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(54) Title: METHOD FOR OBTAINING IMMUNO-SUPPRESSIVE DENDRITIC CELLS

(57) Abstract: The present invention relates to methods for producing immuno-suppressive dendritic cells. The present invention further relates to the use of such cells for treating patients suffering from autoimmune diseases, hypersensitivity diseases, rejection on solid-organ transplantation and/or Graft-versus-Host disease.

Method for obtaining immuno-suppressive dendritic cells

FIELD OF THE INVENTION

The present invention relates to methods for producing immuno-suppressive dendritic cells. The present invention further relates to the use of such cells for treating patients undergoing solid-organ transplantation and/or suffering from Graft-versus-Host-disease, autoimmune diseases, and hypersensitivity diseases. The present invention in particular relates to a method of preferentially producing immuno-suppressive dendritic cells relative to immuno-stimulatory dendritic cells

BACKGROUND OF THE INVENTION

Dendritic cells (DC) are recognized to be potent antigen presenting cells for the initiation and control of cellular immunologic responses in humans. Since DC can either be immuno-stimulatory or immuno-suppressive, depending on which set of their potential properties they express at the moment of interaction with responsive specific clones of T cells, they are considered profoundly important pivotal players in

T cell-mediated immune reactions. As a broad, but widely held generalization, immature DC are more "tolerogenic" than their more mature counterparts, while mature DC are thought to be more "immunogenic" than their immature precursors. The capacity of DC, generated ex vivo from monocytes and armed with specific antigen, to function effectively in either immunologic direction, is dependent on their viability and vigor after being returned to the patient. It is logically concluded that the balance between counteractive immuno-stimulatory and immune-suppressive DC will be a major determinant of both the direction and potency of DC-dependent therapeutic immune responses.

The purpose of this invention is to facilitate production of DC populations particularly conducive to the generation of powerful and clinically relevant immune responses. Despite the tremendous promise of DC-based therapy, such as efforts to enhance anti-cancer immunity, clinical results have generally been disappointing. For example, Provenge, the recently first FDA-approved immunotherapy for a solid tumor, adapting the conventional method of ex vivo production of DC from blood monocytes, has yielded merely a four-month improvement in survival of patients with advanced prostate cancer. Further, Provenge has so far not been shown to diminish the size of the treated prostate cancer. That conventional method of inducing DC, as well as Extracorporeal Photopheresis (ECP), an FDA-approved therapy for the "liquid" malignancy Cutaneous T Cell Lymphoma (CTCL), is encumbered by production of relatively heterogeneous DC populations under conditions which handicap the in vivo vigor, and viability, of the resulting DC. By employing more physiologic conditions to the production of therapeutic DC, the present invention enables production of more maturationally synchronized DC, whose survival and vigor are not inhibited by factors inherent to the method by which they are produced. Moreover, this method is applicable to both human and animal leukocytes.

DC prime both $CD8^+$ cytotoxic T-cell (CTL) and $CD4^+$ T helper (Th1) responses. DC are capable of capturing and processing antigens and migrating to the regional lymph nodes to present the captured antigens and induce T-cell responses. In humans, DC are a relatively rare component of peripheral blood (<1 % of leukocytes). However, large quantities of DC can be differentiated by laboratory procedures from $CD34^+$ precursors or blood monocytes.

For the afore-described properties, DC have been identified as an important cellular agent for eliciting effective anti-tumor immune responses. The idea is to generate dendritic cells, which present tumor-specific antigens on their MHC Class I and MHC Class II complex and can be (re)introduced into a patient to thereby launch an immune-attack against the tumor. However, generation of such immuno-stimulatory dendritic cells usually requires differentiation of $CD34^+$ precursors or blood monocytes using complex and rather expensive cytokine cocktails. In those standard methods, the cytokines are employed at concentrations very much higher (often by orders of magnitude) than those encountered in vivo under physiological conditions. Therefore, one proffered reason for the overall disappointing clinical results from DC-based immuno-modulation is that DC produced by the common cytokine method may not function effectively at the far lower cytokine concentrations actually in patients. DC produced ex vivo at markedly supra-physiologic concentrations of growth factors, such as cytokines, are selected to be dependent on conditions which are reproduced in the in vivo environment in patients.

In addition to the afore-mentioned immuno-stimulatory dendritic cells, other types of dendritic cells, namely tolerogenic dendritic cells, which may also be designated as immuno-suppressive or immuno-inhibiting dendritic cells have been described. These types of dendritic cells play an important role in maintaining immune tolerance and they have been discussed for therapeutic settings such as solid-organ transplantation, Graft-versus-Host-disease, autoimmune diseases, and hypersensitivity diseases (see e.g. Hu et al. *Immunology*, (2010), 132, 307-314).

Classical Extracorporeal Photopheresis (ECP) has been used successfully to treat cutaneous T-cell lymphoma (CTCL) in subsets of patients. In ECP, patients suffering from CTCL receive the photoactivatable compound 8-methoxypsoralen (8-MOP). Patients are then leukapheresed to obtain buffy coats and these buffy coats are passed through a continuous closed circuit ultraviolet exposure device to irradiate the leukapheresed buffy coats and thereby lethally damage exposed lymphocytes. In this manner, 8-MOP is induced to covalently crosslink base pairs of DNA. The concept of ECP is to destroy proliferating metastatic T-cells of CTCL and to then to intravenously re-introduce the dying cells to the patient. It has been learned that this process additionally leads to conversion of passaged blood monocytes to DC without the need for stimulation by addition of exogenous cytokines. These ECP-induced DC are furthermore assumed to internalize, process and display antigens from the tumor cells, which were destroyed by the combination of 8-MOP and UV irradiation. It has been hypothesized that reintroduction of these loaded dendritic cells to the patient account for at least part of the success of ECP when treating CTLC. In fact ECP-like processes, in which neither 8-MOP nor UV light irradiation are used, but in which extracorporeal blood sample comprising monocytes are passed under shear stress through an ECP device have also been assumed to initiate monocyte differentiation into DC.

However, it has also been found that the ECP or ECP-like process leads to truncated, i.e. immuno-suppressive or tolerogenic DC, likely contributing heavily to ECP's clinical efficacy in the treatment of Graft versus Host Disease which commonly follows post-bone marrow stem allotransplants. The precise mechanistic aspects of ECP on differentiation of monocytes into either immuno-stimulatory or immuno-suppressive DC have remained elusive (for review of the ECP process see Girardi et al. (2002), *Transfusion and Apheresis Science*, 26, 181-190).

The present ECP and ECP-like processes are thus conceived to lead to complex mixtures of immuno-stimulatory and immuno-suppressive DC. Of course, from *inter alia* a clinical perspective, it would be important to understand how the ECP and ECP-like processes can be modified to to overcome these limitations and how one can obtain purposively and selectively preferentially immuno-suppressive relative to immuno-stimulatory DC and vice versa. Further, the classical ECP process is, in principle an in vivo method as the obtained dendritic cell mixtures are reinfused into the patient. It would, however, be desirable to have methods available that allow preferential production of immuno-suppressive over immuno- stimulatory DC and vice versa outside the human or animal body.

Thus, there is a continuing need for methods that allow predictable and reproducible production of individual-specific, i.e. autologous immuno-suppressive dendritic cells, which upon re-introduction into a patient allow for treatment of e.g. autoimmune disease such as multiple sclerosis or Graft-versus-Host disease.

OBJECTIVES AND SUMMARY OF THE INVENTION

One objective of the present invention is to provide methods for producing immuno-suppressive dendritic cells.

Another objective of the present invention is to provide methods for producing immuno-suppressive antigen-presenting cells.

Another objective of the present invention is to provide methods for producing immuno-suppressive dendritic cells or immuno-suppressive antigen-presenting cells in an extracorporeal amount of blood obtained from a patient.

A further objective of the present invention is to provide methods for producing immuno-suppressive dendritic cells or immuno-suppressive antigen-presenting cells

in an extracorporeal amount of blood obtained from a patient without the need for cytokine cocktails.

Yet another objective of the present invention relates to the use of such immuno-suppressive dendritic cells or immuno-suppressive antigen-presenting cells for treating patients undergoing solid-organ transplantation and/or suffering from Graft-versus-Host-disease, autoimmune diseases, and hypersensitivity diseases.

These and other objectives as they will become apparent from the ensuing description hereinafter are solved by the subject matter of the independent claims. Some of the preferred embodiments of the present invention form the subject matter of the dependent claims. Yet other embodiments of the present invention may be taken from the ensuing description.

The present invention is based to some extent on data presented hereinafter, which for a miniaturized and scalable device allowed (i) to mimic some aspects of the classical ECP procedure and (ii) to elucidate the cellular and molecular mechanism and biophysical conditions of induction of differentiation of monocytes into immuno-stimulatory autologous dendritic cells in an extracorporeal amount of blood. This data shows that the activation of platelets and binding of monocytes to such activated platelets under conditions of shear force is essential for obtaining immuno-stimulatory autologous dendritic cells. As is shown by the experiments described hereinafter, these immuno-stimulatory autologous dendritic cells can be characterized by expression of molecular markers indicative of immuno-stimulatory autologous dendritic cells. The data also shows that conditions that lead to an increased expression of Glucocorticoid-induced Leucine Zipper (GILZ) will favorably allow monocytes to differentiate into immuno-suppressive dendritic cells. These immuno-suppressive dendritic cells, which show an increased expression of GILZ as consequence of treating monocyte-derived immature dendritic cells with 8-MOP and exposing to UVA, will show an even stronger expression of GILZ upon

contact with apoptotic leukocytes. Further, such dendritic cells show increased expression of IL-10 and decrease production of various pro-inflammatory cytokines and chemokines as can be taken form a reduced IL-10 to IL-12p70 ratio. These findings thus allow for a rationalized approach to obtain immuno-suppressive dendritic cells by thus carefully selecting the properties of the devices to be used and the parameters of the process. The findings of the present invention allows preferential production of immuno-suppressive dendritic cells relative to immuno-stimulatory dendritic cells and thus overcome the limitations of the classical ECP procedure because, in the classical ECP, procedure the lack of understanding what type of dendritic cells are produced and how their production can be manipulated to some extent prevents the extension of using this method for other than the authorized applications (see Girardi et al. (2002), *Transfusion and Apheresis Science*, 26, 181-190). Moreover, other than for the classical ECP procedure as used in the device obtainable from Therakos, the present invention allows to obtain such immuno-stimulatory dendritic cells in an experimental setting, where the extracorporeal amount of blood is not in a continous connection with the body. The data *inter alia* suggests that the process of obtaining immuno-stimulatory dendritic cells includes a global monocyte activation step and a subsequent monocyte to immuno-stimulatory antigen-presenting cell (e.g. dendritic cell) differentiation step. These steps seem to be initially dependent on physical activation of monocytes with the physical forces occurring during e.g. initial purification or enrichment of monocytes being sufficient for activation even though passage of e.g. initially activated monocytes through devices as described herein allow improvement of activation and differentiation. Further, the data suggest that once monocyte activation has occurred, differentiation is channeled into the direction of immuno-suppressive antigen-presenting dendritic cells by applying photoactivatable agents such as 8-MOP and UV-A thereby increasing expression of GILZ. The present data suggest that these processes take place in the absence of e.g. apoptosis of cytotoxic T-cells or other foreign cells such that the immuno-suppressive antigen-presenting dendritic cells can be obtained before loading them with e.g. a desired antigen.

Some of the embodiments, which are based on this data, are described in more detail hereinafter.

In a first aspect, the invention relates to a method for differentiation of monocytes contained in an extracorporeal quantity of a mammalian subject's blood sample into immuno-suppressive dendritic cells, said method comprising at least the steps of:

a) subjecting said extracorporeal quantity of said mammalian subject's blood sample to a physical force such that said monocytes are activated and induced to differentiate into immuno-suppressive dendritic cells, which are identifiable by at least one molecular marker, wherein said at least one molecular marker is indicative of immuno-suppressive dendritic cells.

Suitable molecular markers indicative of immuno-suppressive dendritic cells include GILZ and/or IL-10. Thus, dendritic cells as they are obtainable by the methods described herein, which show an increased expression of GILZ and/or IL-10 are considered to be immuno-suppressive dendritic cells. GILZ is a preferred molecular marker being indicative for immuno-suppressive dendritic cells. It is to be understood that an increased expression is to be determined in comparison to the monocytes before they are subjected to the methods described herein. Such immuno-suppressive dendritic cells may also show an GILZ-induced reduced IL-10 to IL-12p70 ratio.

