thus blocking negative regulatory feedback loops in DCs. This was accomplished by genetically engineering DCs either by over-expressing immune stimulatory genes in addition to exposing them to DC maturation agents before or after genetic

engineering, such as LPS/IFN-γ, or by knocking down immune-suppressive genes using RNA interference. The expression of a multitude of immune stimulatory or immune suppressive genes may be modulated following the same basic principle. The feasibility of this approach by over-expressing the immune-stimulatory CD40L molecule or by knocking down the immune-suppressive molecules IL-10 and IDO is shown in the example section. Combinations of over-expression and knock down may enhance the potency of a DC immune medicine but follow the same basic logic. A T-lymphocyte suppressive DC immune medicine for the treatment of pathological over-activity of the immune system may be designed in analogy to the T-lymphocyte stimulatory DC immune medicine by genetically engineering a DC initially exposed to a maturation stimulus, such as LPS/IFN-γ, by genetically engineering the resulting smDCs to over-express immune suppressive molecules and/or knock down immune stimulatory molecules in the DC.

According to the novel T-lymphocyte stimulatory or suppressive DC immune medicine based on genetic engineering that is described in the present invention, partially matured smDCs are manipulated by introducing into said DCs nucleic acid molecules encoding the at least one immune stimulatory or immune suppressive molecule and/or nucleic acid molecules, preferably ribonucleic acid molecules (e.g. siRNA), to inhibit or prevent the expression of at least one immune suppressive or immune stimulatory molecule.

The expression of immune stimulatory as well as immune suppressive molecules in DCs may be influenced or induced by various methods, whereby it is preferred to modulate said expressions by introducing nucleic acid molecules as outlined above. For instance, the nucleic acid molecule transfer can be achieved with lentiviral gene transfer vehicles as well as liposome-mediated transfection. The same principle, however, will hold true when other viral vectors, such as retro viruses or adeno viruses, or non-viral vectors, such as gene gun or poly-cationic technologies, or when any other gene transfer is/are employed.

Several strategies have been developed to introduce foreign genes into cells, including direct injection of

plasmids or DNA liposome complexes and infection with modified viruses. However, safety and efficacy are important considerations in the development of therapy protocols that use such gene transfer methods. For example, proteins that are therapeutic in the context of one tissue may be harmful in another. Accordingly, transcriptionally targeted vectors that can restrict the expression of a therapeutic sequence to appropriate cells are particularly desirable. Furthermore, in some cases there may be a therapeutic window for certain proteins, such that levels of expression below or above certain thresholds may be ineffective or toxic, respectively. Therefore, it would also be desirable to create constructs and devise methods that allow exogenous control of expression, so that levels of a therapeutic protein can be raised or lowered according to therapeutic need.

Conventional viral and non-viral based gene transfer methods can be used to introduce nucleic acids encoding the respective molecules into the DCs of the present invention or, alternatively, nucleic acids that inhibit transcription or translation of said molecules, such as siRNAs or anti-sense RNAs. Non-viral vector delivery systems include DNA plasmids, naked nucleic acid, and nucleic acid complexed with a delivery vehicle such as a liposome. Viral vector delivery systems include DNA and RNA viruses, which have either episomal or integrated genomes after delivery to the cell. For a review of gene delivery procedures, see Anderson, Science 256:808-813 (1992); Nabel & Felgner, TIBTECH 11:211-217 (1993); Mitani & Caskey, TIBTECH 11:162-166 (1993); Dillon, TIBTECH 11:167-175 (1993); Miller, Nature 357:455-460 (1992); Van Brunt, Biotechnology 6(10): 1149-1154 (1988); Vigne, Restorative Neurology and Neuroscience 8:35-36 (1995); Kremer & Perricaudet, British Medical Bulletin 51(1):31-44 (1995); Haddada et al., in Current Topics in Microbiology and Immunology Doerfler and Bohm (eds) (1995); and Yu et al., Gene Therapy 1:13-26 (1994).

Small interfering RNA molecules can also be used. In mammalian cells, introduction of long dsRNA (>30 nt) often initiates a potent antiviral response, exemplified by non-specific inhibition of protein synthesis and RNA degradation.

The phenomenon of RNA interference is described and discussed, e.g., in Bass, Nature 411:428-29 (2001); Elbahir et al., Nature 411:494-98 (2001); and Fire et al., Nature 391:806-11 (1998), where methods of making interfering RNA also are discussed. The siRNAs sequences used in the present invention are preferably less than 100 base pairs, typically 30 bps or shorter, and are made by methods known in the art. Exemplary siRNAs according to the invention could have up to 29 bps, 25 bps, 22 bps, 21 bps, 20 bps, 19 bps, 15 bps, 10 bps, 5 bps or any integer thereabout or therebetween.

According to a preferred embodiment of the present invention, the precursors for the manufacturing of immature DCs are obtained from skin, spleen, bone marrow, thymus, lymph nodes, umbilical cord blood or, most preferably, from peripheral blood. The DCs used in the method according to the present invention can be directly isolated from a respective source or derived from progenitor cells. The person skilled in the art knows respective methods. For example, DC precursors and immature DCs can be isolated by collecting anti-coagulated peripheral blood, haematopoietic stem cells, by leukocyte apheresis, or by preparation of buffy coats, rosetting, centrifugation, density gradient centrifugation (e. g., using Ficoll (such as FICOLLPAQUE), PERCOLO (colloidal silica particles (15-30 nm diameter) coated with non-dialyzable polyvinylpyrrolidone (PVP), sucrose, and the like), differential lysis of cells, filtration etc.. In certain embodiments, a leukocyte population may be prepared, such as, for example, by collecting blood from a subject, defribrinating it, removing the platelets, and lysing the red blood cells. DC precursors, monocytes, or myeloid progenitor or stem cells may be used to differentiate iDCs. Monocytes can optionally be enriched from peripheral blood by, for example, taking advantage of their capacity to adhere to plastic surfaces, centrifugation through a density gradient, monoclonal antibody panning, counter flow centrifugation and the like. If the DCs obtainable by the method according to the present invention are used to treat individuals, the iDCs can be obtained from the individual to be treated or from a healthy individual HLA-matched to the individual to be

treated.

DC progenitors can be cultured and differentiated in suitable culture media. Suitable tissue culture media include e.g. RPMI 1640 and DMEM. The tissue culture media can be supplemented with human autologous or pooled donor serum but not serum of any bovine source, amino acids, vitamins, cytokines, such as GM-CSF and IL-4 or IL-13, or IFN-γ, and divalent cations to promote differentiation of the cells. The progenitor cells may be preferably cultured also in serum-free clinical grade media. A typical cytokine combination used with dendritic cell culture medium comprises GM-CSF and IL-4 or IL-13, or IFN-γ.

In order to apply the maturation stimulus to the DCs that drives them into the smDC differentiation status (before or after genetic engineering or at the stage of a DC precursor cell such as a monocyte or a haematopoietic stem or precursor cell) that is the preferred status for a the DC immune medicine of the present invention, genetic engineering, an effective amount of at least one DC maturation agent is contacted with the iDCs. The at least one DC maturation agent is preferably selected from the group consisting of heat-inactivated or formalin-treated Bacillus Calmette-Guerin (BCG), preferably cell wall constituents of BCG, BCG-derived lipoarabidomannans or BCG components, lipopolysaccharide (LPS) derived from E. coli, or inactivated Gram positive or Gram negative microorganisms, an imidazoquinoline compound, preferably an imidazoquinoline-4-amine compound, in particular 4-amino-2-ethoxymethyl-x-dimethyl-1H-imidazol [4,5-c]quinolin-1-ethanol or 1-(2-methylpropyl)-1H-imidazo [4,5-c] quinolin-4-amine, or derivatives thereof (see e.g. WO00/47719), a synthetic double-stranded polyribonucleotide, preferably polyI:C, natural double-stranded RNA or RNA viruses or fragments of RNA, or synthetic analogues, or a synthetic or natural nucleic acid molecule comprising un-methylated CpG motifs. The majority of these compounds are TLR agonists (see table 1 for a comparison). In the present invention it is particularly preferred to use LPS as dendritic cell maturation agent. However, in principle, it is also feasible to use any TLR agonists alone or in combination with IFN-γ. In principle,

it is also possible to expose iDCs to cocktails of cytokines for maturation that typically include but are not limited to tumour necrosis factor α (TNF-α), IL-1, IL-6, and prostaglandin E6, or parts of that combination. Furthermore, it is possible to trigger the CD40/CD40L signalling pathway. This may be done by contacting iDCs with recombinant CD40L molecules or fusion proteins comprised of the CD40L domain and another protein, such as IgG-Fc, in soluble form or immobilised at a surface, e.g. the culture dish or a nano-particle, or with primary cells or cell lines genetically engineered to express CD40L, or with activated T-lymphocytes that physiologically up-regulate expression of CD40L. The CD40/CD40L signal may be applied in any combination with TLR agonists, inflammatory cytokines. Of course any combination of at least two of said maturation agents may be used according to the present invention. The at least one (preferably at least 2, 3, 5, 10) dendritic cell maturation agent is preferably contacted with the dendritic cells in the presence of IFN-γ.

According to another preferred embodiment of the present invention, the iDCs prior to genetic engineering step c) are contacted with effective amounts of at least one dendritic cell maturation agent for at least 2 hours, preferably for at least 6 hours, in particular for at least 12 hours, and for a maximum of up to 24 hours. The maturation time depends on various parameters (e.g. DC maturation agent). The contact time and the other parameters have to be chosen so that the iDCs mature only partially into smDCs using methods known in the art. Cell surface markers can be detected in assays familiar to the art, such as flow cytometry and immunohistochemistry. The cells can also be monitored for cytokine production (e.g. by ELISA, another immune assay, or by use of an oligonucleotide arrays or protein arrays).

The at least one molecule capable of mediating maturation of iDCs into IL-12 releasing smDCs is preferably selected from the group consisting of LPS in the presence of IFN-γ in order to ready the DCs for the step of genetic engineering in order to manufacture a novel T-lymphocyte stimulatory or suppressive DC immune medicine with improved

features. The at least one molecule capable of enabling the DCs to maintain their immune stimulatory phenotype characterised e.g. by the secretion of IL-12 beyond the physiologic immune stimulatory window of approximately 24 hours, thus conferring to them superior features in comparison with smDCs, is CD40L, typically but not necessarily in the presence of IFN-γ. We here elected to use an approach that is based on enabling smDCs to artificially express CD40L, which they normally don't, using genetic engineering methods outlined above. It is, however, conceivable, to express CD40L not from the DC itself but rather from an accessory primary cell or cell line, an activated T-lymphocyte, or to use soluble or immobilised recombinant CD40L molecules or fusion proteins. According to another preferred embodiment of the present invention, the at least one molecule that interferes with the expression of DC molecules that mediates T-lymphocyte suppressive activity is selected from the group consisting of interleukin 10 (IL-10) and indoleamine 2,3-dioxygenase (IDO). The molecule that mediates T-lymphocyte suppressive activity may also be selected from the molecules listed in tables 2 and 3 of the example section, whereby molecules showing two-fold over-expression in the DNA micro array expression profiling data are preferred, but molecules showing a six-fold or higher over-expression are particularly preferred.