In addition, immuno-suppressive dendritic cells can be distinguished from immuno-stimulatory autologous dendritic cells in that they do not show an increased expression of molecular markers, which are indicative of immuno-stimulatory autologous dendritic cells. It is to be understood that an expression is to be determined in comparison to the monocytes before they are subjected to the methods described herein

Molecular markers indicative of immuno-stimulatory autologous dendritic cells are described hereinafter and may be taken from e.g. Table 1. These molecular markers may be grouped according to their know function as e.g. molecular markers of antigen-presenting cells, molecular markers of cellulara adhesion etc. Preferred molecular markers the expression of which is considered indicative of immuno-stimulatory dendritic cells include PLAUR, NEU1, CD80, CCR7, LOX1, CD83, ADAM Decysin, FPRL2, GPNMB, ICAM-1, HLA-DR, and/or CD86. Markers like HLA-DR, PLAUR and ICAM-1 may be considered to be indicative of global monocyte activation while increased expression of e.g. CD83 and ADAM-Decysin seems indicative of monocyte to dendritic cell differentiation. Thus, if these molecular markers show an increased expression and particularly if GILZ expression is not increased, dendritic cells as they are obtainable by the methods described herein will be considered to be immuno-stimulatory autologous dendritic cells. In turn, dendritic cells as they are obtainable by the methods described herein will be considered immuno-suppressive dendritic cells if they show an increased expression of GILZ and/or IL-10 and particularly if molecular markers taken from Table 1 do not show an increased expression.

In one embodiment of this first aspect, activation of monocytes is *inter alia* achieved in that said extracorporeal quantity of said mammalian subject's blood sample is subjected to a physical force by passing or cycling said extracorporeal quantity of said mammalian subject's blood sample through a flow chamber of a device, which allows adjustment of the flow rate of said extracorporeal quantity of said mammalian subject's blood sample through said flow chamber of said device such that a shear force is applied to said monocytes contained within said mammalian subject's blood sample.

Thus, activation of monocytes and induction of differentiation into immuno-suppressive dendritic cells can be achieved and influenced by varying the flow forces of the extracorporeal quantity of the mammalian subject's blood sample through the

flow chamber of such a device, by varying the geometry of the flow path of the flow chamber, by varying the dimensions of the flow chamber, by varying the temperature of the flow chamber and thus of the extracorporeal quantity of the mammalian subject's blood sample, by changing the biophysical and geometric surface properties of the flow path, by allowing the exposure of the extracorporeal quantity of the mammalian subject's blood sample in the flow chamber to visible or UV light, by adding DNA-cross linking agents such as 8-MOP, etc..

As is shown hereinafter, activation of monocytes and induction of differentiation into immuno-suppressive dendritic cells is mediated e.g. by interaction of monocytes with activated platelets and/or specific plasma components in a situation where the monocytes experience physical force which may be provided by a device as described hereinafter.

In another embodiment of this first aspect, the present invention thus relates to activation of monocytes, which experience a physical force and which interact with activated platelets and/or plasma components such as fibrinogen or fibronectin. Activation may be a process of subsequent steps including the steps of (i) immobilizing plasma components such as fibrinogen or fibronectin either as isolated components or as part of the extracorporeal quantity of the mammalian subject's blood sample in the flow chamber of said device (ii) passing platelets, which may be obtained as a purified fraction from the extracorporeal quantity of the mammalian subject's blood sample or as part of the extracorporeal quantity of the mammalian subject's blood sample, through the flow chamber such that the platelets can interact with and become activated by the plasma components and (iii) passing monocytes, which may be obtained as a purified fraction from the extracorporeal quantity of the mammalian subject's blood sample or as part of the extracorporeal quantity of the mammalian subject's blood sample, through the flow chamber such that the monocytes can interact with and become activated by the activated platelets and/or the plasma components.

Thus, in addition and/or alternatively to the above described parameters and variable touching on the architecture of and the conditions under which the device is operated, activation of monocytes and induction of differentiation into immuno-suppressive dendritic cells can be achieved and influenced by varying the nature, purity and concentrations of the plasma components, the nature, purity and concentration of the platelets, the order of steps by which the plasma components and/or the platelets are passed through and/or disposed on the flow chamber, the density by which the flow chamber is coated with the plasma components and/or the platelets, the flow forces of the extracorporeal quantity of the mammalian subject's blood sample and in particular the platelets and/or the monocytes are passed through the flow chamber of such a device, the temperature and/or time at which the extracorporeal quantity of the mammalian subject's blood sample and in particular the platelets and/or the monocytes are passed through the flow chamber of such a device, etc., the nature, purity and concentrations of additional factors such as 8-MOP and/or cytokines are added to the extracorporeal quantity of the mammalian subject's blood sample and in particular to the monocytes, etc.

It needs, however, to be understood that while such devices may be particularly effective in inducing monocyte activation and differentiation into immuno-suppressive dendritic cells in the presence of a photo-activatable agent such as 8-MOP and UVA, physical forces which monocytes experience during initial purification or enrichment such as during Ficoll-Hypaque enrichment as described hereinafter may already be sufficient to activate monocytes and to induce their differentiation. Similarly even though activated platelets and/or specific plasma components may be helpful in increasing monocyte activation and differentiation into antigen-presenting cells such as dendritic cells they may not be absolutely necessary. In order to effect monocyte activation and differentiation into antigen-presenting cells such as dendritic cells the invention thus contemplates as a mimimal requirement the application of physical forces. Once the activation and

differentiation process has started application of photoactivatable agents such as 8-MOP and UVA or other means that increase GILZ expression (such as dexamethasone) can be used to selectively channel the differentiation process towards immuno-suppressive antigen-presenting cells such as dendritic cells.

In the above and ensuing aspects and embdodiment, the extracorporeal quantity of the mammalian subject's blood sample and in particular the monocytes thus may or may not be obtained by leukapheresis.

Additionally or alternatively to these embodiments, the invention in particular also relates to such methods which are conducted under conditions which favor an increased expression of GILZ and/or an increased number of $CD4^+CD25^+Foxp3^+$ cells and/or a down-regulations of CD80, CD86 and CD83. The invention thus relates to e.g. methods, which are conducted in the presence of a photoactivatable agent such as 8-MOP and with exposure to light such as UV-A. In one embodiment, the invention correspondingly relates to methods which are conducted under conditions which avoid activation of monocytes and induction of immuno-stimulatory autologous dendritic cells as can be identified by determining expression of GILZ and/or IL-10 and/or of the molecular markers of Table 1. In one embodiment, the invention thus does not relate to e.g. methods, which are conducted in the absence of a photoactivatable agent such as 8-MOP and without exposure to light such as UV-A

Another embodiment relates to methods as described hereinafter for obtaining , autologous and/or allogenic immuno-suppressive antigen-presenting cells, which can be used e.g. in treating autoimmune diseases, hypersensitivity diseases, rejection of solid organ transplant, and Graft-versus-Host Disease.

Another embodiment relates to methods as described hereinafter for producing immune-suppressive antigen presenting cells and preferably immuno-suppressive

dendritic cells which may be mammalian cells. Even though some of the preferred embodiments of the invention relate to producing human immuno-suppressive antigen presenting cells and preferably human immuno-suppressive allogeneic dendritic cells, the present invention considers also to use the methods for producing animal immuno-suppressive antigen presenting cells and preferably animal immuno-suppressive dendritic cells such as for mice, rats, etc. These embodiments of the invention provide useful animal models and thus scalability of the methods and results described herein from e.g. mice to man. Moreover, as there are genetically identical lines of animals such as mice available, animal immuno-suppressive antigen presenting cells and preferably animal immuno-suppressive dendritic cells such as mice immuno-suppressive antigen presenting cells and preferably mice immuno-suppressive dendritic cells may be introduced either in the individual from which the extracorporeal amount of blood sample was taken and thus be autologous in the strict sense or be introduced in a genetically identical individual. This will allow e.g. testing for any unexpected effects of these cells.

It is to be understood that the methods described hereinafter have been shown to produce immune-suppressive cells, which due to their molecular markers seem to be related to if not correspond to cells that are commonly named dendritic cells. Thus the immune-suppressive cells according to the invention have been named immune-suppressive dendritic cells. However, dendritic cells are representatives of a broader class of cells, which may be designated as antigen-presenting cells. Thus, the methods as described hereinafter generally refer to the production of immune-suppressive antigen-presenting cells with immune-suppressive dendritic cells being preferred.

A second aspect of the invention relates to autologous and/or allogenic immuno-suppressive dendritic cells or autologous and/or allogenic immuno-suppressive antigen-presenting cells obtainable by a method as described hereinafter for use in

treating autoimmune diseases, hypersensitivity diseases, rejection of solid organ transplant, and Graft-versus-Host Disease.

Further embodiments will be described hereinafter.

FIGURE LEGENDS

Figure 1 Effect of platelet density on number of monocyte-platelet interactions and subsequent monocyte phenotype. Monocytes were passed through parallel plates coated with platelets at low, medium, or high density. (A) The number of monocyte-platelet interactions increased substantially for plates coated with higher densities of platelets. (B) After overnight incubation, monocytes which were exposed to high levels of platelets were significantly more likely to develop a phenotype consistent with DC differentiation, as assessed by expression of membrane CD83 and HLA-DR (high versus medium or low density: $p < 0.0001$; medium versus low density: $p < 0.005$). Data shown are the means (+/- SD) of at least 6 independent experiments. lpf, low power field.

Figure 2 Gene expression following exposure to platelets. Monocytes were exposed to high or low levels of platelets in flow. Following overnight incubation, cells were assessed for differences in gene expression using RT-PCR. Figure 2 shows gene expression changes in monocytes exposed to high levels of platelets relative to those exposed to low levels. Seven genes associated with DC-differentiation and/or function were found to be upregulated, while three were downregulated. Of the genes downregulated, GPNMB and FPRL2 have known functions in decreasing cytokine production and inhibiting DC maturation, respectively. Of the genes upregulated, all have either pro-immune functions or

miscellaneous roles in DC biology. See text for specific description of genes. Data shown are the means (+/- SD) of 2 independent experiments.

Figure 3 Platelet influence on monocyte differentiation in static conditions. Monocytes were co-cultured for 18 hours with low, medium, or high concentrations of platelets in static conditions lacking flow. Under these conditions, there was no observable platelet influence on DC differentiation; all conditions resulted in low, baseline levels of cells expressing DC markers. Furthermore, activating platelets with thrombin in culture (blue line) did not cause a discernible difference in monocyte differentiation relative to those cultures containing platelets not activated by thrombin (red line).

Figure 4 Plasma protein influence on platelet adhesion to plates. Platelets were passed through plates coated with fibrinogen, plasma, fibronectin, or RMPI at the shear stress level indicated by the x-axis. Platelets in flow adhered optimally to fibronectin. For all proteins, platelet adhesion occurred maximally between 0.5 and 1.0 dyne/cm^2 lpf, low power field. Data shown are the means (+/- SD) of at least 2 independent experiments.

Figure 5 Plasma protein influence on platelet adhesion to plates coated with Fibrinogen (A) or Fibronectin (B). Platelets were either untreated (baseline), or pretreated with either RGD fragments (+RGD) or gamma fragments (+Gamma) and their subsequent adhesion to fibrinogen (left panel) and fibronectin (right panel) was assessed. Platelet binding to fibrinogen was decreased by gamma fragments ($p < 0.05$), while binding to fibronectin was decreased by RGD peptides ($p < 0.001$). lpf, low power field. Data shown are the means (+/- SD) of at least 2 independent experiments.

Figure 6 Proteins involved in monocyte-platelet interactions. Monocytes were passed between platelet-coated plates at a wall shear stress of 0.5 dyne/cm2 under the conditions indicated by the x-axis: platelets were either pretreated with anti-P-selectin (P-) or an isotype control (P+); monocytes were either pretreated with RGD peptides (RGD-) or a control fragment (RGD+). Monocyte-platelet interactions were quantified under each set of conditions using digital microscopy, and are expressed in the figure as a fraction of the maximum seen under conditions of P+/RGD+. Interactions were divided into those lasting less than 3 second (short duration, black bars) and those lasting greater than 3 seconds, including stable binding (long-duration, gray bars). All conditions which involved blocking with anti-P-selectin (P-) resulted in a significant decrease in both short and long duration interactions (**, $p < 0.01$); Blocking only RGD (RGD-) resulted in a significant decrease in long-duration interactions (*, $p < 0.05$) but no change in short-duration interactions. Data shown are the means (+/- SD) of 3 independent experiments.