According to a preferred embodiment of the present invention the at least one antigen is selected from the group

a) consisting of tumour antigens, viral antigens, bacterial antigens, or any other human microbial or parasitic pathogens; or

b) consisting of environmental antigens that cause allergy, auto-antigens against which an immune response was initiated that causes disease, or transplantation antigens.

In order to produce novel T-lymphocyte stimulatory or suppressive DC immune medicines with improved features based on smDCs, which are able to induce a specific enhanced immune response or enhanced immune suppression against an antigen in an individual, the iDCs are preferably loaded with at least one antigen before contacting them with the preferred LPS/IFN-γ stimulus to manufacture smDCs followed by genetic

engineering. Antigen loading is necessary to instruct T-lymphocytes against what antigen they need to become active or which antigen they are supposed to tolerate. Antigens for the charging of DCs may be derived from diseased tissue, such as tumour antigen or viral antigens from virally infected cells. They may be a fragment of or an entire dead or living microorganism or a dead or living prokaryotic human or animal cell, e.g. a human or animal tumour cell. An antigen may be a recombinant protein, or a synthetic peptide, a DNA-based viral or non-viral recombinant expression vector or natural or synthetic RNA coding for an antigen. Alternatively, antigens may be environmental antigens that have triggered an immune dysfunction such as an allergy, an auto-antigen against which a pathologic autoimmune response has caused disease, or an antigen that determines organ or stem cell transplant rejection, such as MHC molecules. It is worth noting that, in case of a T-lymphocyte suppressive DC immune medicine for tolerance induction against an allogeneic transplant, loading might not be necessary, as the organ or stem cell donor DCs carry the same MHC molecules as the transplant. Obviously, in these latter situations a DC immune medicine will be designed in a way that it suppresses immunity against the allergen, transplantation antigen, or auto-antigen. In order to deliver the antigen to the DC, various methods may be used such as passive exposure that allows the DC to phagocytose the protein or peptide antigen, an antigenic protein complex, cells or cell membranes expressing antigens or antigenic peptides, texosomes, liposomes containing antigens or antigenic peptides, nucleic acids encoding antigens or antigenic peptides (possibly incorporated in plasmids or viral vectors), or total RNA from a tumour cell. These methods have been disclosed, for instance, in W099/03499. Such vehicles may be of viral or non-viral origin or may be nano-particles. Antigens may be tumour antigens, viral antigens, bacterial antigens, etc., more generally, any peptide or polypeptide against which an immune response or reaction is sought. In this respect, DCs may be sensitised to one or several antigens according to various techniques known in the art. The term "sensitized" indicates that the antigen or a portion thereof

is exposed at the surface of the DCs, preferably in complex with molecules of the major histocompatibility complex (MHC). In principle, DCs could be inoculated into a patient without prior loading with an antigen and enabled for taking up an antigen in vivo, e.g. by injection directly into a tumour or into it's surroundings, into a metastasis, or into the draining lymphaytic system including lymph nodes and primary and/or secondary lymphoid tissue. Essentially, only the presence of the antigen and its presentation to a T-lymphocyte determines the DC immune medicine but not the way the antigen reaches the DC. An overview of DC loading techniques is given in RM Steinman & J Banchereau (Nature, Volume 449/27 September 2007, page 419-426) and the references therein.

The antigen-loaded and genetically engineered DC of the present invention may be used to therapeutically modulate immune responses in various immunological dysfunctions depending on the antigen loaded into said cells as well as the functional status the DC is in physiologically, by use of various signalling molecules such as DC maturation agents, or by genetic engineering of the DC. Such dysfunctions may include but are not limited to cancer, which may be pictured as a failure of the immune system to reject transformed and mutated cells; infectious disease, for example in the context of severe and otherwise untreatable microbial infections or in immune-compromised individuals, particularly during organ or stem cell transplantation. Other immune dysfunctions that may be treated by such a DC immune medicine may result from immunological hyper-activity, for example against environmental antigens resulting in allergies, or in situations where the immune system attacks its host causing autoimmune diseases. Finally, a DC immune medicine may be designed based on the methods of the present invention that interferes with the rejection of an organ or stem/precursor cell transplant including induced progenitor cells (iPS) generated by genetic engineering of other cells, thus facilitating the acceptance of the graft by its host. According to a preferred embodiment of the present invention, the at least one antigen is selected from the group consisting of tumour antigens, viral antigens, and bacterial antigens.

The genetically engineered DCs according to the present invention may be loaded with any antigen against which an immune response in an individual should be induced, suppressed, or prevented. Particularly preferred are tumour antigens.

The novel genetically engineered DC immune medicine with improved T-lymphocyte stimulatory or suppressive capacity according to the present invention can be preserved, e.g. by cryopreservation either before maturation as iDCs, following partial maturation as smDCs, before or after genetic engineering as improved DCs prior to administration to a patient. Cryopreservation agents which can be used include but are not limited to dimethyl sulfoxide (DMSO), glycerol, polyvinylpyrrolidone, polyethylene glycol, albumin, dextran, sucrose, ethylene glycol,i-erythritol, D-ribitol, D-mannitol, D-sorbitol, i-inositol, D-lactose, choline chloride, amino acids, methanol, acetamide, glycerol monoacetate and inorganic salts.

A further aspect of the present invention relates to a pharmaceutical composition comprising the novel genetically engineered DC immune medicine with improved T-lymphocyte stimulatory or suppressive capacity according to the present invention. The DCs of the present invention can be formulated with physiologically acceptable carriers, excipients, buffers, and/or diluents using methods and compositions well known to the skilled artisan.

The novel genetically engineered DC immune medicine with improved T-lymphocyte stimulatory or suppressive capacity may be administered directly to a subject in need of immune modulation. Typically, about 10^2 to about 10^{10} cells are suspended in a pharmaceutically acceptable carrier. If an individual suffering from cancer is treated, the cells are preferably injected into a disease free lymph node, preferably into the inguinal region but any tumour free or tumour bearing (metastatic) lymph node will serve the purpose, into the tumour directly or into a region, near to, adjacent to, or in circulatory or lymphatic contact with the tumour or tumour bed, or into metastatic disease. The DC immune medicine may be applied subcutaneously or intradermally into the skin to allow

migration into lymph nodes. In principle, it is also possible to inject the DC immune medicine into the blood stream, either as a single shot or as an infusion over a longer period of time, into the peripheral blood or via a catheter into a blood vessel (artery or vein) that supplies a diseased organ or region of the body, or the portal vein or a pulmonary vein or artery, and the like. Implanted release devices may be used that deliver a continuous stream of the DC medicine into the tumour or a metastasis, a lymph node, the blood stream, or the skin.

The novel genetically engineered DC immune medicine with improved T-lymphocyte stimulatory or suppressive capacity of the present invention can be administered by any means appropriate for the formulation and mode of administration. For example, the cells can be combined with a pharmaceutically acceptable carrier and administered with a syringe, a catheter, a cannula, and the like. As above, the cells can be formulated in a slow release matrix. When administered in this fashion, the formulation can be administered by a means appropriate for the matrix used. Other methods and modes of administration applicable to the present invention are well known to the skilled artisan.

Compositions of the present invention can be used alone in the treatment of an individual, or the compositions can be used in combination with any other method to treat a tumour. For example, the methods of the present invention can be used in combination with surgical resection of a tumour; prior to, simultaneous with, or subsequent to radiation therapy and/or chemotherapy (cytotoxic drugs, apoptotic agents, antibodies, and the like); cryo-therapy; brachy-therapy; other forms of immune therapy (ex vivo expanded tumour antigen specific T-lymphocytes, NK cells, cytokines and growth factors, antibodies specific for tumour antigens, or targeting structures of the tumour tissue that are critical for tumour cell survival, such as blood vessels, etc.); gene therapy using viral or non-viral vectors, and the like. Furthermore, the DC immune medicine of the present invention can be co-administered with another agent, which agent acts as an adjuvant to the maturation of the dendritic cell and/or the

processing of antigen within the tumour or region near or adjacent to the tumour. Any and all of these methods can also be used in any combination. Combination treatments can be concurrent or sequential and can be administered in any order as determined by the treating physician.

Another aspect of the present invention relates to the use of a dendritic cell according to the present invention for the manufacture of a medicament to treat and/or prevent cancer and/or microbial or parasitic infections; or to treat and/or prevent allergies, autoimmune disease, or stem cell or organ transplant rejection. The partially matured dendritic cells according to the present invention may be preferably employed in cancer prevention and/or cancer treatment. In such a case the dendritic cells are loaded with at least one tumour antigen. For example, but not by limitation, the cells can be administered directly into a tumour, into the tumour bed subsequent to surgical removal or resection of the tumour, peri-tumorally, into a draining lymph node in direct contact with the tumour, into a blood vessel or lymph duct leading into, or feeding a tumour or organ afflicted by the tumour, e.g., the portal vein or a pulmonary vein or artery, and the like.

The administration of the partially mature dendritic cells of the invention may be applied either simultaneous with or subsequent to other treatments for the tumour, such as chemotherapy or radiation therapy. Further, the partially mature dendritic cells of the invention can be co-administered with another agent, which agent acts as an adjuvant to the maturation of the dendritic cell and/or the processing of antigen within the tumour or region near or adjacent to the tumour. In addition, the dendritic cells can also be formulated or compounded into a slow release matrix for implantation into a region in or around the tumour or tumour bed such that cells are slowly released into the tumour, or tumour bed, for contact with the tumour antigens.

According to a preferred embodiment of the present invention the medicament is administered to an individual prior to, simultaneous with, or subsequent to radiation therapy and/or anti-tumour or anti-microbial chemotherapy, or

any therapy aimed at treating allergies, autoimmune diseases, or stem cell or organ transplant rejection. The dendritic cells according to the present invention may be employed in combination with other cancer therapies in order to achieve an even more beneficial effect.

Another aspect of the present invention relates to the use of a dendritic cell according to the present invention for the manufacture of a medicament to treat and/or prevent immunological disease caused by a pathologic over-reaction of the immune system against environmental antigens, such as allergens, or against autoantigens in the course of an autoimmune disease.

Said medicament is preferably administered to an individual prior to, simultaneous with, or subsequent to other modalities aimed at treating or preventing allergies or autoimmune disease.

A further aspect of the present invention relates to the use of a dendritic cell according to the present invention for the manufacture of a medicament to treat and/or prevent the immunologic rejection of an allogeneic stem cell transplant, preferably used in the treatment of haematological malignancies, or to treat and/or prevent rejection of an allogeneic organ transplant.

Said medicament is preferably administered to an individual prior to, simultaneous with, or subsequent to other modalities aimed at treating or preventing the rejection of an allogeneic stem cell or organ transplant.

The present invention is further illustrated by the following figures and example, however, without being restricted thereto.

Fig. 1 shows developmental plasticity of a DC in a schematic representation of the kinetics of a DC differentiation process.

Fig. 2 shows a quality control of the smDC1 basic design.

Fig. 3 shows the results of a CD40L gene transfer.

Fig. 4 shows the quantity and quality of IL-12 and IL-10 secretion.

Fig. 5 shows the potential for cytolytic activity

(square, CD40L transgenic DCs; diamond, GFP transgenic DCs; triangle, control DCs).

Fig. 6 shows the immune stimulatory capacity of LPS-activated DCs blocked for IL-10 expression.

Fig. 7 shows the immune stimulatory capacity of LPS-activated DCs with silenced IDO expression.

Fig. 8 shows the experimental design of the DC expression profiling experiments.

Fig. 9a shows examples for improved proliferative responses after knocking down the expression of target molecules in DCs identified in expression profiling experiments using RNA interference. Fig. 9b shows additional examples of genes that after knocking down their expression in DCs with siRNA result in an improved stimulatory capacity of such genetically engineered DCs for allogeneic lymphocytes as indicated compared to control siRNA transfection or un-transfected DCs as indicated.

EXAMPLE: Method for manufacturing a T-lymphocyte stimulatory or suppressive DC immune medicine by genetic engineering.

Leukocyte apheresis

Leukocytes were collected using an Amicus leukocyte apheresis device (Baxter, Deerfield, IL) from healthy volunteers and patients suffering from various neoplasias treated in the context of clinical trials that were approved by the responsible institution's review boards. All individuals gave their informed consent to these studies according to the World Medical Association Declaration of Helsinki. Cell numbers and subsets were determined on a Sysmex cell counter (Sysmex, Bornbarch, Germany) and/or by flow cytometry.

Monocyte enrichment

Monocytes were enriched by plastic adherence as described previously using AIM-V (Invitrogen, Carlsbad, CA) supplemented with 1% human pooled AB plasma (Octaplas, Octapharma, Vienna, Austria) or CellGro medium (CellGenix, Freiburg, Germany). For the in-line procedures we followed the instructions provided by the manufacturers. Using the Elutra cell separator (Gambro BCT, Lakewood, CO), monocytes were

enriched from the leukocyte apheresis product by loading into the elutriation chamber while maintaining the centrifuge speed at 2400 rpm. Thereafter, the centrifuge speed and the flow of elutriation media (PBS/HSA Baxter, New Jersey, NJ) was held constant for cell fractionation. Alternatively, selection of monocytes was done with the CliniMACS cell selection system (Miltenyi, Bergisch Gladbach, Germany) that uses CD14-coated magnetic beads to retain monocytes in a magnetic column. Another option for monocyte enrichment is depletion of T and B-lymphocytes for the enrichment of monocytes was done using the Isolex 300i Magnetic Cell Selector (Nexell, Irvine, CA). Lymphocytes were retained in a magnetic column by connecting them to CD2 and CD19 coated magnetic beads, and collecting the flow-through. The final products of all enrichment procedures were characterised by flow cytometry.

Flow cytometry

Leukocyte apheresis and monocyte enrichment products were analysed for total leukocytes, T-lymphocytes, B-lymphocytes, monocytes, and granulocytes by antibody labelling with anti-CD45-FITC, anti-CD3-PerCP, anti-CD19-APC, anti-CD14-APC, and anti-CD15-FITC (BD Pharmingen San Diego, CA), respectively, using the Trucount system (Becton Dickinson, New Jersey, NJ). Labelled cells were analysed on a FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA). The appropriate isotype control antibodies were included in the analysis.

DC manufacturing

Monocytes isolated by the respective enrichment procedures described above were cultured at a density of $1x10^6$ monocytes/cm^2 either in AIM-V medium supplemented with 2% pooled human AB plasma or in CellGro medium at 37°C in a humidified incubator for 6 days. The culture medium was supplemented with 1000 U/ml human GM-CSF and 300 U/ml human IL-4 (both from CellGenix, Freiburg, Germany) and replaced with the same volume of AIM-V/2%OP or CellGro plus GM-CSF and IL-4 on day 3. Maturation was carried out on day 6 by adding 50 ng/ml IFN-γ (Boehringer Ingelheim, Vienna, Austria) and lipopolysaccharide (LPS, E. coli strain O111:B4, Calbiochem, San Diego, CA, USA), ranging from 1-1000 ng/ml, to the culture

for 6 hours to generate semi-mature (sm) DCs that subsequently were frozen; patient's DC vaccines were manufactured with clinical grade LPS (US Pharmacopeia, Bethesda, MD).

DC immune phenotyping

The maturation status of the DCs was determined using the following antibodies: anti-CD86-APC (BD Pharmingen, San Diego, CA), anti-CD80-PE (Immunotech, Beckman Coulter, Fullerton, CA), anti-CD83-APC (all three from BD Pharmingen, San Diego, CA), anti-MHC I-PE, anti-MHC II-FITC (both from Dako Cytomation, Carpinteria, CA), and anti-CD45-PerCP (BD Pharmingen, San Diego, CA). The viability of the DCs was measured by propidium iodide staining (Sigma, St. Louis, MO). Cells were analysed using a FACS Calibur flow cytometer. The appropriate isotype control antibodies were included in the analysis.

IL-12 detection by ELISA

IL-12 concentrations in the supernatant of the DC cultures were measured as described previously.

Allogeneic mixed leukocyte reactions

Allogeneic responder PBMCs were isolated by gradient centrifugation from peripheral blood. Stimulating DCs (10000, 2000, or 400) were placed in triplicates with 10^5 responder cells in 200 µl AIM-V medium supplemented with 2% pooled human plasma on a 96 well round bottom plate. For a positive reference, 10^5 responder cells were stimulated with 100 ng/ml Staphylococcal enterotoxin A/B (SEA/SEB, Toxin Technologies Inc., Sarasota, FL). On day 4 the co-culture was incubated for another 18 hours with 1 µCi of tritium thymidine solution (NEN Life Science Products, Boston, MA). Finally, the cells were harvested with a Skatron (Lier, Norway) harvester. The incorporated tritium thymidine was counted using a Trilux-plate reader (Wallac Oy, Turku, Finland). Alternatively, allogeneic PBMCs were labelled with CFSE (Molecular Probes, Eugene, OR) and mixed with DCs in a ratio of 1/5, 1/10, 1/20, 1/40, and 1/80. For the controls, no DCs or SEA/SEB was added. Finally, the PBMCs were labelled with anti-CD3-PerCP and analysed using a FACS Calibur flow cytometer. The percentage of CD3 positive CFSE negative T-lymphocytes was determined.

Lentiviral gene transfer into smDCs

Using ViraPower™ Lentiviral Expression System (from Invitrogen) lentiviral particles were generated by co-transfection of 293FT producer cell line with pLP-plasmids encoding for viral structural proteins, polymerase and reverse transcriptase (pLP/VSVG, pLP-1, pLP-2) and plasmids containing GFP or CD40L. 72 hours after co-transfection, the whole supernatant was harvested and 100x concentrated by ultra-centrifugation. DCs were cultivated and matured under the conditions outlined above. DCs were harvested 48 hours or 6 hours after initiation of maturation, respectively. Pre-matured smDCs were then transduced with lentiviral particles (250 µl 100x concentrated lentiviral supernatant/1x10^6 DC) in combination with 6 µg/ml Polybrene (from Sigma-Aldrich) plus IL-4, GM-CSF, and IFN-γ in standard concentrations. For IL-12 quality control supernatant was taken after 24 hours, and expression of GFP/CD40L was measured after 48 hours following standard procedures.

RNA interference in DCs

DCs are manufactured according to the standard procedures outlined above. On day 6, 10^6 DCs are transfected with 100 pmol gene-specific siRNA using a transfection reagent (Dharmacon) according to the manufacturer's instructions. Twelve hours after transfection, DCs are stimulated with LPS/IFN-γ for 6 hours. All uses are in analogy to the methods outlined above.

Results

DC immune medicines currently in use employ monocyte-derived DCs that are charged with an antigen of any nature, as outlined in the introduction, and exposed to a maturation stimulus that has the capacity to trigger the release of IL-12 from the DC. A DC phenotype characterised by IL-12 secretion has the capacity to induce a type 1 polarisation of the immune system that supports cytolytic immunity. This implies that a stimulatory DC immune medicine needs to be applied to the patient during the time window of IL-12 secretion to allow presentation of the antigens from the DCs to the T-lymphocytes in the presence of IL-12 (Fig. 1). The novel genetically engineered DC immune medicine with improved T-lymphocyte stimulatory or suppressive capacity overcomes this limitation,

as the genetic manipulations of the DCs allow the immune stimulatory window to remain open for a longer time period.

In the following examples, genetically engineered DC immune medicines have been manufactured and studied. Lentiviral gene transfer or liposome-based transfection to deliver DNA or RNA into DCs was used, but it may be assumed that any nucleic acid delivery technology may serve in that capacity. As an example of over-expression of an immune stimulatory gene in a DC immune medicine, DCs were engineered using lentiviral gene transfer to express the CD40L molecule. Functional studies confirmed that such an engineered DC immune medicine has an enhanced potential to stimulate immune responses. Furthermore, it is demonstrated that the knock down of the immune suppressive molecules IL-10 and IDO also enhances the stimulatory capacity by engineering DCs with siRNA molecules designed for RNA interference for IL-10 and IDO. In order to identify further DC molecules involved in immune suppressive feedback loops, whole genome DNA expression profiling using DNA micro arrays were conducted. Based on cluster analysis that grouped genes with an expression profile similar to that of IL-10 or IDO, a list of genes was found that has the potential to negatively regulate immune responses. Knocking down these genes in a DC immune medicine will thus improve its immune stimulatory capacity. Therefore, a DC immune medicine genetically engineered for allowing specific modulation of defined immune system components enables the treatment of associated immune system dysfunctions. Finally examples of the functional consequences of knocking down target genes expressed with kinetics similar to IL-10 or IDO in the DC expression profiling experiments using RNA interference are shown. In co-cultures with allogeneic T-lymphocytes an improved capacity of such genetically engineered DCs to trigger proliferation indicative for enhanced T-lymphocyte stimulation were observed.

Fig. 2 shows the quality control of the smDC1 basic design that is used for genetic engineering. The DC immune medicine has to meet defined quality control criteria. Panel A shows the purity, viability, and yield of DCs manufactured from peripheral blood monocytes. Such monocytes are collected

by leukocyte apheresis, and monocytes are enriched by counter flow centrifugation (elutriation). In the presence of IL-4 and GM-CSF, monocytes differentiate in vitro within six days into iDCs. The iDCs are charged with antigen and subsequently exposed to a maturation stimulus comprised of LPS and IFN-γ for 6 hours and frozen. At this stage they are called semi-mature, as, although they are irreversibly committed to continue their maturation, they do not yet show the typical phenotypic and functional characteristics of mDCs. Most importantly, at that stage (the immune stimulatory window, approximately 0-24 hours after initiation of maturation, figure 1) DCs trigger immunity whereas at later stages (the immune suppressive window, approximately 24-48 hours after initiation of maturation, figure 1). However, in clinical application, they are injected into the patients at this differentiation stage where they complete their maturation and trigger immune responses (before 24 hours), but subsequently (at approximately 24 hours) also enter - according to their physiologic developmental program triggered by the maturation stimulus - an immune suppressive stage, which we aim to prevent by genetic engineering of the DC immune medicine.

For quality control, one aliquot of the DC immune medicine is thawed and re-cultivated for 2 days in order to let the DCs complete their maturation process. During these two days, they secrete cytokines, most importantly IL-12 (early after maturation) and IL-10 (late after maturation) (panel B; shown is mean ± SEM from three individuals). Also, they show changes in the expression pattern of critical DC membrane molecules (panel C). Finally, DCs are subjected to an alloMLR potency test (panel D) by co-cultivation with CFSE-labelled allogeneic PBMCs at the indicated ratios, which triggers cell division that is associated with a dilution of the CFSE and a reduction of fluorescence. The bar graphs show the mean ± SEM percentage of proliferating cells from three individuals.