Figure 7 Effect of p-Selectin exposure on monocyte integrins. Plastic plates were coated with platelets at the relative density indicated by the x-axis. Platelets were then pretreated with anti p-selectin (dashed line) or an isotype control (gray line), or received no pretreatment (black line). Monocytes were passed through the plates at 0.5 dyne/cm2 and then immediately assessed by flow cytometry for expression of active β1 integrins. The y-axis indicates the percent of monocyte which bound an antibody directed at an epitope only exposed when the integrin is in the open confirmation. Data shown are the means (+/- SD) of 3 independent experiments.

Figure 8 Effect of P-selectin exposure on monocyte phenotype after overnight incubation. Platelet-coated plates were either untreated (first column), or pretreated with an isotype control (second column) or anti-P-selectin (third column). Monocytes were passed through the plates at 0.5 dyne/cm2 then incubated overnight. The y-axis indicates the percent of monocytes which developed a phenotype consistent with DC differentiation, i.e., membrane HLA-DR+/CD83+. Data shown are the means (+/- SD) of 3 independent experiments.

Figure 9 Proposed mechanism for induction of monocyte-to-DC differentiation. Based on data presented in this manuscript, the following sequence of events is postulated: (1) plasma fibrinogen coats the plastic surface of the flow chamber; (2) through their αIIbβ3 receptor, unactivated platelets bind to the gamma-component of immobilized fibrinogen; (3) platelets become activated and instantaneously express preformed P-selectin and other surface proteins; (4) passaged monocytes transiently bind P-selectin via PSGL-1, causing partial monocyte activation and integrin receptor conformational changes; (5) partially-activated monocytes, now capable of further interactions, bind additional platelet-expressed ligands, including those containing RGD domains; (6) finally, so influenced, monocytes efficiently enter the DC maturational pathway within 18 hours. Note that, in-vivo, step (1) above may be replaced physiologically by inflammatory signals from tissue acting on local endothelium, causing it to recruit and activate platelets in a similar manner.

Figure 10: Expression of GILZ is rapidly down-regulated as monocytes differentiate into immature MoDC, and up-regulated after exposure to dexamethasone. A.) GILZ mRNA expression in CD11c+ MoDC is presented as a fold change relative to freshly isolated monocytes. B.) Median fluorescence intensities for intracellular and cell surface markers

after 0 and 36 hr. C.) GILZ mRNA expression in CD11c+ MoDC after 24 hr is presented as a fold change relative to MoDC receiving no dexamethasone. D.) GILZ mRNA expression in CD11c+ MoDC is presented as a fold change relative to MoDC at time 0 hr. E.) GILZ mRNA expression in CD11c+ MoDC after 24 hr is presented as a fold change relative to untreated MoDC. F.) GILZ mRNA expression in CD11c+ MoDC is presented as a fold change relative to untreated MoDC. All data are expressed as mean ± standard deviation for a minimum of 3 independent experiments. For differential gene expression: * ≥ 2.5-fold change and $p < 0.05$, ** ≥ 2.5-fold change and $p < 0.01$, *** ≥ 2.5-fold change and $p < 0.001$

Figure 11: 8-MOP plus UVA light up-regulates GILZ in immature MoDC in a dose-dependent fashion. A.) GILZ expression is presented as a function of the 8-MOP concentration at 1 J/cm2 and 2 J/cm2 of UVA light. GILZ mRNA expression in CD11c+ MoDC 24 hr after PUVA treatment is presented as a fold change relative to MoDC receiving no 8-MOP. B.) GILZ expression is presented as a function of the 8-MOP concentration multiplied by the UVA dose. C.) The percentage of early apoptotic CD11c+ cells after 24 hr. D.) The percentage of late apoptotic CD11c+ cells after 24 hr E.) Dot plots of CD11c+-gated cells for UVA doses of 1 J/cm2 and 2 J/cm2 are shown for 1 representative experiment of 4. The percentage of CD11c+ cells displaying Annexin-V+/7-AAD- or Annexin-V+/7-AAD+ phenotypes are indicated. The percentage of F.) CD11c+ cells and G.) CD3+ cells expressing early and late apoptotic markers were quantified 24 hr after treatment with 8-MOP (100 ng/mL) and UVA light (1 J/cm2). All data represent mean ± standard deviation of at least 4 independent experiments. For differential gene expression: * ≥ 2.5-fold change and $p < 0.05$, ** ≥ 2.5-fold change and $p < 0.01$

Figure 12: 8-MOP plus UVA light down-regulates CD83, CD80 and CD86 and up-regulates HLA-DR in immature MoDC in a dose-dependent manner. Relative fluorescence intensities for membrane expression of A.) HLA-DR and CD83, and B.) CD80 and CD86 are presented as a function of the 8-MOP concentration (0 to 200 ng/mL) multiplied by the UVA dose (1 or 2 J/cm2) 24 hr after PUVA treatment. Untreated MoDC served as controls and were assigned an RFI value of 1. Data represent mean ± standard deviation of 4 independent experiments. *$p < 0.05$, **$p < 0.01$

Figure 13: Immature MoDC exposed to apoptotic lymphocytes up-regulate GILZ. A.) GILZ mRNA expression in CD11c+ MoDC 24 hr after co-culture is presented as a fold change relative to untreated MoDC that were cultured alone. B.) GILZ mRNA expression in CD11c+ MoDC 24 hr after co-culture is presented as a fold change relative to untreated MoDC that were cultured alone. C.) Relative fluorescence intensity for intracellular GILZ 24 hr after co-culture. Relative fluorescence intensities post- to pre-LPS stimulation for D.) CD80 and CD86 and E.) HLA-DR and CD83 were calculated as follows: (MFItreated after LPS – MFItreated before LPS) / (MFIuntreated after LPS – MFIuntreated before LPS). Data represent mean ± standard deviation for at least 4 independent experiments. For differential gene expression: * ≥ 2.5-fold change and $p < 0.05$

Figure 14: MoDC expressing GILZ increase production of IL-10, and decrease production of various pro-inflammatory cytokines and chemokines. 24 hr after LPS stimulation, culture supernatants were harvested for cytokine quantification by magnetic bead multiplex immunoassays for A.) IL-10, and the pro-inflammatory cytokines B.) IL-12p70 and IFN-γ, C.) IL-6 and TNF-α. The same analysis was performed for the pro-inflammatory chemokines D.) IL-8, and E.) MCP-1, MIP-1β and

RANTES. Data are presented as mean ± standard deviation of 3 independent experiments. * $p < 0.05$ compared to the untreated MoDC group.

Figure 15: siRNA-mediated knockdown of GILZ abolishes the increased IL-10 to IL-12p70 ratio characteristic of tolerogenic dendritic cells. A.) GILZ mRNA expression is presented as fold change compared to untreated MoDC that were cultured alone. * ≥ 2.5-fold change and $p < 0.05$. B.) Quantification of IL-10 and IL-12p70 protein levels in culture supernatants after LPS stimulation. Data represent mean ± standard deviation of 3 independent experiments. *$p < 0.05$, compared to identically treated MoDC not transfected with siRNA.

Figure 16: depicts the flow of monocytes in a classical ECP process in the presence of UVA and 8-MOP. The monocytes in the middle experience lower UVA exposure than the monocytes towards the surfaces of the channels.

Figure 17: depicts the design of the channels of the device used in a classical ECP process.

Figure 18: a) to d) depict different geometries of the flow chamber of a device that may be used for the methods of the invention.

Figure 19: A) depicts the geometry of a device used in some of the examples. B) depicts the geometry of an alternative device.

Figure 20: depicts increase of expression of HLA-DR upon physical activation of monocytes through a device of Figure 19

Figure 21: depicts increase of FSC/SSC complexity upon physical activation of monocytes through a device of Figure 19

Figures 22: depicts increase of FSC/SSC complexity upon physical activation of monocytes by passing through a device of Figure 19

Figure 23: depicts increase of expression of HLA-DR, CD86, ICAM-1, PLAUR and or FSC/SSC complexity upon physical activation of monocytes through a device of Figure 19

Figure 24: depicts increase of expression of GILZ upon physical activation of monocytes through a device of Figure 19 and application of 8-MOP and UVA

DETAILED DESCRIPTION OF THE INVENTION

Before the invention is described in detail with respect to some of its preferred embodiments, the following general definitions are provided.

The present invention as illustratively described in the following may suitably be practiced in the absence of any element or elements, limitation or limitations, not specifically disclosed herein.

The present invention will be described with respect to particular embodiments and with reference to certain figures but the invention is not limited thereto but only by the claims.

Where the term "comprising" is used in the present description and claims, it does not exclude other elements. For the purposes of the present invention, the term

"consisting of" is considered to be a preferred embodiment of the term "comprising of". If hereinafter a group is defined to comprise at least a certain number of embodiments, this is also to be understood to disclose a group, which preferably consists only of these embodiments.

For the purposes of the present invention, the term "obtained" is considered to be a preferred embodiment of the term "obtainable". If hereinafter e.g. an antibody is defined to be obtainable from a specific source, this is also to be understood to disclose an antibody, which is obtained from this source.

Where an indefinite or definite article is used when referring to a singular noun, e.g. "a", "an" or "the", this includes a plural of that noun unless something else is specifically stated. The terms "about" or "approximately" in the context of the present invention denote an interval of accuracy that the person skilled in the art will understand to still ensure the technical effect of the feature in question. The term typically indicates deviation from the indicated numerical value of ±20 %, preferably ±15 %, more preferably ±10 %, and even more preferably ±5 %.

Furthermore, the terms "first", "second", "third" or "(a)", "(b)", "(c)", "(d)" or "(i)", "(ii)", "(iii)", "(iv)" etc. and the like in the description and in the claims, are used for distinguishing between similar elements and not necessarily for describing a sequential or chronological order. It is to be understood that the terms so used are interchangeable under appropriate circumstances and that the embodiments of the invention described herein are capable of operation in other sequences than described or illustrated herein.

In case the terms "first", "second", "third" or "(a)", "(b)", "(c)", "(d)" or "(i)", "(ii)", "(iii)", "(iv)" etc. relate to steps of a method or use or assay there is no time or time interval coherence between the steps unless indicated otherwise, i.e. the steps may be carried out simultaneously or there may be time intervals of seconds, minutes, hours,

days, weeks, months or even years between such steps, unless otherwise indicated in the application as set forth herein above or below.

Technical terms are used by their common sense. If a specific meaning is conveyed to certain terms, definitions of terms will be given in the following in the context of which the terms are used.

As already mentioned, the present invention is based to some extent on data presented hereinafter, which for a miniaturized device allowed (i) to mimic some aspects of the classical ECP procedure and (ii) to elucidate the cellular and molecular mechanism of induction of differentiation of monocytes into immuno-stimulatory dendritic cells in an extracorporeal amount of blood. As is shown by the experiments described hereinafter, these immuno-stimulatory autologous dendritic cells can be characterized by expression of molecular markers indicative of immuno-stimulatory autologous dendritic cells. The data also shows that conditions that lead to an increased expression of Glucocorticoid-induced Leucine Zipper (GILZ) will favorably allow monocytes to differentiate into immuno-suppressive dendritic cells. For the purposes of the present invention such immuno-suppressive dendritic cells are also designated as immuno-inhibiting autologous dendritic cells, tolerogenic autologous dendritic cells or truncated autologous dendritic cells. This data shows that e.g. the sequential activation of platelets and binding of monocytes to such activated platelets under conditions of shear force is essential for differentiation of monocytes into dendritic cells and, depending on the specific conditions chosen, in particular into immuno-stimulatory or immuno-suppressive dendritic cells. Further, these findings immediately allow for a rationalized approach to obtain immuno-stimulatory and immuno-suppressive dendritic cells. Given that one can mimic and dissect the series of molecular events leading to the formation of immuno-stimulatory autologous dendritic cells and immuno-suppressive dendritic cells obtained in the classical ECP process, one can now design devices and more particularly flow chambers, which allow to further dissect the molecular events

leading to differentiation of monocytes into immuno-stimulatory autologous dendritic cells and immuno-suppressive dendritic cells on a scale suitable for research purposes, but also which allow to obtain immuno-stimulatory autologous dendritic cells and immuno-suppressive dendritic cells for therapeutic purposes. This will be explained in further detail.

In the classical ECP procedure, 2.5 L to 6 L blood is typically obtained from patients suffering from CTLC by apheresis such as leukaphereses. This extracorporeal amount of blood, which typically is processed by apheresis such as leukaphereses to give a final volume of about 200 ml to 500 ml comprising leukocytes including monocytes as well as plasma components, platelets and cancerous T-cells, is then passed under shear stress through a photopheresis device having transparent plastic channels together with the photoactivatable drug 8-MOP. This extracorporeal amount of blood comprising 8-MOP is then irradiated by exposing the transparent channels to UV-A having a wavelength of 315 to 380 nm. The irradiated extracorporeal amount of blood is then re-introduced into the patient. The beneficial effects of this procedure on the course of CTLC in some of the treated patients was originally hypothesized to result from the destruction of cancerous T-cells. Based on this hypothesis, it was assumed that patients would have to undergo repeated cycles of ECP. However, for some of the patients beneficial long-term effects were observed making repeated treatment superfluous and, in the following, interesting and partially non-reconcilable effects were found, which could explain some of the positive outcomes of ECP for CTLC treatment.

For example, as is described in US 6,524,855 induction of DC was observed in the extracorporeal amount of blood and it was hypothesized that some of the beneficial effects of ECP on CTLC resulted from cancer-specific antigens that were shed by cancerous T-cells as a consequence of the 8-MOP induced apoptosis of these cells and loading of DC, which had started to differentiate, with these antigens. The re-introduction of the extracorporeal amount of blood comprising such cancer-antigen

loaded autologous DC was assumed to provide a vaccination-like long-term lasting therapeutic effect. However, at the same time it was observed that so-called "truncated" DC were formed during the ECP procedure, which did not provide an immuno-stimulatory effect, but rather the opposite, namely an immuno-suppressant effect. The induction of such different types of DC with opposing effects by the same process was puzzling and, from a practical perspective, posed hurdles as to a rationalized use of ECP for obtaining immuno-stimulatory or immuno-suppressant DC. Further, the need for apheresis such as leukaphereses to obtain a sufficient amount of extracorporeal blood is another factor negatively affecting treatment quality for patients.

The data presented hereinafter suggest that shear stress is in principle responsible for the induction of DC. By using the miniaturized model device as described hereinafter, it was shown that induction of immuno-stimulatory DC occurs even if substantially lower amounts of extracorporeal blood, which has not been obtained by apheresis such as leukaphereses, are used, and if 8-MOP is not added to the extracorporeal amount of blood and even if no irradiation with UV-A takes place. Thus, induction of DC occurred despite omission of central steps of the classical ECP procedure. However, shear stress seems to be one factor that is crucial for obtaining DC as such. Other crucial steps for the induction of dendritic cell formation seem to be the activation of platelets by plasma components and the activation of monocytes by such activated platelets. The data further suggests that, if shear-stress induced induction of dendritic cell formation takes place in the presence of 8-MOP and irradiation with UVA, expression of the Glucocorticoid-induced Leucine Zipper (GILZ) is increased, which in turn activates a pathway leading to formation of truncated, i.e. immuno-suppressive, tolerogenic DC (see Example 2). The fact that shear-stress induced induction of immuno-stimulatory DC could be achieved by applying shear stress without the addition of 8-MOP and without irradiation with UV-A further suggests that in the classical ECP procedure due to the dimensions of the plastic channels some of the initially shear-stress induced DC were

not effectively irradiated (maybe because they were moving in the middle of the channels of the ECP device) with the consequence that these DC could further develop into immuno-stimulatory DC (see Figure 16). This previous data was obtained using a device having the general architecture of Figure 17. However, in the classical ECP and ECP-like procedures, mixtures of immuno-stimulatory autologous and immuno-suppressive autologous DC were obtained. Based on the data presented hereinafter, it is now possible to e.g. dispense with some of the requirements of the ECP and ECP-like processes of the prior art, e.g. to use large amounts of blood which needs to be processed by apheresis such as leukaphereses. Further, one can now deliberately adapt the process parameters and the design of the device, which is used to exert a physical force on monocytes, to deliberately obtain either immuno-stimulatory autologous or immuno-suppressive autologous DC. For example, by selecting the design and dimensions of the flow chamber, one can make sure that basically all DC are contacted with 8-MOP and irradiated with UVA such that immuno-suppressive DC will form. Moreover, as the molecular markers being indicative of immuno-suppressive DC and of immuno-stimulatory autologous DC as they are obtainable by the methods described herein have been deciphered, one can further purify dendritic cell populations, which depending on the chosen method parameters will be substantially already only immuno-suppressive DC or immuno-stimulatory autologous DC, by e.f. FACS analysis to homogeneity.

The methods as described hereinafter may be performed without the need of molecular cocktails to achieve maturation and differentiation of monocytes into immuno-suppressive dendritic cells. Further, as the invention is based on inducing differentiation of monocytes contained in an extracorporeal quantity of mammalian subject's blood sample, the differentiation process is not limited to the molecular events which can be triggered by typical cytokine cocktails. Rather, dendritic cells as obtainable with the methods described hereinafter seem to have more complex molecular patterns, which seem representative of a broader functionality of these dendritic cells.

In a first aspect, the invention thus relates to a method for inducing differentiation of monocytes contained in an extracorporeal quantity of a mammalian subject's blood sample into immuno-suppressive dendritic cells, said method comprising at least the steps of:

a) subjecting said extracorporeal quantity of said mammalian subject's blood sample to a physical force such that said monocytes are activated and induced to differentiate into immuno-suppressive dendritic cells, which are identifiable by at least one molecular marker, wherein said at least one molecular marker is indicative of immuno-suppressive dendritic cells.

As has already been mentioned, the methods described hereinafter have been shown to produce immune-suppressive cells, which due to their molecular markers seem to be related to if not correspond to cells that are commonly named immune-suppressive or tolerogenic dendritic cells. Thus the immune-suppressive cells according to the invention have been named immune-suppressive dendritic cells. However, dendritic cells are representatives of a broader class of cells, which may be designated as antigen-presenting cells. Thus, the methods as described hereinafter generally refer to the production of immune-suppressive antigen-presenting cells with immune-suppressive dendritic cells being preferred.

The term "immuno-suppressive dendritic cells", which is used synonymous to tolerogenic, immuno-inhibiting, or truncated dendritic cells, thus refers to cells derivable from monocytes by treating the monocytes contained in an extracorporeal quantity of said mammalian subject's blood sample as it is described herein and identifiable by molecular markers as described in the following. These molecular markers include GILZ, IDO (Indoleamine) , KMO (kynurenine 3-hydroxylase), transforming growth factor beta (TGFß), and/or IL-10 (Interleukin 10), with GILZ being a preferred molecular marker. Thus, dendritic cells as they are obtainable by the methods described herein, which show an increased expression of GILZ, IDO,

KMO, TGFß, and/or IL-10, preferably of GILZ are considered to be immuno-suppressive dendritic cells. It is to be understood that an increased expression is to be determined in comparison to the monocytes before they are subjected to the methods described herein. Such immuno-suppressive dendritic cells may also show an GILZ-induced increased IL-10 to IL-12p70 ratio or a decreased production of various pro-inflammatory cytokines and chemokines such as IL-12, TNF-α, and IL-6. The preferred molecular marker, which is considered indicative for immune-suppressive dendritic cells, is currently GILZ. As will be explained further below in more detail the immuno-suppressive dendritic cells may be autologous and/or allogenic immuno-suppressive dendritic cells. Autologous immuno-suppressive dendritic cells may be produced by the methods described herein for treatment of e.g. autoimmune diseases. Allogenic immuno-suppressive dendritic cells may be produced by the methods described herein for treatment of e.g. Graft-versus-Host disease. It needs to be understood that where immuno-suppressive antigen-presenting cells such as dendritic cells are mentioned herein, this refers to immuno-suppressive antigen-presenting cells such as dendritic cells which have the capacity of displaying e.g. disease-specific antigens in their surfaces after these cells have been contacted with such antigens. It is to be further understood that immuno-suppressive dendritic cells as obtainable by the methods described herein and identifiable by the molecular markers described herein in one embodiment may be considered as dendritic cells which have already differentiated enough and internalized and even display e.g. antigens from e.g. cells being involved in an autoimmune disease or antigens of cells from a recipient in e.g. a Graft-versus-Host disease application, such that they can be considered more generally to be immuno-suppressive autologous and/or allogenic antigen-presenting cells. These immuno-suppressive autologous and/or allogenic antigen-presenting cells will nevertheless be of a tolerogenic type and thus suppress an immune answer against the displayed antigens. The term "immuno-suppressive autologous and/or allogenic dendritic cells" in one embodiment thus encompasses immuno-suppressive autologous and/or allogenic antigen-presenting dendritic cells. However, the process can also be conducted in a way such that the dendritic cells

express molecular markers indicative of immuno-suppressive autologous and/or allogenic dendritic cells, which have not yet internalized and displayed antigens, because e.g. the cells were obtained from a sample of a donor that does not suffer from an auto-immune disease or Graft-versus-host disease. However, the insight provided by the data and conclusions of the present invention allows to make immuno-suppressive antigen-presenting cells such as dendritic cells, which then can be loaded with antigens characteristic an auto-immune disease or with antigens from a donor in a transplantation setting, which have been obtained separately and not concurrently with the immuno-suppressive antigen-presenting cells such as dendritic cells. Such antigens may then be co-incubated with immuno-suppressive antigen-presenting cells such as dendritic cells to effect their presentation in the subsequent therapeutic setting.

That dendritic cells, which overexpress GILZ, IDO, KMO, PDL1, PDL2, TGFß and/or IL-10, preferably GILZ and which have been obtained by activating monocytes using processes described herein, show properties reminiscent of tolerogenic dendritic cells can be taken *inter alia* from the observation that such dendritic cells expressing GILZ are resistant to full maturation as it can be induced by e.g. LPS and be determined by increased expression of HLA-DR, CD83, CD80 and CD86. Further, the upregulation of GILZ and the decreased production of various pro-inflammatory cytokines and chemokines such as IL-12, TNF-α, and IL-6 are reminiscent of the known effects of dexamethasone, which can be used to obtain tolerogenic dendritic cells (Cohen et al., *Blood* (2006), 107(5), 2037-2044).

Thus, immuno-suppressive dendritic cells can additionally be distinguished from immuno-stimulatory autologous dendritic cells in that they do not show an increased expression of molecular markers, which are indicative of immuno-stimulatory autologous dendritic cells. It is to be understood that an expression is to be determined in comparison to the monocytes before they are subjected to the methods described herein.

The term "immuno-stimulatory autologous dendritic cells" refers to dendritic cells, which can be obtained by activating monocyte using the methods described herein and which show an increased expression of molecular markers, which are indicative of immuno-stimulatory autologous dendritic cells. These molecular markers, which are indicative of immuno-stimulatory autologous dendritic cells, have been discussed in the literature for dendritic cells, which can present antigens by way of MHC I and MHC II. It is to be understood that immuno-stimulatory autologous dendritic cells as obtainable by the methods described herein and identifiable by the molecular markers described herein may be considered as dendritic cells which have already differentiated enough and internalized and even display e.g. tumor-specific antigens from apoptotic cells such as cytotoxic T-cells, which are contained in the extracorporeal quantity of a respective mammalian subject's blood sample, or e.g. viral or bacterial antigens, which are contained in the extracorporeal quantity of a respective mammalian subject's blood sample, such that they can be considered to be immuno-stimulatory autologous antigen-presenting dendritic cells. However, the process can also be conducted in a way such that the dendritic cells express molecular markers indicative of immuno-stimulatory dendritic cells, which have not yet internalized and display antigens. The term "immuno-stimulatory autologous dendritic cells" in one embodiment thus encompasses immuno-stimulatory autologous antigen-presenting dendritic cells. It is to be understood that the designation of immuno-stimulatory dendritic cells as immuno-stimulatory autologous dendritic cells depends on whether the obtained immuno-stimulatory dendritic cells are later re-introduced into the same donor. This can be done in a continous or discontinous fashion where the dendritic cells are cultivated for extended periods of time before they are re-introduced into the donor. All of the issues discussed hereinafter for immuno-stimulatory dendritic cells refer to immuno-stimulatory autologous dendritic cells. It is, however, to be understood that the discussion of such embodiments always includes the scenario where the processes are used to make immuno-stimulatory dendritic cells as such and where only the later administration

of these cells will make them potentially immuno-stimulatory autologous dendritic cells.

The present invention allows preferential production of immuno-suppressive DC relative to immuno-stimulatory DC. The preferential production of immuno-suppressive dendritic cells relative to immuno-stimulatory dendritic cells means that starting from an extracorporeal amount of blood sample, more immuno-suppressive dendritic cells than immuno-stimulatory DC may be selectively obtained compared to a situation where e.g. the same extracorporeal amount of blood sample was subjected to a classical ECP procedure. Even though production of immuno-suppressive DC will be produced preferentially relative to immuno-stimulatory DC, immuno-stimulatory DC may be still present after the methods in accordance with the invention have been performed. Nevertheless, the present invention provides the parameters and variables that can be manipulated to skew production of one dendritic cell population over the other. Further, the eventually remaining immuno-stimulatory dendritic cells may be removed by e.g. affinity purification against molecular markers that are indicative of immuno-stimulatory dendritic cells.