The initial stimulus is also necessary for the initiation of the immune suppressive feedback loops. In general, the stronger the activation in response to a specific stimulus, the stronger the feedback signalling will be in

order to down-modulate the immune activation to its baseline level, thereby preventing an immune response from getting out of control and causing auto-immune diseases. Thus, it was found that the maturation stimulus LPS/IFN-γ results in the highest amounts of IL-12 release, but also in the highest amounts of IL-10 release.

Table 2: Specifications of the basic design of smDC1 for cancer vaccination.

Test	**Specification**	**Test**	**Specification**
Purity	70-100%	**IL-12**	>100 pg/ml
Viability	70-100%	**alloMLR**	
Phenotype		DC:MNC = 1:5	>30%
CD80	60-100%	DC:MNC = 1:10	>30%
CD86	60-100%	DC:MNC = 1:20	>15%
MHC I	60-100%	Negative control	<10%
MHC II	60-100%	**BACTEC**	NEG
CD83	60-100%	**Mycoplasma**	NEG
CD14	0-40%	**HIV 1/HIV 2**	NEG
IgG control	<1%	**HBV/HCV**	NEG

Description of a stimulatory DC immune medicine enhanced via over-expression of the CD40L molecule.

In order to broaden the immune stimulatory window of the DC that is characterised by the secretion of IL-12 (Fig. 1), DCs were genetically engineered to over-express CD40L. This molecule is normally expressed from activated T-lymphocytes and interacts with CD40 on DCs transmitting a critical activating signal into the DC. This experiment was designed as an example of the transfer of an activating molecule into the DC immune medicine. In principle, however, an identical procedure may be used for other stimulatory molecules or, in order to design a suppressive DC immune medicine, immune suppressive molecules may be over-expressed from a DC. Specifically, the rationale for CD40L gene transfer into DCs was

(i) allowing the DCs to become independent from activating T-lymphocytes to deliver the CD40L signal to the DC;

(ii) it was hypothesised and found in the present

experiments that, because of the continuous presence of CD40L on the DC itself by expression from a constitutively active promoter, the DC was enabled to secrete IL-12 for a much longer time period than when the DCs were subjected to a conventional maturation stimulus such as LPS/IFN-γ;

(iii) the total amount of IL-12 secreted from DCs was considerably higher compared to the LPS/IFN-γ or the CD40L/IFN-γ stimulus alone, and the kinetics of IL-12 secretion had been qualitatively different starting sooner after the stimulus was applied, thus broadening the immune stimulatory window of DC differentiation;

(iv) even 48 hours after exposure to LPS/IFN-γ, when the DCs had exhausted their capacity to secret IL-12, CD40L gene transfer enabled DCs to start a second phase of IL-12 secretion (Fig. 3).

Fig. 3 shows a CD40L gene transfer. In panel A the expression of GFP or CD40L after lentiviral gene transfer in 6 hours smDCs and 48 hours mDCs is shown. All measurements shown here were done 48 hours after exposure of pre-matured DCs to the lentiviral vector. Expression of CD40L from DCs caused enhanced secretion of IL-12 compared to iDCs, DCs exposed to LPS/IFN-γ alone, or GFP engineered 6 hours smDCs and 48 hours mDCs (panel B). The enhanced IL-12 release upon GFP gene transfer is probably caused by the viral double-stranded RNA that signals via TLRs (see table 1) that are expressed in 6 hour-smDC but not any more in 48 hour-LPS/IFN-γ mDCs. The expression profile of functionally important DC membrane molecules (panel C) was un-altered by lentiviral gene transfer into DCs (black histograms, immature DCs; white histograms, mDCs).

The secretion of the cytokines IL-12 and IL-10 was qualitatively and quantitatively different in DCs that were exposed to LPS/IFN-γ, CD40L/IFN-γ, or a combination of both (figure 4). The secretion of IL-12 was almost twice as high when the combination stimulus was applied, compared to the CD40L/IFN-γ stimulus alone, and also considerably higher compared to the LPS/IFN-γ stimulus alone. In addition, IL-12 secretion from DCs exposed to the LPS/IFN-γ/CD40L combined stimulus was already clearly detectable at considerable

amounts after 12 hours, whereas LPS/IFN-γ and CD40L/IFN-γ triggered biologically relevant levels of IL-12 secretion only between 12 and 24 hours after exposure to the initial maturation signal. This observation is in line with the goal of the present invention of broadening the immune stimulatory window of DC differentiation in order to improve the stimulatory capacity of a DC immune medicine. The maximum expression of IL-10 was similar when DCs were exposed to LPS/IFN-γ/CD40L or CD40L/IFN-γ alone, but were lower when only LPS/IFN-γ was used for DC maturation. However, the immune suppressive cytokine IL-10 was already detectable after 12 hours at biologically relevant levels after CD40L/IFN-γ signalling, whereas the combination stimulus LPS/IFN-γ/CD40L showed kinetics similar to those of only LPS/IFN-γ matured DCs. Early release of IL-10 as after CD40L/IFN-γ stimulation negatively interferes with the immune stimulatory window of DC differentiation and should, in the case of designing an immune stimulatory DC medicine, be avoided. It is concluded that the net effect in the balance between immune stimulatory and immune suppressive capacity of the combination stimulus LPS/IFN-γ/CD40L, considering the secretion pattern of IL-12 and IL-10, is clearly towards improved immune stimulatory capacity compared to applying LPS/IFN-γ or CD40L/IFN-γ alone. As opposed to earlier publications the DCs used here will receive a combination of maturation stimuli. Physiologically, a phase in which the DC can activate T-lymphocytes (characterised by IL-12 secretion) and a second phase, in which the DC will suppress the activity of T-lymphocytes (characterised by IL-10 secretion and tryptophan depletion by the activity of the enzyme IDO), will be triggered by contacting iDCs with an adequate maturation stimulus, such as LPS/IFN-γ (figure 1). Here it is demonstrated that the initial exposure to LPS/IFN-γ (or another TLR agonist in the presence of IFN-γ), followed by genetic engineering of the DCs to over-express molecules, such as CD40L maintains a DC phenotype capable of T-lymphocyte activation and prevents the DC from assuming the suppressive phenotype (figure 4). The secretion of IL-12 is maintained for longer than the physiologic time window of 20-24 hours when the genetic engineering is done 6

hours or 48 hours after the initial maturation via the TLR signalling pathway in the presence of IFN-γ.

Fig. 4 shows the quantity and quality of IL-12 and IL-10 secretion. DCs were exposed to the indicated maturation stimuli in the presence of IFN-γ. The concentrations of IL-12 and IL-10 in the culture supernatant were measured at the indicated time points.

Of particular importance in the present design of a genetically engineered T-lymphocyte stimulatory DC immune medicine is that IL-12-secreting DCs have the capacity, via type 1 polarisation of an immune response, to trigger cytolytic immunity. Thus, the potential of T-lymphocytes exposed to CD40L transgenic DCs to trigger cytolytic immune responses by analysing the content of granzyme B in CD8 positive CTLs was further investigated (Fig. 5). Indeed, it was found that co-cultivation of CTLs with CD40L transgenic DCs resulted in clearly enhanced expression of granzyme B compared to control GFP transgenic DCs or un-transduced mDCs. This is a strong indicator of the improved cytolytic potential of such CTLs and thus provides evidence that CD40L expression from a DC immune medicine has improved immune stimulatory capacity.

Fig. 5 shows the potential for cytolytic activity. The total percentage of CTLs was only slightly increased when PBMCs were co-cultivated with CD40L transgenic DCs (left panel, squares) compared to GFP transgenic DCs (diamonds) and un-transduced mDCs (triangles). When analysing the granzyme B expression in CTLs co-cultivated with CD40L transgenic DCs, a clear increase was found (right panel, squares) compared to GFP transgenic DCs (diamonds) and un-transduced mDCs (triangles).

Description of a DC immune medicine with improved stimulatory capacity for T-lymphocytes by engineering for knocking down the expression of the immune suppressive cytokine IL-10

Based on the hypothesis that a DC immune medicine in which the expression of molecules mediating immune suppression is knocked down, experiments were devised to block IL-10 gene expression in DCs by RNA interference using a pool of 4

target-specific siRNAs (Fig. 6). This resulted in very consistent and reproducible knock down of IL-10 expression in LPS/IFN-γ activated DCs, leading to a higher IL-12 secretion compared to control silenced mDCs. This observation hints at an autocrine pathway based on IL-10 secreted from a DC binding to IL-10 receptors on the same DC resulting in down modulated IL-12 production. Other than that, no immune phenotypic differences between genetically engineered and normal DCs, as assessed by CD80, CD86, MHC class I, and II expression, were found. Most importantly, in an alloMLR, a considerably greater potency of a DC immune medicine engineered for suppression of IL-10 secretion to activate T-lymphocytes compared to control experiments was observed.

Additionally, the percentage of CD25+FoxP3+ cells in the CD4+ T-cell population, supposedly a population of regulatory T-cells (Tregs) that suppresses immune responses, was reduced, probably due to the IL-10 silencing in LPS/IFN-γ activated DCs.

Fig. 6 shows the immune stimulatory capacity of LPS/IFN-γ-activated DCs, blocked for IL-10 expression by genetic engineering. Twelve hours before the activation with LPS/IFN-γ, DCs were transfected with a pool of four IL-10 specific siRNAs or an unspecific control siRNA. Isolated allogeneic CD3+ T-cells were then stimulated with 6 hour-LPS-matured DCs (mDCs) either IL-10 (black bars) or control-silenced (white bars) in a 1:3 = DC:T-cell ratio. CD4+, CD8+, (panel A) and CD4+CD25+FoxP3+ T-cells (panel B) were analysed on day 6 of co-cultivation using the Trucount system and a FACS LSRII flow cytometer. The immune phenotype as well as IL-10 and IL-12 secretion were measured 48 hours after LPS/IFN-γ activation by flow cytometry and ELISA, respectively (panel C). The immune phenotypic analysis compares LPS/IFN-γ-activated DCs (white histogram) with iDCs (black histogram) in IL-10-silenced DCs (panel D) or control-silenced DCs (panel E).

Description of a T-lymphocyte stimulatory DC immune medicine engineered for knocking down the expression of the immune suppressive enzyme IDO

siRNA was used to knock down the expression of the

known immune suppressive effector molecule IDO (figure 7). In order to optimise the transfection of siRNA and the efficiency of IDO knock down, first HeLa cells, activated with IFN-γ, were used. Subsequently, DCs were transfected under optimised conditions. In both, HeLa cells and DCs, the expression of IDO as demonstrated in Western blot experiments could be silenced. IDO silenced DCs, DCs transfected with a scrambled control siRNA, and iDCs were used as stimulators in an alloMLR potency assay. It was observed that the stimulatory potency of IDO silenced DCs was considerably greater compared to DCs transfected with scrambled siRNA or iDCs. This held true for CD8+ CTLs as well as CD4+ Th-cells.

Fig. 7 shows the immune stimulatory capacity of LPS/IFN-γ-activated DCs with silenced IDO expression. First, efficient IDO knockdown in HeLa cells (panel A) as well as DCs (panel B) was demonstrated using Western blotting experiments. In order to investigate the stimulatory potency of IDO silenced DCs on CD8+ CTLs (panel C) and CD4+ Th lymphocytes (panel D), PBMCs were co-cultivated with IDO-silenced DCs (squares), control silenced DCs (diamonds, scra = sequence scrambled), or iDCs (triangles). In all cases the stimulatory capacity of IDO-silenced DCs was superior over the controls.

Immune suppressive molecules

Whole genome DNA micro arrays were used to generate expression profiles of DCs exposed to the maturation stimulus LPS/IFN-γ, to CD40L/IFN-γ signalling, or to a combination of LPS/IFN-γ/CD40L signalling, as well as the appropriate controls (Fig. 8).

Fig. 8 shows DC expression profiling. DCs were exposed to the indicated maturation stimuli or were left immature. RNA was extracted at the indicated time points and subjected to expression profiling using whole genome DNA micro arrays. The results of the expression profiling was analysed using CarmaWeb (Comprehensive R based Microarray Analysis, Bioinformatics Graz and the Tyrolean Cancer Research Institute, Austria). All data were grouped into 20 clusters that used the basic algorithm of the CarmaWeb software platform and identified the clusters that contained IDO and IL-10. The genes in these clusters have an expression profile

similar to that of the two known immune suppressive DC molecules, which led to the conclusion that they have a function in the immune regulation of a DC that is also immune suppressive (tables 3 and 4).

Table 3: IDO expression cluster. According to the CarmaWeb algorithm, the genes listed have an expression profile that resembles that of IDO (gene name INDO) suggesting a function similar to that of IDO (numbers are log with base 2 relative to immature DCs).

Unique ID	*Name*	*6 hours LPS IFN-γ vs. 6 hours iDC M*	*12 hours LPS CD40L IFN-γ vs. 12 hours iDC M*	*24 hours LPS CD40L IFN-γ vs. 24 hours iDC M*	*48 hours LPS CD40L IFN-γ vs. 48 hours iDC M*
210118_s_at	IL1A	12,5712385	12,656363	11,625489	9,828246
1405_i_at	CCL5	9,775104	10,949544	12,230392	11,143438
1552995_at	IL27	10,449822	10,585213	10,862312	8,519849
1554997_a_at	PTGS2	11,7640085	9,820589	11,614364	11,624819
1555759_a_at	CCL5	12,916491	12,965171	12,63708	12,723415
1556378_a_at	LOC401530	5,897853	10,2600975	12,640888	13,004906
1570388_a_at	LOC401530	3,8191023	9,072843	11,445329	11,711242
202269_x_at	GBP1	8,517265	8,2832775	8,787927	8,968534
202411_at	IFI27	9,403024	9,560578	9,054876	8,233445
203828_s_at	IL32	9,310799	8,968001	10,09564	9,038413
203915_at	CXCL9	13,417887	13,097824	12,633152	11,7289915
204439_at	IFI44L	11,743961	9,8876705	7,0858197	6,4474745
204470_at	CXCL1	10,931133	8,087279	8,933327	5,9426484
204533_at	CXCL10	9,392692	10,44239	10,786983	11,601183
204655_at	CCL5	9,542912	11,558153	11,215681	11,169104
204698_at	ISG20	12,59375	10,52378	9,431844	8,965937
204748_at	PTGS2	12,489249	7,144587	7,9113717	8,436964
205013_s_at	ADORA2A	10,245063	8,496423	10,054197	9,867236
205067_at	IL1B	12,89035	7,7892747	11,259029	11,700619

205207_at	IL6	7,463433	7,6129217	8,461436	8,870339
205476_at	CCL20	12,470199	12,587071	13,649405	12,875935
205569_at	LAMP3	6,9127846	9,671546	9,207934	10,519818
205599_at	TRAF1	8,448494	9,332896	9,679705	11,050019
205680_at	MMP10	6,9289026	9,5307	11,131636	11,908984
205681_at	BCL2A1	12,63274	12,276044	12,321126	12,00514
205692_s_at	CD38	11,776373	10,918448	8,890723	6,572375
205890_s_at	UBD	11,738066	11,718061	12,080868	11,735866
206025_s_at	TNFAIP6	12,46147	9,442592	10,791083	10,010692
206026_s_at	TNFAIP6	8,55364	8,492605	8,934455	8,366126
206337_at	CCR7	8,625213	10,63397	11,733897	11,844164
206341_at	IL2RA	7,962748	9,035077	10,360973	8,479506
206765_at	KCNJ2	4,7675686	9,681816	10,2331705	9,70106
206881_s_at	LILRA3	9,8311825	9,066225	9,314402	5,396609
207113_s_at	TNF	12,704884	13,268166	11,278352	8,395802
207160_at	IL12A	9,43968	13,149242	13,380418	8,626079
207176_s_at	CD80	8,31917	7,76368	8,680011	8,573913
207375_s_at	IL15RA	9,771918	10,090428	10,584916	10,606205
207536_s_at	TNFRSF9	6,231527	7,7779512	9,701627	9,752483
207901_at	IL12B	12,714132	13,101075	13,353125	7,458026
209813_x_at	TRGV9	3,9887655	10,498012	10,522085	8,133455
210029_at	***INDO***	***12,789924***	***10,134047***	***12,052269***	***12,43933***
210072_at	CCL19	7,2848625	10,570788	12,461668	11,943225
210163_at	CXCL11	13,109848	12,912714	11,681597	10,053444
210511_s_at	INHBA	12,3267975	12,957313	10,577578	10,25125
211122_s_at	CXCL11	13,135339	13,034375	12,199861	9,8954315
211269_s_at	IL2RA	7,485799	9,023119	10,643187	10,435802
213497_at	ABTB2	6,1966333	8,42709	9,347927	9,879564
215806_x_at	TRGC2	4,249278	10,289696	10,347067	7,9237046
217546_at	MT1M	6,80711	8,103879	9,402974	8,6524935
219159_s_at	SLAMF7	12,195147	10,547217	8,994919	6,4161515
219424_at	EBI3	10,374818	11,288197	12,070472	10,607756
220054_at	IL23A	6,8979635	12,20336	13,634168	11,491903
222838_at	SLAMF7	12,751745	12,511322	10,977879	8,82794
226560_at	SGPP2	10,281151	9,231938	9,82061	8,582578
227140_at	INHBA	12,689469	10,28581	10,5587015	10,436948
227180_at	ELOVL7	8,1362	8,21396	9,105779	9,419119
229437_at	BIC	9,607157	11,00861	11,168569	11,648725
229625_at	GBP5	11,195785	11,012673	7,085479	8,598587

231577_s_at	GBP1	7,464914	8,356869	8,556055	8,40867
235229_at		8,6328745	8,228744	8,894646	8,620824
238439_at	ANKRD22	7,496807	7,9435267	8,688104	10,303036
238581_at	GBP5	9,960986	9,9203	7,430702	7,571562
238725_at		5,625349	7,5093164	9,704128	10,179434
240287_at	LOC341720	10,947632	11,357612	9,703499	4,2557507
242814_at	SERPINB9	9,147152	9,357069	9,150615	9,138746
33304_at	ISG20	11,309338	7,729137	8,4003	6,2511134
39402_at	IL1B	12,057478	6,510902	10,422022	11,546757
228439_at	MGC20410	6,443625	4,5576925	5,1437	2,8559468
232078_at	PVRL2	6,135475	4,746409	3,8879604	4,522022
1561908_a_at	HS3ST3B1	7,0928926	4,458722	3,670319	3,8414023
204141_at	TUBB2	5,3551335	4,7444496	4,0537806	5,1056914
207275_s_at	ACSL1	5,8632264	5,869882	4,1483216	4,095168
210563_x_at	CFLAR	5,100706	5,0670333	5,419278	4,1996965
210564_x_at	CFLAR	5,380331	4,9984617	5,3778234	4,160804
218400_at	OAS3	6,8762517	6,1737237	4,410526	2,8219512
220132_s_at	CLEC2D	4,4514236	6,669927	5,78131	1,7744006
222303_at	ETS2	4,60927	5,654717	5,4186363	3,4308143
229221_at	CD44	7,1867433	5,771355	3,1319969	4,7465234
230499_at	BIRC3	4,71687	4,9918036	5,3390183	5,366412
232682_at	DSU	4,6348085	5,7252564	5,5562453	3,688244
243296_at	PBEF1	6,237873	4,170852	4,781295	5,1768007
243894_at	SLC41A2	7,1203766	4,7231464	4,238267	4,4368286
1554539_a_at	RHOF	5,002441	4,1752563	5,4232445	5,0010266
1563357_at	SERPINB9	6,368476	5,3010783	3,9647322	5,4146433
202509_s_at	TNFAIP2	5,195548	5,9835477	4,9414105	3,823941
203287_at	LAD1	4,5966916	6,1679506	5,8620677	3,8349202
204715_at	PANX1	6,0651593	4,6488233	5,01669	4,461601
204794_at	DUSP2	5,4158106	3,7610755	4,858317	6,9610953
207389_at	GP1BA	6,946879	6,472854	5,3776803	2,2710993
209039_x_at	EHD1	5,992218	4,9087305	4,788946	5,520831
209928_s_at	MSC	4,9872894	5,956739	4,365724	5,714541
215078_at	SOD2	6,952513	7,3351035	3,1302252	3,7524989
216336_x_at	MT1M	4,864737	4,9637637	6,200034	5,143623
219716_at	APOL6	7,687471	5,4070616	4,6342998	3,8593137
221779_at	MICAL-L1	5,128767	5,6324835	6,08239	4,3245826

226189_at		7,0649176	5,7119026	5,3064666	3,6578546
227014_at	LOC57168	7,947885	4,03786	4,678014	4,4947524
232304_at	PELI1	6,7769756	5,062101	4,4606757	5,45498
234985_at	LOC143458	5,5271115	4,7101035	5,720184	4,8197145
242649_x_at	C15orf21	6,9237723	5,3964243	4,532885	4,963293
1559391_s_at	B4GALT5	4,598228	7,0806437	5,126474	4,786352
200629_at	WARS	6,951068	5,0352116	4,546803	4,863137
202688_at	TNFSF10	6,8965373	5,708206	4,570576	5,1128407
202748_at	GBP2	5,793167	6,4275317	4,901077	4,8227687
203685_at	BCL2	7,285017	5,9438853	4,1533656	4,8756795
204015_s_at	DUSP4	8,879749	6,673204	3,3034465	4,2480526
204926_at	INHBA	5,6395097	7,8953505	4,531301	3,1433787
206157_at	PTX3	7,3603053	5,5506916	5,1639824	4,220827
209803_s_at	PHLDA2	5,2155585	4,638463	6,690672	5,015275
209939_x_at	CFLAR	5,3425546	5,3650227	6,3261905	4,451903
211302_s_at	PDE4B	9,388175	4,2461367	4,496878	4,7590027
215671_at	PDE4B	6,8900924	4,9770446	4,6578245	5,777932
216705_s_at	ADA	6,1992345	4,8220434	5,4637165	5,23145
218943_s_at	DDX58	7,6221895	6,2795057	4,79867	4,15005
219014_at	PLAC8	4,8591843	4,901381	8,194876	3,2458937
221087_s_at	APOL3	6,84244	5,7354436	4,5675263	5,530723
221185_s_at	IQCG	6,629468	3,8091857	6,4129505	5,9112988
222812_s_at	RHOF	5,2598224	5,562695	5,265753	5,8453097
239876_at	NFKB1	6,668459	5,936471	4,785418	5,0308824
240013_at		5,927128	5,32223	5,1699376	5,818874
242234_at	BIRC4BP	5,92746	5,946681	4,6361747	6,306454
35150_at	CD40	5,5139947	5,5919642	6,1309977	5,227399
1553713_a_at	RHEBL1	5,2900615	5,984809	5,9541264	5,9824634
1570253_a_at	RHEBL1	5,2768035	6,012619	5,687341	5,871174
202687_s_at	TNFSF10	7,8631916	5,812222	4,413325	5,5924144
204415_at	G1P3	4,8981137	6,400349	6,9897156	3,8297942
205483_s_at	G1P2	6,6436443	6,099025	5,6568613	4,5633087
206975_at	LTA	7,6997614	8,081627	5,2525034	2,2437675
214228_x_at	TNFRSF4	8,910944	6,6099143	3,4565573	5,0546947
215346_at	CD40	5,0572295	5,593753	6,163759	5,6992526
219211_at	USP18	8,285303	7,0497055	5,0085244	3,340998