As is described in the examples, molecular marker which are indicative of immuno-stimulatory autologous dendritic cells obtainable by the methods described herein were identified by subjecting monocytes contained in the extracorporeal quantity of mammalian subjects' blood samples derived either from healthy volunteers to the process using a miniaturized device (see markers 88 to 99 of Table 1). Further, as is also described in the example, molecular markers, which are indicative of immuno-stimulatory autologous dendritic cells, were identified by subjecting monocytes contained in the extracorporeal quantity of mammalian subjects' blood samples derived either from healthy volunteers or from patients suffering from CTCL to an ECP process. It is also described in the example, how molecular markers, which are indicative of immuno-suppressive dendritic cells, were identified by subjecting monocytes contained in the extracorporeal quantity of mammalian subjects' blood

samples derived either from healthy volunteers or from patients suffering from GvH disease (GvHD)to an ECP process (see markers 1 to 87 of Table 1). The dendritic cells were then isolated and up-regulated expression of molecular markers, which are known or suspected to play a role in immuno-stimulatory dendritic cells, was analyzed.

Some of the markers identified for the ECP process, which is assumed to lead to a complex mixture of immune-stimulatory and immune-suppressive dendritic cells, are the same as they were observed for the dendritic cells obtained by the process with the miniaturized device, which should lead to immune-stimulatory dendritic cells only. Thus to the extent that the ECP process leads to up-regulation of molecular markers, which can be associated with dendritic cell function, it seems justified to assume that these markers will also be suitable to identify immune-stimulatory dendritic cells as they are obtainable by the processes described herein such as with the miniaturized device. A set of overall 99 molecular markers was identified as being upregulated for immuno-stimulatory autologous dendritic cells obtainable by methods described herein. This set may be extended by further molecular markers in the future through comparable analysis

Thus, the data of examples 1 and 3 lead to a set of 99 markers, which are considered indicative of immuno-stimulatory autologous dendritic cells. These markers are summarized in Table 1.

Table 1

No.	Marker	NCBI Gene ID No.	mRNA REF	SEQ ID No.
1	ABCA1	19	NM_005502.3	1
2	ACVR1B	91	NM_004302.4	2
3	ANPEP	290	NM_001150.2	3
4	AQP9	366	NM_020980.3	4
5	ATP6V0B	533	NM_001039457.1	5
6	BASP1	10409	NM_001271606.1	6
7	BEST1	7439	NM_001139443.1	7
8	CD63	967	NM_001257389.1	8

9	CD68	968	NM_001040059.1	9
10	CDCP1	64866	NM_022842.3	10
11	CPM	1368	NM_001005502.2	11
12	CRK	1398	NM_005206.4	12
13	CSF2RA	1438	NM_001161529.1	13
14	CTNND1	1500	NM_001085458.1	14
15	CTSB	1508	NM_001908.3	15
16	CXCL16	58191	NM_001100812.1	16
17	EMP1	2012	NM_001423.2	17
18	ENG	2022	NM_000118.2	18
19	EPB41L3	23136	NM_012307.2	19
20	FLOT1	10211	NM_005803.2	20
21	GNA15	2769	NM_002068.2	21
22	GPNMB	93695	NM_053110.4	22
23	GPR137B	83924	NM_031999.2	23
24	GPR157	269604	NM_177366.3	24
25	HEXB	3074	NM_000521.3	25
26	HOMER3	9454	NM_001145721.1	26
27	ICAM1	3383	NM_000201.2	27
28	IL1R1	3554	NM_000877.2	28
29	IRAK1	3654	NM_001025242.1	29
30	ITGA5	3678	NM_002205.2	30
31	ITGB8	3696	NM_002214.2	31
32	KCTD11	147040	NM_001002914.2	32
33	LAMP2	3920	NM_001122606.1	33
34	LEPROT	54741	NM_001198681.1	34
35	LGALS3	3958	NM_001177388.1	35
36	LILRB4	11006	NM_001081438.1	36
37	MARCKSL1	65108	NM_023009.6	37
38	MCOLN1	57192	NM_020533.2	38
39	MFAP3	4238	NM_001135037.1	39
40	MGAT4B	11282	NM_014275.4	40
41	MR1	3140	NM_001194999.1	41
42	MRAS	22808	NM_001085049.2	42
43	MSR1	4481	NM_002445.3	43
44	NEU1	4758	NM_000434.3	44
45	NPC1	4864	NM_000271.4	45
46	OLR1 (LOX1)	4973	NM_001172632.1	46
47	OMG	4974	NM_002544.4	47
48	P2RX4	5025	NM_001256796.1	48
49	PI4K2A	55361	NM_018425.2	49
50	PLAUR	5329	NM_001005376.2	50
51	PMP22	5376	NM_000304.2	51
52	PPAP2B	8613	NM_003713.4	52
53	PSEN1	5663	NM_000021.3	53
54	PVRL2	5819	NM_001042724.1	54
55	RAB13	5872	NM_002870.2	55
56	RAB8B	51762	NM_016530.2	56
57	RAB9A	9367	NM_001195328.1	57
58	RALA	5898	NM_005402.3	58
59	RHEB	6009	NM_005614.3	59

60	RNASE1	6035	NM_002933.4	60
61	SC5DL	6309	NM_001024956.2	61
62	SDC2	6383	NM_002998.3	62
63	SEMA6B	10501	NM_032108.3	63
64	SIRPA	140885	NM_001040022.1	64
65	SLC17A5	26503	NM_012434.4	65
66	SLC1A4	6509	NM_001193493.1	66
67	SLC22A4	6583	NM_003059.2	67
68	SLC31A1	1317	NM_001859.3	68
69	SLC35E3	55508	NM_018656.2	69
70	SLC39A6	25800	NM_001099406.1	70
71	SLC6A6	6533	NM_001134367.1	71
72	SLC6A8	6535	NM_001142805.1	72
73	SLC7A11	23657	NM_014331.3	73
74	STX3	6809	NM_001178040.1	74
75	STX6	10228	NM_005819.4	75
76	TM9SF1	10548	NM_001014842.1	76
77	TMBIM1	64114	NM_022152.4	77
78	TMEM33	55161	NM_018126.2	78
79	TNFRSF10B	8795	NM_003842.4	79
80	TNFRSF11A	8792	NM_001270949.1	80
81	TNFRSF1A	7132	NM_001065.3	81
82	TNFRSF1B	7133	NM_001066.2	82
83	TNFSF14	8740	NM_003807.3	83
84	TNFSF9	8744	NM_003811.3	84
85	TRIP10	9322	NM_004240.2	85
86	TRIP6	7205	NM_003302.2	86
87	YKT6	10652	NM_006555.3	87
88	DC-LAMP (LAMP3)	27074	NM_014398.3	88
89	CLEC5A	23601	NM_013252.2	89
90	SPC2 (PCSK2)	5126	NM_002594.3	90
91	THBS1	7057	NM_003246.2	91
92	CD14	929	NM_000591.3	92
93	CD40	958	NM_001250.4	93
94	CD80	941	NM_005191.3	94
95	CCR7	1236	NM_001838.3	95
96	CD83	9308	NM_001251901.1	96
97	ADAM Decysin	27299	NM_014479.3	97
98	FPRL2 (FPR3)	2359	NM_002030.3	98
99	CD86	942	NM_006889.4	99

Of the 87 genes (markers 1 to 87) that represent surface markers /functional mediators of immunostimulatory DC function, 66 were found to be uniquely identified in the ECP-induced process (plate passaged, overnight cultured, see example) dendritic cells, after comparison to expression databases for “classical” dendritic cells. These are: ABCA1 , ACVR1B, ATP6V0B, BASP1, BEST1, CPM,

CRK, CSF2RA, CTNND1, CTSB, CXCL16, ENG, FLOT1, GNA15, GPR137B, GPR157, HEXB, HOMER3, ICAM1, IRAK1, ITGA5, ITGB8, KCTD11, LAMP2, LEPROT, MARCKSL1, MCOLN1, MFAP3, MGAT4B, MR1, MRAS, MSR1, NEU1, OLR1, OMG, PI4K2A, PLAUR, PMP22, PVRL2, RAB13, RAB8B, RAB9A, RALA, RNASE1, SC5DL, SEMA6B, SIRPA, SLC1A4, SLC22A4, SLC31A1, SLC35E3, SLC39A6, SLC6A6, SLC6A8, STX3, STX6, TM9SF1, TMBIM1, TMEM33, TNFRSF10B, TNFRSF11A, TNFRSF1A, TNFRSF1B, TNFSF14, TNFSF9, YKT6.

Immuno-suppressive dendritic cells can thus be distinguished from immune-stimulatory dendritic cells by determining expression of at least one molecular marker for the immuno-stimulatory autologous dendritic cells obtainable by the methods described herein and by comparing its expression for monocytes contained within the extracorporeal quantity of a mammalian subject's blood sample. If no increased expression of these markers is observed for presumed immuno-suppressive dendritic cells vs. monocytes, this is indicative of the differentiation of monocytes to immuno-suppressive dendritic cells.

Immuno-suppressive dendritic cells, in addition to increased expression of GILZ (SEQ ID No.: 100), , IDO (SEQ ID No.: 101), KMO (SEQ ID No.: 102), TGFß (SEQ ID No.: 103) and/or IL-10 (SEQ ID No.: 104), preferably of GILZ are thus identifiable by determining expression of at least one molecular marker for the immuno-stimulatory autologous dendritic cells obtainable by the methods described herein and by comparing its expression for monocytes contained within the extracorporeal quantity of a mammalian subject's blood sample. If no increased expression of such molecular markers for dendritic cells vs. monocytes is observed, this is indicative of the differentiation of monocytes to immuno-suppressive autologous and/or allogenic dendritic cells.

Immuno-stimulatory autologous dendritic cells are identifiable by determining expression for at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50 or more molecular markers selectable from Table 1. For example, one may identify immuno-stimulatory autologous dendritic cells by determining expression for at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21 or 22 molecular markers selectable from the group comprising PLAUR, NEU1, CTSB, CXCL16, ICAM1, MSR1, OLR1, SIRPA, TNFRSF1A, TNFSF14, TNFSF9, PMB22, CD40, LAMP3, CD80, CCR7, LOX1, CD83, ADAM Decysin, FPRL2, GPNMB and/or CD86. More preferably, one may identify immuno-stimulatory autologous dendritic cells by determining expression for at least 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 molecular markers selectable from the group comprising PLAUR, NEU1, CD80, CCR7, LOX1, CD83, ADAM Decysin, FPRL2, GPNMB and/or CD86. The most preferred markers which are considered indicative of immuno-stimulatory autologous dendritic cells are PLAUR, NEU1, CD80, CD83, and/or CD86. The data and conclusions presented herein suggest that the process of obtaining immuno-stimulatory dendritic cells seems to include a global monocyte activation step and a monocyte to immuno-stimulatory antigen-presenting cell (e.g. dendritic cell) differentiation step. These different steps seem to be traceable by molecular markers as described above and by Forward Scattering/Side Scattering Complexity (FSC/SSC Complexity) which is determinable by FACS analysis. The molecular markers may moreover be be grouped according to their know function as e.g. molecular markers of antigen-presentation, molecular markers of cellular adhesion etc.. HLA-DR, CD86, and CD 80 may be considered to representative of antigen-presentation. PLAUR, and ICAM-1 may be considered to representative of cell adhesion. Markers like HLA-DR, PLAUR and ICAM-1 as well as FSC/SSC complexity may be moreover considered to be indicative of global monocyte activation while increased expression of e.g. CD83, ADAM-Decysin, CD40, CD80, LAMP-3, and CCR7 seems indicative of monocyte to dendritic cell differentiation.

The immuno-stimulatory suppressive dendritic cells as they are obtainable by the methods described herein can thus not only be positively identified by molecular markers, which are indicative of immune-suppressive dendritic cells such as GILZ, IDO, KMO, TGFß, and/or IL-10, but also by the absence of up-regulation of molecular markers, which are indicative of immune-stimulatory dendritic cells such as PLAUR, CD80 and CD83. Further both of these cell types, i.e. immune-suppressive and immune-stimulatory dendritic cells can be distinguished from the monocytes, which are subjected to a physical force to induce the differentiation process thereof, by determining the expression of molecular markers which are considered indicative of monocytes such as CD33 , CD36, and/or FCGR1a (Receptor for IgGFc fragment 1A). If it is found that the expression of these factors is down-regulated compared to expression of the monocytes, before they have been subjected to a physical force and process as described herein, then this is considered indicative that the monocytes have entered the maturation and differentiation pathway towards immune-stimulatory and/or immuno-suppressive dendritic cells. The distinction between these later two dendritic cell population can then be made by determining expression of molecular markers such as PLAUR, CD80, CD83 and GILZ.