223887_at	GPR132	6,7607636	4,543277	5,509192	7,0652533
226702_at	LOC129607	7,0691476	5,9127674	5,569863	5,045494
227816_at	LOC400572	5,9134483	6,345112	6,439589	5,0362144
231578_at	GBP1	7,4657335	6,917391	3,8039267	6,1784716
232213_at	PELI1	7,4485292	5,587166	5,212285	6,0972896
200628_s_at	WARS	9,368469	5,516358	4,64247	5,419954
202800_at	SLC1A3	6,087409	6,9154663	7,895211	3,3894732
204014_at	DUSP4	8,585252	6,8769712	4,5652914	4,590569
204070_at	RARRES3	5,2620735	6,591536	6,2708507	5,70036
204747_at	IFIT3	8,602514	7,492393	5,6343427	3,3676317
212458_at	SPRED2	5,5534644	7,490529	6,08654	4,736595
212641_at	HIVEP2	6,433779	5,7410865	6,5065494	6,1581783
231779_at	IRAK2	8,596796	4,4581327	5,576258	6,910897
1559777_at		5,605326	8,543147	6,0953383	4,9331293
200986_at	SERPING1	9,492846	8,300841	4,229471	3,6452117
203708_at	PDE4B	11,223041	4,007264	4,071895	4,2633905
204286_s_at	PMAIP1	9,400938	5,3456664	4,7092376	6,7266498
205153_s_at	CD40	6,071054	6,5306187	6,4112535	6,4513636
222934_s_at	CLEC4E	8,080958	8,404435	6,981838	2,577743
224225_s_at	ETV7	7,935058	6,064494	6,699362	5,50029
226474_at	NOD27	5,9191046	7,2645817	5,1934004	7,0305643
227262_at	HAPLN3	7,1910353	6,615262	5,1981425	7,5915074
230127_at		7,3191285	7,9864817	5,3266	6,0064306
244780_at	SGPP2	8,127058	5,944443	6,879924	5,023631
1569095_at		7,318123	8,3700075	6,2253203	4,625397
201601_x_at	IFITM1	7,6313257	7,573814	6,0775313	4,9257803
202643_s_at	TNFAIP3	6,250073	5,1900063	6,5136204	7,95266
202760_s_at	PALM2-AKAP2	9,142822	7,253937	5,7028017	5,2498336
204285_s_at	PMAIP1	10,275632	5,124492	4,698287	7,937848
204363_at	F3	5,9613805	7,1758485	7,078458	5,787101
208747_s_at	C1S	6,224019	6,8833885	6,2983246	6,745276
209723_at	SERPINB9	10,339775	5,989251	4,825143	6,5091825
214329_x_at	TNFSF10	6,1703367	6,8099036	6,2552257	7,0860806
216598_s_at	CCL2	7,959111	5,9420986	7,541988	5,4770517
218656_s_at	LHFP	5,799256	5,6268	7,0819764	6,887301
221680_s_at	ETV7	7,4525156	6,8642745	6,899107	6,077394
227677_at	JAK3	5,4577355	5,5538926	6,847657	6,6245513
229450_at	IFIT3	7,4833083	7,133669	7,3032265	5,366725
235574_at	GBP4	8,758058	7,3968883	5,5135446	6,7058306

1554519_at	CD80	7,0503464	5,5513606	6,9412417	7,924613
1555689_at	CD80	8,324632	7,511187	6,2935348	6,2964225
204224_s_at	GCH1	7,3962016	6,945448	5,8722777	7,290103
205114_s_at	CCL3	8,18418	6,780873	7,019624	5,952113
206508_at	TNFSF7	6,858301	7,575267	8,330129	4,665465
209722_s_at	SERPINB9	8,607762	7,3330297	5,989785	6,4565625
213524_s_at	G0S2	7,547209	7,6664352	6,440924	6,2842937
222292_at	CD40	8,530226	8,246122	6,060198	6,302727
223798_at	SLC41A2	9,232196	8,087529	3,90797	7,397384
242907_at	GBP2	7,746566	8,2072735	5,8188763	6,584134
1555465_at	MCOLN2	9,518959	8,71965	6,8508315	4,539327
200953_s_at	CCND2	7,411466	6,296918	7,8509226	6,8842726
201860_s_at	PLAT	8,657469	7,604994	6,370206	6,5896416
208303_s_at	CRLF2	8,685696	8,653402	8,276672	3,62456
238727_at	LOC440934	7,688413	7,8426127	7,593639	5,8430877
239186_at	MGC39372	7,809087	6,8687167	7,7233596	6,9605627
222221_x_at	EHD1	7,799284	6,630239	7,478602	7,7974253
222326_at	PDE4B	8,487402	7,4390974	5,838442	8,485796
223767_at	GPR84	9,556347	9,886792	8,25461	2,6693628
214022_s_at	IFITM1	8,305978	9,906662	6,8351192	5,3545113
221241_s_at	BCL2L14	7,763052	8,927696	7,4124217	6,0943
209037_s_at	EHD1	6,202972	6,62678	6,968862	8,198914
222802_at		9,193985	9,879644	6,7598433	5,578077
234306_s_at	SLAMF7	11,054989	9,386422	7,5571413	4,0329967
219584_at	PLA1A	7,8830075	8,5753975	7,899431	6,5355268
232593_at	LINCR	7,426831	7,1173863	7,508543	8,483282
235175_at	GBP4	8,098684	8,014557	6,7604356	8,305143
238567_at	SGPP2	9,60297	7,9624267	8,190959	5,9755754
202270_at	GBP1	7,857806	7,5784187	7,864753	7,7254214
206058_at	SLC6A12	7,0730734	7,6346216	7,868434	7,8353267
209270_at	LAMB3	6,3819847	7,637574	8,068874	8,396816
223217_s_at	NFKBIZ	6,579339	5,8985357	7,933263	9,0830765
230110_at	MCOLN2	7,0183334	8,743037	8,322358	6,695663
235116_at	TRAF1	7,515788	6,6126266	7,946375	8,608736
239196_at	ANKRD22	6,893366	7,2058015	7,997893	8,679558
1557359_at	LOC285758	5,7492123	6,7752757	6,428899	8,264215
202833_s_at	SERPINA1	6,047883	6,2670393	8,372422	8,15069
220655_at	TNIP3	4,227455	9,260285	8,14861	3,8333952
210354_at	IFNG	7,0490704	9,9158745	9,995628	4,7299943

239331_at		4,9562793	7,907742	6,738407	7,5105863

Table 4: IL-10 expression cluster. According to the CarmaWeb algorithm, the genes listed have an expression profile that resembles that of IL-10 (gene name IL10) suggesting a function similar to that of IL-10 (numbers are log with base 2 relative to immature DCs).

Unique ID	Name	6 hours LPS IFN-γ vs. 6 hours iDC M	12 hours LPS CD40L IFN-γ vs. 12 hours iDC M	24 hours LPS CD40L IFN-γ vs. 24 hours iDC M	48 hours LPS CD40L IFN-γ vs. 48 hours iDC M
1556300_s_at		0	4,323714	1,5688843	0,257391
1556378_a_at		0	3,3170495	-0,124789566	1,3912028
1556883_a_at	LOC401530	0	4,490707	-0,14703345	2,7003522
202291_s_at	LOC401528	0	5,571205	2,098167	0,038769286
202878_s_at	MGP	0	3,306639	0,3353752	1,2330873
204475_at	C1QR1	0	5,03655	0,2590128	0,17097393
204614_at	MMP1	0	3,4892087	2,2649622	0,08403955
205676_at	SERPINB2	0	3,6301327	0,1309929	0,7396309
223287_s_at	CYP27B1	0	2,983712	1,7371364	0,5753527
224773_at	FOXP1	0	3,3603613	0,35192382	0,9197203
227812_at	NAV1	0	3,3981404	1,6189107	0,044692483
235042_at	TNFRSF19	0	3,450752	0,328667	1,5720363
235444_at	CMYA1	0	3,0461197	-0,042442646	1,3964777
241860_at	FOXP1	0	2,671234	0,61670065	1,4493694
1556582_at	STK17B	0	2,6907892	1,2484756	1,1807224
1564028_s_at	LOC440536	0	2,750025	0,67192906	1,4337206
1566480_x_at	FLJ40722	0	2,810023	1,4379538	0,6065171
1570388_a_at	FLJ31795	0	3,911918	0,027789168	1,8137968
202877_s_at	LOC401530	0	3,3630056	0,6407456	1,4440103
	C1QR1				

204602_at	DKK1	0	3,921798	3,6861098	0,3208148
204932_at	TNFRSF11B	0	4,2726865	3,2933848	0,21878207
215268_at	MACF1	0	2,6788146	1,2724367	0,8143623
216497_at	LOC120364	0	2,3947499	1,1637448	0,9242658
216867_s_at	PDGFA	0	2,1128201	1,2416315	1,132686
220655_at	TNIP3	0	3,474797	0,8904967	1,8436728
221870_at	EHD2	0	2,571253	0,94932306	1,2732899
224771_at	NAV1	0	3,8266947	1,5089604	1,4963266
238712_at		0	4,2949014	1,8307322	2,142599
239311_at	DHX57	0	2,27365	1,3914022	1,0178465
1556318_s_a					
t	CAND1	0	2,3933206	0,9396068	1,3715496
227732_at	ATXN7L1	0	3,4273734	0,6846624	2,2728565
239060_at	EHD1	0	1,997289	0,32936123	1,8916218
206176_at	BMP6	0	2,2903044	-0,18439728	2,1745071
207386_at	CYP7B1	0	3,9868062	0,7309004	2,746021
210229_s_at	CSF2	0	3,1023788	-0,1405712	2,5077276
				-	
215750_at	KIAA1659	0	3,0428922	0,006880157	2,480364
225025_at	IGSF8	0	1,905534	0,49440768	1,845558
227345_at	TNFRSF10D	0	2,1187048	0,36650723	2,0576801
236738_at	LOC401097	0	2,4518428	1,685922	1,7134967
242517_at	GPR54	0	1,9395804	0,21328141	2,186202
228910_at	CD82	0	3,5779603	1,2853656	2,5263379
229307_at	ANKRD28	0	3,1102884	0,8216141	2,5533133
231832_at	GALNT4	0	2,2378843	0,72167736	2,091024
37005_at	NBL1	0	2,158463	1,2050192	2,0193584
227410_at	FAM43A	0	3,1527455	1,5260311	2,2625198
228625_at	CITED4	0	2,2035446	0,8985138	2,1428196
240432_x_at		0	2,0101619	1,3507649	2,0001297
203074_at	ANXA8	0	2,754555	0,79692614	2,6273892
206009_at	ITGA9	0	1,9594706	0,54135984	2,3522627
235438_at		0	4,0041165	0,70460325	3,542835
1560869_a_a					
t		0	2,5460286	1,5980372	2,5416172
223525_at	DLL4	0	3,5447857	0,5724994	3,72409
232090_at	DNM3	0	4,302288	3,442975	2,8309567
203904_x_at	CD82	0	2,0282779	1,3777583	2,4270318
225645_at	EHF	0	4,428559	2,14202	3,7522347