As mentioned above, the method as described hereinafter may be performed without the need of molecular cocktails to achieve maturation and differentiation of monocytes into immuno-suppressive autologous and/or allogenic dendritic cells. Such cocktails may comprise factors such as e.g. dexamethasone IL-10, IL-4 or TGF-ß. However, in one embodiment it is considered to add such maturation cocktails to the immuno-suppressive autologous and/or allogenic dendritic cells as they are obtainable by methods in accordance with the invention, e.g. to push the differentiation towards a certain dendritic cell profile that can be achieved with such cocktails.

Given that one now has the understanding and correspondingly the tools, e.g. the molecular markers at hand to distinguish between immuno-suppressive autologous

and/or allogenic dendritic cells and immuno-stimulatory autologous dendritic cells, one can now deliberately vary both the design of the device and the flow chamber through which the extracorporeal quantity of a mammalian subject's blood sample and thus the monocytes are passed to experience a physical force, and the parameters at which the process of inducing differentiation of monocytes into immuno-suppressive autologous and/or allogenic dendritic cells is performed, to purposively enable differentiation of monocytes into immuno-suppressive autologous and/or allogenic dendritic cells.

As mentioned above, an extracorporeal quantity of a mammalian subject's blood sample is passed through a flow chamber of a device, such that a shear force is applied to said monocytes contained within said mammalian subject's blood sample. Alterations of the design of the device and the flow chamber which have an influence on the differentiation of monocytes into immuno-suppressive autologous and/or allogenic dendritic cells include variation of flow forces, variation of the geometry of the flow path of the flow chamber, variation of the dimensions of the flow chamber, the possibility to adjust temperature, the possibility of exposure of the extracorporeal quantity of the mammalian subject's blood sample in the flow chamber to visible or UV light, etc.. Application of a physical force may not only be achieved by e.g. passing an extracorporeal amount of blood sample through a flow chamber, but also by placing such an extracorporeal amount of blood sample in e.g. an EVA plastic bag as obtainable from Macopharma and gently moving or shaking this blood sample-filled bag (see e.g. Andreu et al., (1994), *Trans. Sci.*, 15(4), 443-454)

As also mentioned above and shown hereinafter, activation of monocytes and induction of differentiation into immuno-suppressive autologous and/or allogenic dendritic cells is dependent on interaction of monocytes with activated platelets and/or specific plasma components in a situation where the monocytes experience physical force which may be provided by a device as described hereinafter. Variation of process parameters thus include varying the nature, purity and concentrations of

plasma components; the nature, purity and concentration of platelets; the order of steps by which plasma components and/or platelets are passed through and/or disposed on the flow chamber; the density by which the flow chamber is coated with plasma components and/or platelets, the flow forces of the extracorporeal quantity of the mammalian subject's blood sample and in particular the platelets and/or the monocytes are passed through the flow chamber of such a flow chamber, the temperature and/or time at which the extracorporeal quantity of the mammalian subject's blood sample and in particular the platelets and/or the monocytes are passed through the flow chamber of such a device, etc., the nature, purity and concentrations of additional factors such as 8-MOP and/or cytokines are added to the extracorporeal quantity of the mammalian subject's blood sample and in particular to the monocytes, etc.

Factors relating to the design of the device and the flow chamber as well as to process parameter will now be discussed in more detail as regards their relevance for the differentiation of monocytes into immuno-suppressive autologous and/or allogenic dendritic cells. It is to be understood that for any of the embodiments discussed in the following differentiation of monocytes into immuno-suppressive autologous and/or allogenic dendritic cells is achieved wherein immuno-suppressive autologous and/or allogenic dendritic cells are identifiable by determining expression of at least GILZ, IDO, KMO, TGFß, and/or IL-10, preferably of GILZ and/or by determining expression of molecular markers of Table 1. If e.g. expression of GILZ is increased and if expression of molecular markers of Table 1 is not increased, this is considered indicative that monocytes have differentiated into immuno-suppressive autologous and/or allogenic dendritic cells. Further, for all embodiments discussed in the following it is to be understood that monocytes that are contained in an extracorporeal quantity of a mammalian subject's blood sample are subjected to a physical force such as shear stress in order to allow them to differentiate into dendritic cells, e.g. upon interaction with activated platelets and/or plasma components.

In one embodiment of the first aspect, the invention relates to a method of inducing differentiation of monocytes contained in an extracorporeal quantity of a mammalian subject's blood sample into immuno-suppressive autologous and/or allogenic dendritic cells, wherein said extracorporeal quantity of said mammalian subject's blood sample is subjected to a physical force by passing said extracorporeal quantity of said mammalian subject's blood sample through a flow chamber of a device, which allows adjustment of the flow rate of said extracorporeal quantity of said mammalian subject's blood sample through said flow chamber of said device such that a shear force is applied to said monocytes contained within said mammalian subject's blood sample.

In another embodiment of the first aspect, the invention relates to a method of inducing differentiation of monocytes contained in an extracorporeal quantity of a mammalian subject's blood sample into immuno-suppressive autologous and/or allogenic dendritic cells, wherein said extracorporeal quantity of said mammalian subject's blood sample is subjected to a physical force by passing said extracorporeal quantity of said mammalian subject's blood sample through a flow chamber of a device, which allows adjustment of the flow rate of said extracorporeal quantity of said mammalian subject's blood sample through said flow chamber of said device such that a shear force is applied to said monocytes contained within said mammalian subject's blood sample, and wherein said flow chamber of said device has a design allowing to apply a shear force to said monocytes contained within said mammalian subject's blood sample.

In another embodiment of the first aspect, the invention relates to a method of inducing differentiation of monocytes contained in an extracorporeal quantity of a mammalian subject's blood sample into immuno-suppressive autologous and/or allogenic dendritic cells, wherein said extracorporeal quantity of said mammalian subject's blood sample is subjected to a physical force by passing said extracorporeal

quantity of said mammalian subject's blood sample through a flow chamber of a device, which allows adjustment of the flow rate of said extracorporeal quantity of said mammalian subject's blood sample through said flow chamber of said device such that a shear force is applied to said monocytes contained within said mammalian subject's blood sample, and wherein said device additionally allows for adjustment of at least one parameter selected from the group comprising temperature, and light exposure.

In another embodiment of the first aspect, the invention relates to a method of inducing differentiation of monocytes contained in an extracorporeal quantity of a mammalian subject's blood sample into immuno-suppressive autologous and/or allogenic dendritic cells, wherein said extracorporeal quantity of said mammalian subject's blood sample is subjected to a physical force by passing said extracorporeal quantity of said mammalian subject's blood sample through a flow chamber of a device as mentioned before and wherein said monocytes are activated and induced to differentiate into immuno-suppressive autologous and/or allogenic dendritic cells through interaction with activated platelets and/or plasma components.

For all of these embodiments and for all of the embodiment discussed in the following, it is preferred to subject the monocyte contained within the extracorporeal amount of a mammalian's blood sample to a physical force such as shear stress in the presence of DNA-cross linking agent such as 8-MOP and exposing the monocytes to light, preferably to UVA light such that an increased expression of GILZ is effected. The design and dimensions of the flow chamber for all of these embodiments should be preferably selected such that substantially all of the monocytes are in contact with a DNA-cross linking agents such as 8-MOP and that substantially all of the monocytes are exposed to light, preferably to UVA.

For example, in one embodiment of the first aspect, the invention relates to a method of inducing differentiation of monocytes contained in an extracorporeal quantity of a

mammalian subject's blood sample into immuno-suppressive autologous and/or allogenic dendritic cells, wherein said method comprises at least the steps of:

a) applying said extracorporeal quantity of said mammalian subject's blood sample comprising at least monocytes to a device, which is configured to provide for a flow chamber through which said extracorporeal quantity of said mammalian subject's blood sample can be passed,

b) activating platelets, which may be comprised within said extracorporeal quantity of said mammalian subject's blood or which may be provided separate from said mammalian subject's blood sample comprising at least monocytes,

c) treating said extracorporeal quantity of said mammalian subject's blood sample comprising at least monocytes in said device by applying a physical force to the monocytes contained within said extracorporeal quantity of said mammalian subject's blood sample such that said monocytes are activated and induced to differentiate into immuno-suppressive autologous and/or allogenic dendritic cells by binding to said activated platelets obtained in step b).

In another embodiment of the first aspect, the invention relates to a method of inducing differentiation of monocytes contained in an extracorporeal quantity of a mammalian subject's blood sample into immuno-suppressive autologous and/or allogenic dendritic cells, wherein said method comprises at least the steps of:

a) applying said extracorporeal quantity of said mammalian subject's blood sample comprising at least monocytes to a device, which is configured to provide for a flow chamber through which said extracorporeal quantity of said mammalian subject's blood sample can be passed,

b) passing plasma components, which may be comprised within said extracorporeal quantity of said mammalian subject's blood sample or which may be provided separate from said mammalian subject's blood sample,

c) treating said extracorporeal quantity of said mammalian subject's blood sample comprising at least monocytes in said device by applying a physical force to the monocytes contained within said extracorporeal quantity of said mammalian subject's blood sample such that said monocytes are activated and induced to differentiate into immuno-suppressive autologous and/or allogenic dendritic cells by binding to said plasma components obtained in step b).

In yet another embodiment of the first aspect, the invention relates to a method of inducing differentiation of monocytes contained in an extracorporeal quantity of a mammalian subject's blood sample into immuno-suppressive autologous and/or allogenic dendritic cells, wherein said method comprises at least the steps of:

a) applying said extracorporeal quantity of said mammalian subject's blood sample comprising at least monocytes to a device, which is configured to provide for a flow chamber through which said extracorporeal quantity of said mammalian subject's blood sample can be passed,

b) passing plasma components, which may be comprised within said extracorporeal quantity of said mammalian subject's blood or which may be provided separate from said mammalian subject's blood sample,

c) activating platelets, which may be comprised within said extracorporeal quantity of said mammalian subject's blood sample or which may be provided separate from said mammalian subject's blood sample comprising at least monocytes,

d) treating said extracorporeal quantity of said mammalian subject's blood comprising at least monocytes in said device by applying a physical force to the monocytes contained within said extracorporeal quantity of said mammalian subject's blood sample such that said monocytes are activated and induced to differentiate into immuno-suppressive autologous and/or allogenic dendritic cells by binding to said activated platelets and/or plasma components obtained in steps b) and c).

In yet another embodiment of the first aspect, the invention relates to a method of inducing differentiation of monocytes contained in an extracorporeal quantity of a mammalian subject's blood sample into immuno- suppressive autologous and/or allogenic dendritic cells, wherein said method comprises at least the steps of:

a) optionally passing platelets-rich plasma through a a device, which is configured to provide for a flow chamber through which said extracorporeal quantity of said mammalian subject's blood sample can be passed,

b) applying said extracorporeal quantity of said mammalian subject's blood sample comprising at least monocytes to a device, which is configured to provide for a flow chamber through which said extracorporeal quantity of said mammalian subject's blood sample can be passed,

c) treating said extracorporeal quantity of said mammalian subject's blood comprising at least monocytes in said device by applying a physical force to the monocytes contained within said extracorporeal quantity of said mammalian subject's blood sample such that said monocytes are activated and induced to differentiate into immuno-suppressive autologous and/or allogenic dendritic cells optionally by binding to said platelets-rich plasma obtained of step a).

As can be taken from the experiment described herein, the method for inducing differentiation of monocytes contained in an extracorporeal quantity of a mammalian subject's blood into immuno-suppressive autologous and/or allogenic dendritic cells works optimal, if platelets which are comprised within said extracorporeal quantity of said mammalian subject's blood are activated and if the extracorporeal quantity of said mammalian subject's blood comprising at least monocytes in said device is treated by applying a physical force to the monocytes contained within said extracorporeal quantity of said mammalian subject's blood such that said monocytes are activated and induced to differentiate into dendritic cells by binding to said activated platelets. However, activation of monocytes may also be achieved by direct

interaction with plasma components, i.e. without interaction with activated platelets. These dendritic cells can then be made embarking on differentiation into immuno-suppressive autologous and/or allogenic dendritic cells by exposing the monocytes additionally to DNA-cross linking agents such as 8-MOP and light such as UVA light.