235737_at	TSLP	0	4,959613	1,5306113	4,4908447
202237_at	NNMT	0	3,6071026	3,2686546	2,8587787
207433_at	***IL10***	***0***	***4,00954***	***1,7226876***	***3,7454016***
214414_x_at	HBA1	0	2,1132698	1,575519	2,6071193
224940_s_at	PAPPA	0	2,786031	2,7599206	0,7074637
212730_at	DMN	0	3,6943512	0,91267496	4,2896175
219874_at	SLC12A8	0	2,8926702	3,182794	1,9976637
224646_x_at	H19	0	2,466048	2,7163086	2,6425989
209324_s_at	RGS16	0	3,3799229	2,467596	3,7553544
243788_at	PHF11	0	2,1383092	2,2994013	2,1082926
202238_s_at	NNMT	0	4,160124	4,563192	3,5705357
224997_x_at	H19	0	2,8080769	3,1897178	3,0020173
236176_at		0	2,051894	0,7956073	3,2491539
44790_s_at	C13orf18	0	2,1295624	2,4644802	2,7274456
206825_at	OXTR	0	2,7509916	0,5947344	4,1146626
207442_at	CSF3	0	2,2279809	1,6218964	3,2061028
216575_at		0	2,1809235	1,0562297	3,4115193
237559_at	GPR55	0	2,0120387	1,6486369	3,2241027
				-	
200951_s_at	CCND2	0	0,061742224	2,055891	5,531777
204163_at	EMILIN1	0	-0,08650574	2,116541	3,2442234
215646_s_at	CSPG2	0	1,0869541	0,9552003	4,6304297
220442_at	GALNT4	0	1,6785349	2,2396698	2,8896742
223194_s_at	C6orf85	0	1,4545181	1,8726805	3,2625294
				-	
227703_s_at	SYTL4	0	0,012060306	2,8051267	3,7273147
				-	
1552393_at	FLJ25421	0	0,008524944	0,79469424	4,0263605
1552394_a_a t	FLJ25421	0	0,4518348	1,1220381	3,568555
1553785_at	RASGEF1B	0	0,8244259	3,0841393	2,744691
1554079_at	GALNTL4	0	0,80384594	2,1117246	3,2543478
1559777_at		0	1,373273	1,964059	4,745006
1562433_at	FLJ10489	0	0,26058874	3,4475367	4,6152167
1568949_at	PITPNC1	0	0,004084121	2,9905202	3,3454092
1569095_at		0	1,0567621	1,0456768	3,5029418
200783_s_at	STMN1	0	0,2741603	1,4904935	3,2696452
202403_s_at	COL1A2	0	0,83871436	2,151641	3,4803479
202431_s_at	MYC	0	1,0226223	2,5720317	3,490832

202998_s_at	LOXL2	0	0,4333325	1,7000065	3,451721
203108_at	GPRC5A	0	1,6255034	1,8504707	4,352528
203131_at	PDGFRA	0	-0,45227933	1,4600621	3,411754
203592_s_at	FSTL3	0	0,14812845	2,2247574	3,3438997
203980_at	FABP4	0	-0,009128572	1,3565757	4,341141
204301_at	KIAA0711	0	-0,015199099	1,469634	3,7521276
204411_at	KIF21B	0	0,99417245	2,3202088	3,2713737
204619_s_at	CSPG2	0	1,7562126	1,4990444	4,1206384
204620_s_at	CSPG2	0	1,6183093	1,011578	4,371935
204879_at	PDPN	0	-0,15425473	0,95934194	3,9027925
204904_at	GJA4	0	0,30981743	2,2777586	3,7517166
205100_at	GFPT2	0	1,5140616	1,8844032	5,630774
205289_at	BMP2	0	0,60859656	1,5663037	3,305952
205290_s_at	BMP2	0	0,07147631	1,7073456	4,3988748
205826_at	MYOM2	0	-0,004983342	3,2177415	6,163263
205861_at	SPIB	0	0,7059429	2,4294124	2,704671
205898_at	CX3CR1	0	-0,010416569	2,8846204	5,349967
206027_at	S100A3	0	0,13657278	1,5692319	3,4727564
206090_s_at	DISC1	0	-0,8741116	1,8682606	5,05058
206729_at	TNFRSF8	0	0,27889317	2,6180744	5,8870077
206741_at	LOC51066	0	0,47887027	2,5547576	5,7319098
206859_s_at	PAEP	0	-0,001321607	1,3401538	3,7394311
207510_at	BDKRB1	0	2,0661907	3,4934416	3,781754
209325_s_at	RGS16	0	1,5113075	2,5088499	3,8291175
210095_s_at	IGFBP3	0	0,21584912	2,5991304	3,2872534
211372_s_at	IL1R2	0	-0,6165594	1,6310264	3,7544057
211571_s_at	CSPG2	0	0,91350543	0,87646186	5,6404405
211596_s_at	LRIG1	0	3,8467457	2,826491	5,69805
211597_s_at	HOP	0	0,1277425	1,6993432	6,786917
212143_s_at	IGFBP3	0	-0,19099335	3,7475343	4,0994177
212444_at		0	1,1151347	1,3201189	3,6738355
213139_at	SNAI2	0	0,001255514	3,9536047	5,2359695
215495_s_at	SAMD4	0	-0,6807276	2,1140902	4,5931683
218574_s_at	LMCD1	0	0,004399216	0,005991654	4,832267

218975_at	COL5A3	0	1,8476033	3,2505863	4,3477015
219168_s_at	PRR5	0	0,3802334	1,883373	5,4903607
219181_at	LIPG	0	0,034028087	1,7650458	4,60227
221731_x_at	CSPG2	0	1,1740024	1,1199198	4,3726726
224950_at	PTGFRN	0	0,2711382	2,548231	4,0687737
225571_at	LIFR	0	1,2333707	2,1292431	2,7829444
226621_at	FGG	0	0,91611135	2,2092934	3,1336222
227256_at	USP31	0	0,66912067	1,812497	3,3547237
228245_s_at	OVOS2	0	-0,6036949	1,9032807	5,509818
228367_at	ALPK2	0	1,9285325	1,4045739	3,7516391
228854_at		0	0,013371324	1,4183841	4,6363435
229247_at	FLJ37440	0	9,65E-04	0,14736369	5,156355
229622_at	FLJ43374	0	0,15105823	2,228979	3,3192601
230233_at	RASGEF1B	0	0,44206667	2,0026255	4,1667137
231496_at	FCAMR	0	0,0146722	3,7155752	2,9080575
231867_at	ODZ2	0	0,002545893	-9,53E-04	4,766165
232739_at	SPIB	0	-0,03394005	2,8278906	2,699333
235100_at		0	1,1068798	2,099443	3,164012
237344_at		0	0,14466353	0,72832346	5,1735163
239808_at	PITPNC1	0	0,035855636	0,69854295	4,18631
240770_at	PRP2	0	0,35803238	1,2584826	3,8403585
242691_at		0	0,042725943	2,1449049	3,0019956
40687_at	GJA4	0	0,001706956	2,8572736	4,300132
41469_at	PI3	0	0,08784416	3,8997574	2,944381
47069_at	PRR5	0	-0,30954257	1,9246503	5,094549
52255_s_at	COL5A3	0	2,9138856	3,5991492	5,075999
223503_at	DKFZP566N034	0	0,001206623	0,010989565	4,3298063

In the present DNA micro arrays, also genes were identified induced in DCs upon LPS/IFN-γ or CD40L/IFN-γ signalling with involvement in the regulation of the genes IL-10, TSLP, INDO, IL2RA, CSF-2 and CSF-3, all of which are known to have an immune suppressive effect. In order to identify potential master switches of immune regulation, a network of regulators for those genes was generated with the Pathway Studio software using Resnet 5 (version 1.2 January, 2007), a

database of mammalian pathways and molecular interactions derived from PubMed and 44 open access journals. By uploading the micro array data from the differentially activated DCs to the regulatory network, potential master regulators induced in maturing DCs (table 5) could then be selected.

Table 5: Master switches of immune regulation.

	Immune regulation	*Affymetrix Index*
STAT6	IL10	201332_s_at
LITAF	IL10	200706_s_at
STAT1	IL10, INDO, IL2RA, CSF3	232375_at
IRF4	IL10, CSF2	204562_at
IRF1	IL10, INDO, IL2RA	202531_at
IRF2	Not known	203275_at
REL	IL10	206035_at
NFKB1	IL10, IL2RA	239876_at
STAT3	IL2RA	235680_at
RELA	IL10, IL2RA	209878_s_at
JUNB	IL10, CSF2	201473_at
CEBPB	IL10, CSF3	212501_at
TBX21	IL10, IL2RA	220684_at
JUN	CSF2	201465_s_at
STAT5B	IL2RA	205026_at
STAT5A	IL2RA, CSF2	203010_at
STAT4	Not known	206118_at
ETV6	CSF2	205585_at
EGR1	IL2RA	227404_s_at
NFATC1	IL10	210162_s_at
CREB1	CSF2	214513_s_at
JAK1	TSLP, CSF3	1552611_a_at
JAK3	IL10, TSLP, CSF2	227677_at
LYN	IL10	202626_s_at
MAPKAPK2	IL10	201460_at
MAP2K1	IL10, IL2RA, CSF2	202670_at
CD274	Not known	223834_at
PDL2	IL10	224399_at

PTGR4	IL10, IL2RA	204896_s_at
ITGAX	IL10	210184_at
ADORA2A	IL10	205013_s_at
SerpinA1	IL10	202833_s_at
SerpinE1	CSF2	202627_s_at
AREG	CSF2	205239_at
OSM	CSF3, CSF2	230170_at
SOCS3	Not known	206359_at
IFNG	INDO, IL2RA	210354_at

Fig. 9 shows examples for improved proliferative responses after knocking down the expression of target molecules in DCs identified in expression profiling using RNA interference. It is shown that the genes exhibiting a DC expression kinetic similar to IL-10, IDO, or belonging to the cluster of master switches of immune regulation are involved in negative regulatory immune suppressive feedback loops. Experiments similar to those in which the expression of IL-10 or IDO using RNA interference was knocked down were designed. DCs were transfected with siRNA specific genes from table 3, 4, or 5. Shown in figure 9 are the genes MAPKAPK2, IRF2, PHF11, IRF4, JAK1, CEBPB, and ETV6. After initiation of maturation by a 6 hours exposure with LPS/IFN-g, the genetically engineered DCs were co-cultivated with allogeneic T-lymphocytes for 6 days. The allogeneic T-lymphocytes were labelled with CFSE, a fluorescent dye that enters cells, binds to proteins, and is retained inside the cell; excess CFSE was washed off. With each cell division the fluorescence intensity of the T-lymphocytes was halved, which allowed the assessment of T-lymphocyte proliferation on day 6 of the co-culture. As controls un-transfected DCs or DCs transfected with control siRNA were used. The improvement of the capacity of genetically engineered DCs to stimulate allogeneic T-lymphocytes provides evidence for the involvement of the siRNA-targeted genes in negative regulatory feedback loops. This furthermore indicates that a DC immune medicine that is genetically engineered for the knock down of such genes will have an improved therapeutic effect compared to conventional immune therapeutics.