The steps of activating platelets and the subsequent activation and differentiation of monocytes into dendritic cells will be discussed in the following for the embodiment that (i) plasma components such as plasma proteins are passed through the flow chamber of the device so that these components adhere to the walls of the flow chamber, that (ii) platelets are passed through the flow chamber and are activated by binding to the plasma components and that (iii) monocytes-containing fractions such as an extracorporeal quantity of said mammalian subject's blood comprising at least monocytes are passed through the flow chamber and are activated for differentiation into dendritic cells by binding to the activated platelets. It is, however, to be understood that these activities also occur if the plasma fraction or plasma proteins or fragments thereof, the platelet fraction and the monocytes-containing fraction are passed simultaneously through the channels or channel-like structures as is the case for a whole blood fraction if obtained from the extracorporeal amount of blood as described below. It is further to be understood that the process may be performed even though not with same effectiveness by adhering only plasma components to the walls of the flow chamber and letting monocytes interact with the plasma components. Nevertheless, in the following these aspect will be discussed for a preferred embodiment, i.e. where steps (i), (ii), and (iii) are realized.

As regards the first step, plasma components including proteins like fibrinogen or fibronectin, or fragments thereof like the gamma component of fibrinogen may be provided either as fractions obtained from the extracorporeal amount of blood sample or in purified form from other resources e.g. in the form of recombinantly expressed proteins. Even though it seems that activation of platelets by plasma proteins such as

fibrinogen and fibronectin is sufficient so that recombinantly expressed forms of these proteins are sufficient, it can be preferred to use plasma fractions which are obtained from the extracorporeal amount of blood sample and comprise these proteins as these plasma fractions have a more complex composition and may comprise all plasma components, which provide for an optimal activation of platelets.

Plasma protein fractions, plasma proteins or fragments thereof may be passed through the flow chamber, which may be made of plastic or non-plastic materials such as glass in order to adhere to the walls of the channels or channel-like structures. There is no requirement that the plasma fractions or plasma proteins are passed through the flow chamber at a specific physical force such as e.g. a specific pressure. However, in order to streamline the process, it is envisaged to pass the plasma fractions or plasma proteins through the flow chamber at a shear stress which is comparable if not identical to the shear stress required for monocyte activation being described in more detail below. In general, the plasma fractions or plasma proteins are first pumped through the flow chamber to coat the surfaces thereof with plasma proteins, including fibronectin and fibrinogen. The flow rate of the plasma protein fractions, plasma proteins or fragments thereof through the flow chamber is controlled to obtain a desired level of protein adherence to the plastic surfaces. If desired, the flow can be stopped for a period of time and the plasma component can "soak" the surfaces of the flow chamber. By controlling the speed and timing of the pump that propels the plasma components through the flow chamber, the degree of coating of can be controlled. In one approach, the plasma fractions or plasma proteins are exposed to the surfaces of the flow chamber structures for a period between about 1 to 60 min, between about 1 to about 30 min, between about 1 to about 20 min, or between about 1 to about 10 min. To enhance plasma protein adherence to the surfaces of the flow chamber, the flow may be temporarily discontinued (for up to about 60 min), before resumption, or the flow rate may be

slowed from the filling rate (up to 100 ml/minute) to as low as 5 ml/minute, during this phase of the procedure.

One can also envisage a scenario, where a device with a flow chamber is used for which the surfaces of the flow chamber have been pre-coated with e.g. purified plasma proteins or fragments thereof such as the gamma component of fibrinogen. Such pre-coated devices may be used if the whole process s conducted in a handheld device comprising a cartridge providing the flow chamber which is configured for e.g. one time use. One can also envisage a scenario, where a device with a flow chamber is used for which the surfaces of the flow chamber have been pre-coated with e.g. platelets-rich plasma.

After the plasma fractions or plasma proteins or fragments thereof have been passed through the channels or channel-like structures and the surfaces thereof have been coated with plasma proteins, the platelet fraction is passed by e.g. pumping into and through the flow chamber. The flow rate and residence time of the platelets within the flow chamber is selected to allow the platelets to bind to the plasma components or proteins or fragments thereof which have adhered before to the surfaces of the flow chamber and to thereby become activated.

The data presented herein suggest that activation of platelets by plasma components is a sequential process in which inactivated platelets first bind to the gamma component of fibronectin, get activated thereby and can then bind to the RGD motif (Arginine, Glycine, Aspartic Acid) which is found in many plasma proteins such as fibronectin or fibrinogen. If purified and/or recombinantly expressed plasma proteins or fragments thereof are used for activation of platelets, it can therefore be envisaged to pre-coat channels or channel-like structures with at least the gamma-component of fibrinogen and optionally additionally with RGD peptides. These plasma protein fragments and peptides may allow for efficient activation of platelets and at the same

time for an optimal control of the coating process of the surfaces of the channels or channel-like structures. Of course, all of these components are present if a plasma fraction obtained from the extracorporeal amount of blood is used for coating and activation.

For efficient binding of the platelets to the plasma components and activation thereby, the flow rate may be adjusted upward or downward compared to the coating step of the plasma components, or flow may be stopped for a period of time, to obtain the desired level of platelets bound to the plasma components. The flow rates for plasma activation will typically be in the range of about 5 ml/min to about 200 ml/min, of about 10 ml/min to about 150 ml/min, of about 10 ml/min to about 100 ml/min, or of about 5 ml/min to about 50 ml/min. Typically, it will be desirable to allow between about 1 to 60 min, between about 1 to about 30 min, between about 1 to about 20 min, or between about 1 to about 10 min for the platelets to bind to the plasma components.

Even though shear stress does not seem to of the same importance for activation of platelets as for activation of monocytes, it can be preferred to pass the platelets fraction through the flow chamber under a shear force of about 0.1 to about 20.0 dynes/cm^2, of about 0.2 to about 15.0 dynes/cm^2, of about 0.3 to about 10.0 dynes/cm^2 such as from about 0.2 to about 0.4, to about 0.5, to about 0.6, to about 0.7, to about 0.8, to about 0.9, to about 1, to about 2, to about 3, to about 4, to about 5, or to about 6 dynes/cm^2. Typical flow rates of the platelets-containing fraction may be in the range of about 5 ml/min to about 200 ml/min, of about 10 ml/min to about 150 ml/min, of about 10 ml/min to about 100 ml/min, or of about 5 ml/min to about 50 ml/min. The flow rates will depend to some extent on the size and geometry of the flow chamber and can particularly be used if flow chamber of the below-mentioned dimensions are used. In general, one will select flow rates to achieve the afore-mentioned shear stress values.

Thus, it is contemplated to pass the platelets-containing fraction through the channels or channel-like structures with a flow rate of about 10 ml/minute to about 200 ml/minute to produce a shear force of about 0.1 to about 10.0 dynes/cm^2.

After the platelets have been passed through the channels or channel-like structures and have been activated by the plasma proteins or fragments thereof, which have been disposed on the surfaces of the channels or channel-like structures thereof, the monocytes-containing fraction, e.g. the extracorporeal quantity of said mammalian subject's blood sample or the below-mentioned leukocyte or buffy coat fraction, which have been obtained from the extracorporeal amount of blood sample, is passed by e.g. pumping into and through the channels or channel-like structures, by applying a physical force. It is to be understood that activation of platelets through interaction with plasma components will lead to adherence of platelets to plasma components.

It is also to be understood that the same events as described above will happen if an extracorporeal quantity of a mammalian subject's blood sample comprising platelets and plasma components is passed through the flow chamber. In this case, plasma components will adhere to the walls to the flow chamber and then activate platelets. However, in this scenario the process may be less controllable and account may be taken of this by increasing the residence time of the extracorporeal quantity of a mammalian subject's blood sample comprising platelets and plasma components in the flow chamber.

It is further to be noticed that instead of activated platelets, factors derived from platelets may be used, which are sufficient to monocytes. These factors include e.g. fibronectin and may also include factors such as P-selectin, Integrin α5β1the C-type lectin receptor, CD61, CD36, CD47 and complement inhibitors such as CD55 and CD59, or TREM-like transcipt-1. Such platelet-derived factors may also be disposed directly on the surfaces of the flow chamber either as e.g. mixtures of purified components or mixtures of components obtained by e.g. lysis of platelets contained

within the extracorporeal quantity of a mammalian subject's blood sample. In this case, the need for e.g. coating the surfaces of the flow chamber with plasma components may be bypassed.

The data presented herein suggest that once platelets have been activated, proteins such as P-selectin and RGD-containing ligands are expressed by the activated platelets, which can then interact with monocytes and activate their differentiation into dendritic cells. Moreover, it was found monocyte activation and dendritic cell induction by activated platelets do not occur under static conditions. Rather monocytes need to be passed through the channels or channel-like structures under application of a physical force. Given that platelets upon activation need about 60 to about 120 min to express factors such as P-selectin, which then activates monocytes, passing of monocytes may be delayed until platelets have started to express these factors, e.g. for about 60 to about 120 min. If an extracorporeal quantity of a mammalian subject's blood sample comprising monocytes, platelets and plasma components is passed through the flow chamber, this time period may have to be adjusted to longer times.

It is to be understood that interaction of monocytes with activated platelets, platelet-derived factors or plasma components is not sufficient for activation and differentiation of monocytes without the application of a physical force at the same time.

Application of a physical force for moving the monocytes-containing fraction through the flow chamber preferably may mean that a monocytes-containing fraction such as the extracorporeal quantity of a mammalian subject's blood sample is moved through the flow chamber under shear stress. Typically, monocytes-containing fraction may be passed through the flow chamber under a shear force of about 0.1 to about 20.0 dynes/cm^2, of about 0.2 to about 10.0 dynes/cm^2, such as from about 0.2 to about 0.3, to about 0.4, to about 0.5, to about 0.6, to about 0.7, to about 0.8, to

about 0.9, to about 1, to about 1.5, or to about 2 dynes/cm^2. Typical flow rates of the monocytes-containing fraction may be in the range of about 5 ml/min to about 200 ml/min, of about 10 ml/min to about 150 ml/min, of about 10 ml/min to about 100 ml/min, or of about 5 ml/min to about 50 ml/min. The flow rates will depend to some extent on the size and geometry of the flow chamber and can particularly be used if channels or channel-like structures of the below-mentioned dimensions are used. In general, one will select flow rates to achieve the afore-mentioned shear stress values.

Thus, it is contemplated to pass the monocytes-containing fraction through the channels or channel-like structures with a flow rate of about 10 ml/minute to about 200 ml/minute to produce a shear force of about 0.1 to about 0.5 dynes/cm^2. In any case it must be made sure that a shear force is generated that allows binding of monocytes to activated platelets and differentiation of such activated monocytes into dendritic cells.

The data presented herein suggests that monocyte-platelet interaction can be divided into short-acting interactions which are arbitrarily defined as contact occurring for less than 3 seconds by detection with a light microscope and long-acting interactions, defined as contact longer than 3 seconds by detection with a light microscope. It seems that the initial short-acting interactions are mediated by P-selectin which is expressed on activated platelets. These initial contacts can then subsequently trigger long-acting interactions mediated by RGD-containing proteins expressed by the activated platelets.

The activation of monocytes and differentiation into dendritic cells may be positively influenced by allowing the monocytes to establish long-acting contacts with platelets, e.g. by giving the monocytes and platelets enough time to interact. As the monocytes flow through the channels or channel-like structures, they alternately bind to and disadhere from the platelets by the shearing force induced by the flow through the flow chamber. The residence time of the monocyte/platelet interaction may be

controlled by varying the flow rate, e.g. by controlling the speed of the pump. For example, the pump may initially be operated at a slow speed/low flow rate to enhance monocyte/platelet interaction, and the speed/flow rate may then be increased to facilitate disadherence and collection of the treated monocytes from the treatment device. It seems that adherence of the monocytes to the platelets may be best accomplished at about 0.1 to about 2 dynes/cm^2, at about 0.1 to about 1 dynes/cm^2, and preferably at about 0.1 to about 0.5 dynes/cm^2,while disadherence and collection of the monocytes may be best accomplished at increased shear levels.

It is to be understood that activation of monocytes leads to immobilization, e.g. by interacting with activated platelets, platelets-derived factors or plasma components. In order to harvest the induced immuno-suppressive autologous and/or allogenic dendritic cells, one may increase the shear stress to e.g. 20 Dynes/cm^2 and/or may treat the immuno-suppressive autologous and/or allogenic dendritic cells with factors allowing disadherence from activated platelets, platelets-derived factors or plasma components by adding factors such as Plavic, Aspirin or other blood thinners.

Temperature is another factor to influence activation of monocytes and their differentiation into dendritic cells. The methods in accordance with the invention may be performed in a range of about 18°C to about 42°C, preferably in a range of about 22°C to about 41°C and more preferably in a range of about 37°C to about 41°C.

One parameter that can also be varied to tune activation of monocytes is the density by which the flow chamber is coated with plasma components and thus with platelets that bind to the plasma components. In general, the denser the surfaces of the flow chamber are coated with plasma components and platelets, the more efficient will be the monocyte activation.