Conclusion

Evidence is provided by the present invention that the features of a DC immune medicine may be modulated by genetic engineering. It was demonstrated that over-expression of immune stimulatory molecules in DCs as well as knock down of immune suppressive molecules results in enhanced immune stimulatory capacity. This may find an application as a DC cancer vaccine or an anti-infectious DC immune medicine, in which the DCs are charged with tumour derived antigens or antigens derived from microbes, exposed to a maturation stimulus, and engineered as described. Additionally, the data presented here imply that an immune suppressive DC medicine may be designed by knocking down immune stimulatory molecules and by over-expression of immune suppressive molecules. Such a suppressive DC immune medicine may have applications in allergy or auto-immunity, but also in transplantation medicine, in order to tolerise the transplant recipient's immune system to the transplanted tissue.

Claims:

1. Pharmaceutical preparation comprising partially matured dendritic cells obtainable by a method comprising the steps of:

a) providing immature dendritic cells or precursor cells thereof or partially matured dendritic cells obtainable by contacting immature dendritic cells with at least one dendritic cell maturation agent to produce partially matured dendritic cells (semi-mature DCs, smDCs) as defined by their capacity to secrete IL-12,

b) manipulating the cells of step a), in particular the partially matured dendritic cells (semi-mature DCs, smDCs) releasing IL-12 of step a), to

(i) over-express at least one immune molecule capable of maintaining the T-lymphocyte stimulatory capacity of dendritic cells characterised by continued IL-12 secretion for at least 24 hours, preferably at least 48 hours, and selected from the group consisting of CD40L by introducing nucleic acid molecules encoding for said at least one molecule; and/or

(ii) inhibit or prevent the expression of at least one T-lymphocyte suppressive molecule acting within dendritic cells exposed to a primary maturation agent such as LPS/IFN-γ or being released from dendritic cells exposed to a primary maturation agent such as LPS/IFN-γ and being selected from the group consisting of interleukin 10 (IL-10), and indoleamine 2,3-dioxygenase (IDO), such as at least one of the genes given in the tables 3, 4 and/or 5, by knocking out the gene or a fragment thereof encoding said at least one T-lymphocyte suppressive molecule or by introducing nucleic acid molecules, preferably ribonucleic acid molecules, to inhibit or prevent the expression of the at least one T-lymphocyte suppressive molecule that is active within the dendritic cell or is delivered from the dendritic cells to T-cells and

c) optionally adding substances to transform precursor cells of dendritic cells into dendritic cells.

2. Preparation according to claim 1, characterised in that the precursors of immature dendritic cells, in particular

monocytes or haematopoietic stem cells, or the immature dendritic cells are obtained from skin, spleen, bone marrow, thymus, lymph nodes, umbilical cord blood, or peripheral blood.

3. Preparation according to claim 1 or 2, characterised in that the at least one dendritic cell maturation agent is selected from the group consisting of agonists of TLRs such as heat-inactivated or formalin-treated Bacillus Calmette-Guerin (BCG), preferably cell wall constituents of BCG, BCG-derived lipoarabidomannans or BCG components, lipopolysaccharide (LPS) derived from E. coli, or inactivated Gram positive or Gram negative microorganisms, an imidazoquinoline compound, preferably an imidazoquinoline-4-amine compound, in particular 4-amino-2-ethoxymethyl-x-dimethyl-1H-imidazol [4,5-c]quinolin-1-ethanol or 1-(2-methylpropyl)-1H-imidazol [4,5-c] quinolin-4-amine, or derivatives thereof, a synthetic double stranded polyribonucleotide, preferably polyI:C, natural double stranded RNA or RNA viruses or fragments of RNA, or synthetic analogues, or a synthetic or natural nucleic acid molecule comprising un-methylated CpG motifs, cytokine combinations, preferably tumour necrosis factor α (TNF-α), IL-1, IL-6 or prostaglandin E6, CD40L, preferably recombinant CD40L or fusion proteins comprising the CD40L domain, or with primary cells or cell lines genetically engineered to express CD40L, or with activated T-lymphocytes that physiologically up-regulate expression of CD40L such as T-lymphocytes.

4. Preparation according to any one of claims 1 to 3, characterised in that prior to the manipulation step b) the immature dendritic cells are contacted with the at least one dendritic cell maturation agent for at least two hours, preferably for at least four hours, more preferably for at least six hours, in particular for at least twelve hours, to a maximum of 24 hours, to give partially matured dendritic cells.

5. Preparation according to any one of claims 1 to 4, characterised in that the immature or the partially matured

dendritic cells are loaded with at least one antigen.

6. Preparation according to claim 5, characterised in that the at least one antigen is selected from the group

a) consisting of tumour antigens, viral antigens, bacterial antigens, or any other human microbial or parasitic pathogens; or

b) consisting of environmental antigens that cause allergy, auto-antigens against which an immune response was initiated that causes disease, or transplantation antigens.

7. Method for producing dendritic cells comprising the steps of:

a) providing immature dendritic cells,

b) contacting said immature dendritic cells with at least one dendritic cell maturation agent to produce partially matured dendritic cells (semi-mature DCs, smDCs) as defined by their capacity to secrete IL-12, and

c) manipulating the partially matured dendritic cells (semi-mature DCs, smDCs) releasing IL-12 of step b) to

(i) over-express at least one immune molecule capable of maintaining the T-lymphocyte stimulatory capacity of the dendritic cells characterised by continued IL-12 secretion for at least 24 hours, preferably at least 48 hours and selected from the group consisting of CD40L by introducing nucleic acid molecules encoding for said at least one molecule; and/or

(ii) inhibit or prevent the expression of at least one T-lymphocyte suppressive molecule acting within the dendritic cell exposed to a primary maturation agent such as LPS/IFN-γ or being released from dendritic cells exposed to a primary maturation agent such as LPS/IFN-γ and being selected from the group consisting of interleukin 10 (IL-10), and indoleamine 2,3-dioxygenase (IDO), such as at least one of the genes given in the tables 3, 4 and/or 5, by knocking out the gene or a fragment thereof encoding said at least one T-lymphocyte suppressive molecule or by introducing nucleic acid molecules, preferably ribonucleic acid molecules, to inhibit or prevent the expression of the at least one T-lymphocyte suppressive molecule that is active within the dendritic cell or is

delivered from the dendritic cells to T-cells.

8. Method according to claim 7, characterised in that the precursors of immature dendritic cells or the immature dendritic cells or partially matured dendritic cells are obtained from skin, spleen, bone marrow, thymus, lymph nodes, umbilical cord blood, or peripheral blood.

9. Method according to claim 7 or 8, characterised in that the at least one dendritic cell maturation agent is selected from the group consisting of agonists of TLRs such as heat-inactivated or formalin-treated Bacillus Calmette-Guerin (BCG), preferably cell wall constituents of BCG, BCG-derived lipoarabidomannans or BCG components, lipopolysaccharide (LPS) derived from E. coli, or inactivated Gram positive or Gram negative microorganisms, an imidazoquinoline compound, preferably an imidazoquinoline-4-amine compound, in particular 4-amino-2-ethoxymethyl-x-dimethyl-1H-imidazol [4,5-c]quinolin-1-ethanol or 1-(2-methylpropyl)-1H-imidazol [4,5-c] quinolin-4-amine, or derivatives thereof, a synthetic double stranded polyribonucleotide, preferably polyI:C, natural double stranded RNA or RNA viruses or fragments of RNA, or synthetic analogues, or a synthetic or natural nucleic acid molecule comprising un-methylated CpG motifs, cytokine combinations, preferably tumour necrosis factor α (TNFα), IL-1, IL-6 or prostaglandin E6, CD40L, preferably recombinant CD40L or fusion proteins comprising the CD40L domain, or with primary cells or cell lines genetically engineered to express CD40L, or with activated T-lymphocytes that physiologically up-regulate expression of CD40L such as T-lymphocytes.

10. Method according to any one of claims 7 to 9, characterised in that prior to the manipulation step c) the immature dendritic cells are contacted with the at least one dendritic cell maturation agent for at least two hours, preferably for at least four hours, more preferably for at least six hours, in particular for at least twelve hours, to a maximum of 24 hours.

11. Method according to any one of claims 7 to 10, characterised in that the immature or the partially matured dendritic cells are loaded with at least one antigen.

12. Method according to claim 11, characterised in that the at least one antigen is selected from the group

a) consisting of tumour antigens, viral antigens, bacterial antigens, or any other human microbial or parasitic pathogens; or

b) consisting of environmental antigens that cause allergy, auto-antigens against which an immune response was initiated that causes disease, or transplantation antigens.

13. Dendritic cells obtainable by a method according to any one of claims 7 to 12.

14. Pharmaceutical composition comprising a dendritic cell according to claim 13.

15. Use of a dendritic cell according to claim 13 or a preparation according to any one of claims 1 to 6 for the manufacture of a medicament to treat and/or prevent cancer and/or microbial or parasitic infections; or to treat and/or prevent allergies, autoimmune disease, or stem cell or organ transplant rejection.

16. Use according to claim 15, characterised in that the medicament is administered to an individual prior to, simultaneous with, or subsequent to radiation therapy and/or anti-tumour or anti-microbial chemotherapy, or any therapy aimed at treating allergies, autoimmune diseases, or stem cell or organ transplant rejection.

17. Use of a dendritic cell according to claim 13 or a preparation according to any one of claims 1 to 6 for the manufacture of a medicament to treat and/or prevent immunological disease caused by a pathologic over-reaction of the immune system against environmental antigens, such as allergens, or against autoantigens in the course of an

autoimmune disease.

18. Use according to claim 17, characterised in that the medicament is administered to an individual prior to, simultaneous with, or subsequent to other modalities aimed at treating or preventing allergies or autoimmune disease.

19. Use of a dendritic cell according to claim 13 or a preparation according to any one of claims 1 to 6 for the manufacture of a medicament to treat and/or prevent the immunologic rejection of an allogeneic stem cell transplant, preferably used in the treatment of haematological malignancies, or to treat and/or prevent rejection of an allogeneic organ transplant.

20. Use according to claim 19, characterised in that the medicament is administered to an individual prior to, simultaneous with, or subsequent to other modalities aimed at treating or preventing the rejection of an allogeneic stem cell or organ transplant.