It has been mentioned above, that platelets are activated by binding to plasma components. The term "activated platelets" in accordance with the invention is used to refer to platelets which show an increased expression of P-selectin, αIIb-β3 integrin and/or RGD-containing proteins such as fibronectin, fibrinogen or vitronectin as a consequence of binding of platelets to plasma components such as fibronectin and/or fibrinogen. Expression may be determined by conventional methods such as RT-PCR, Western-Blotting or FACS analysis. The term "unactivated platelets" in accordance with the invention is used to refer to platelets for which binding to plasma proteins such as fibronectin or fibrinogen cannot be reduced by pre-incubating platelets with the gamma component of fibrinogen.

It has been mentioned above, that monocytes are activated and start to differentiate into dendritic cells by binding to activated platelets under shear stress conditions. The term "activated monocytes" in accordance with the invention is used to refer to monocytes which upon binding to activated platelets under shear stress conditions express increased levels of the open confirmation of β1-integrin and start expressing markers of maturing dendritic cells such as HLA-DR^+/$CD83^+$. As a control to determine whether interaction of monocytes with activated platelets leads to activation and differentiation of dendritic cells one can compare expression of HLA-DR^+/$CD83^+$ after binding of monocytes to activated platelets under shear stress condition either in the absence of anti-P-selectin antibodies (activation) or presence of anti-P-selectin antibodies (control). Expression may be determined by conventional methods such as RT-PCR, Western-Blotting or FACS analysis. If one the additionally applies DNA-cross linking agents such as 8-MOP and exposes the activated monocytes to light such as UVA, one can direct the differentiation process towards immuno-suppressive autologous and/or allogenic dendritic cells and thus to increase expression of GILZ. As one can take from the data of example 2, such cells even if treated with LPS will be resistant to differentiation into immuno-stimulatory autologous dendritic cells and will thus not continue to express HLA-DR, CD83, CD80 and CD86 at high levels, but rather will maintain the tolerogenic state.

After monocytes have been activated by a method in accordance with the invention, they start differentiating into immuno-suppressive autologous and/or allogenic dendritic cells. The term "immuno-suppressive autologous and/or allogenic dendritic cells s" in accordance with the invention is used as mentioned above. These immuno-suppressive autologous and/or allogenic dendritic cells can be identified by increase expression of e.g. GILZ and preferably additionally no increased expression of molecular markers of Table 1.

The experimental findings described herein further immediately suggest various embodiments of this first aspect that can provide for different advantages.

The finding, that activation of monocytes and subsequent induction of differentiation of these monocytes into immuno-suppressive autologous and/or allogenic dendritic cells can be achieved in a miniaturized device, allows to conduct the process with smaller amounts of an extracorporeal blood sample. As mentioned above, the classical ECP procedure requires processing of 2.5 L to 6 L blood, which is typically obtained from patients by apheresis such as leukaphereses, to obtain a final volume of about 200 ml to 500 ml comprising leukocytes including monocytes as well as plasma components and platelets.

However, the methods in accordance with the invention may require substantial lower amount of blood samples thus bypassing the need of apheresis such as leukaphereses or other processes, which are a considerable burden to patients.

Thus, the present invention can be performed without the need for apheresis such as leukaphereses and the whole process of obtaining such immuno-suppressive autologous dendritic cells may be performed in a handheld device.

Thus, in one embodiment of the first aspect of the invention, which may be combined with the above described embodiments, it is contemplated to perform the method in accordance with the first aspect, wherein said extracorporeal quantity of said mammalian subject's blood is not obtained by apheresis such as leukaphereses.

Said extracorporeal quantity of said mammalian subject's blood may be between about 5 ml to about 500 ml, between about 10 ml to about 450 ml, between about 20 ml to about 400 ml, between about 30 ml to about 350 ml, between about 40 ml to about 300 ml, or between about 50 ml to about 200 ml or between about 50 ml to about 100 ml of extracorporeal blood of said mammalian subject to give a final volume between about 1 ml to about 100 ml, between about 1 ml to about 50 ml, between about 1 ml to about 40 ml, or between about 1 ml to about 30 ml an extracorporeal amount of a mammalian's blood sample.

The quantity of extracorporeal blood withdrawn and applied to the device may be whole blood. Alternatively, said extracorporeal quantity of said mammalian subject's blood may be obtained by isolating leukocytes from between about 5 ml to about 500 ml, between about 10 ml to about 450 ml, between about 20 ml to about 400 ml, between about 30 ml to about 350 ml, between about 40 ml to about 300 ml, or between about 50 ml to about 200 ml or between about 50 ml to about 100 ml of extracorporeal whole blood of said mammalian subject.

Said extracorporeal quantity of said mammalian subject's blood may also be obtained by isolating buffy coats from between about 5 ml to about 500 ml, between about 10 ml to about 450 ml, between about 20 ml to about 400 ml, between about 30 ml to about 350 ml, between about 40 ml to about 300 ml, or between about 50 ml to about 200 ml or between about 50 ml to about 100 ml of extracorporeal whole blood of said mammalian subject.

In all of the afore-mentioned cases (whole blood, leukocyte fraction, buffy coats), said extracorporeal amount of blood will typically comprise between about $1x10^4$ to about $1x10^8$ such as about $5x10^6$ mononuclear cells/ml.

The person skilled in the art is familiar how to obtain whole blood, a leukocyte fraction thereof or a buffy coat fraction thereof (see e.g. Bruil et al., *Transfusion Medicine Reviews* (1995), IX (2), 145-166) an include filtration, differential centrifugation. A preferred method relies on filters, as they are available from e.g. Pall. Such filters may be incorporated into the device such that processing of the extracorporeal sample can be done in the handheld device. As a source one can also use e.g. blood of the umbilical cord.

If one uses centrifugation, one may obtain whole blood through a syringe with e.g. a 17 or 18 gauge-gauge needle. Such a whole blood sample may be centrifuged to remove debris and other components. The whole blood sample may then be filtered through common filters, as they are available from Pall.

For obtaining a mononuclear leukocyte fraction, one may obtain a whole blood sample as described and then layer such a sample on e.g. Ficoll-Hypaque. Subsequently a centrifugation step is performed at e.g. about 100g to about 200 g such as 180 g and the mononuclear leukocyte fraction can then be collected from the interface and washed with common buffers such as HBSS. The washed mononuclear leukocyte fraction can then be resuspended in serum-free cell culture medium such as RPMI-1640 medium (GIBCO). Other methods for obtaining mononuclear leukocyte fractions include elutriation, filtration, density centrifugation, etc..

As pointed out above, crucial steps for the induction of dendritic cells formation seem to involve the activation of platelets by plasma components and the activation of monocytes by such activated platelets. In principle, one could pass a whole blood sample through the device under shear stress. The plasma components of such a

sample will then bind to the surfaces of the flow chamber and allow for adherence and activation of platelets within such a sample by plasma-components. The monocytes of such a sample will then bind to the activated platelets and be activated themselves.

Similarly one may obtain combinations of the various components such as a platelet-rich plasma containing fraction which may be obtained by centrifuging a whole blood sample which has been obtained as described above at about 100 g to about 180 g such as about 150 g for about 10 min to about 20 min such as about 15 min to separate the debris of the whole blood sample. The platelet-rich plasma layer is then collected and recentrifuged at about 700 g to about 1000 g such as about 900 g for about 3 min to about 10 min such as about 5 min. The resultant pellet is then resuspended in serum-free cell culture medium.

However, in order to have the best control over the process, it may be desirable to first pass plasma components through the flow chamber and let them adhere, then platelets and then the monocytes-containing fraction. For this approach, it may be desirable to obtain a leukocyte fraction comprising a monocytes- or buffy-coat fraction comprising monocytes, which does not comprise plasma components and which does not comprise platelets. Such plasma- and platelet-free monocytes-containing fractions may be obtained as is described in the art. If leukocyte or buffy-coat fractions are obtained as described above, they will be sufficiently free of plasma or platelets for the purposes of the invention. For this approach, it may also be desirable to have platelet- and/or plasma-fractions.

Thus, the invention contemplates to use platelets which have been separated from the extracorporeal quantity of said mammalian subject's blood before said extracorporeal quantity of said mammalian subject's blood is applied to said device. These platelets may then be passed through the flow chamber, which has been coated with plasma components such as fibronectin.

In another embodiment, the invention considers to use plasma components, which have been separated from the extracorporeal quantity of said mammalian subject's blood before said extracorporeal quantity of said mammalian subject's blood is applied to said device. These plasma components may then be passed through flow chamber so that they can adhere.

Instead of using plasma components which have been obtained from the extracorporeal amount of blood, one may also use plasma components, which have been isolated from other sources such as e.g. by recombinant protein expression. Such plasma components include fibrinogen, fibronectin, P-selectin, and fragments thereof such as the gamma component of fibrinogen.

Even though it may be preferred to use an extracorporeal amount of blood, which has not been obtained by apheresis such as leukaphereses, using an extracorporeal amount of blood, which was obtained by apheresis such as leukaphereses is not excluded by the invention.

Thus, in another embodiment of the first aspect of the invention it is contemplated to perform the method as described above, wherein said extracorporeal quantity of said mammalian subject's blood is obtained by apheresis such as leukaphereses.

Apheresis such as leukaphereses may be performed as is known in the art. Thus, an extracorporeal quantity of blood such as 2.5 L to 6 L may be obtained from a subject and treated by conventional leukaphereses to obtain three fractions, namely the plasma, the platelets and the buffy coats. The plasma, which contains proteins such as fibronectin and fibrinogen, is the lightest blood fraction, and therefore is the first portion of the blood selectively removed from the centrifuge and passaged through channels or channel-like structures. After the plasma has been pumped through the channels or channel-like structures and the surfaces thereof have been coated with

plasma proteins, the second lightest component in the leukaphereses centrifuge, the platelet fraction, is pumped into and through the channels or channel-like structures. The third lightest fraction to be eluted from the leukaphereses centrifuge is the buffy coat, which contains the white blood cells, including the blood monocytes. The buffy coat including the monocytes is then pumped through the channels or channel-like structures. Blood sample may be obtained using the Therakos device, the Spectra cell separator (see Andreu et al., (1994), *Transf. Sci.*, 15(4), 443-454), or the Theraflex device from Macopharma.

Thus, the invention in one embodiment the invention considers to use platelets which have been separated from the extracorporeal quantity of said mammalian subject's blood obtained by apheresis such as leukaphereses before said extracorporeal quantity of said mammalian subject's blood comprising monocytes is applied to said device.

In another embodiment the invention considers to use plasma components, which have been separated from the extracorporeal quantity of said mammalian subject's blood obtained by apheresis such as leukaphereses before said extracorporeal quantity of said mammalian subject's blood comprising monocytes and/or platelets is applied to said device.

Instead of using plasma components which have been obtained from the extracorporeal amount of blood, one may use also either plasma components which have been isolated from other sources such as e.g. by recombinant protein expression. Such plasma components include fibrinogen, fibronectin, or P-selectin. One can also use fragments of plasma proteins such as the gamma component of fibrinogen which corresponds to amino acids 400-411 (SEQ ID NO.: 105, His-His-Leu-Gly-Gly-Ala-Lys-Gln-Ala-Gly-Asp-Val). This gamma component is shown by the data presented herein to be able to activate platelets. It can therefore be preferred to use plasma fractions which at least, if not predominantly comprise fibronectin.

Similarly, it can be preferred to use e.g. recombinantly expressed and/or purified fibronectin or the gamma component thereof to activate platelets.

For both embodiments of the first aspect of the invention where the extracorporeal amount of blood is obtained or not obtained by apheresis such as leukaphereses, it may be considered to pass all three fractions, namely plasma components, platelets and the monocytes-containing fraction at once, e.g. even in the form of a whole blood sample or by using only pre-purified fractions of whole blood, through the flow chamber even though the afore-described sequential passing of these fractions through the flow chamber may provide for better control over the process. Pre-purified fractions of whole blood may be obtained by e.g. centrifuging a blood bag and squeezing out the supernatant, which would be enriched in white blood cells and platelets.

As mentioned the flow rate through flow chamber and thus the resulting shear stress will effect the differentiation of the monocytes into dendritic cells. Further, application of photo-activatable DNA-cross linking agents such as 8-MOP and exposure to light such as UVA light can be used to induce increased expression of GILZ. Aside from the flow rate, the design and the dimensions of the flow chamber may be varied to manipulate and even improve the application of a physical force to the monocytes as well as to make sure that substantially all monocytes can be contacted with DNA-cross linking agents such as 8-MOP and exposed to light such as UVA..

A device having a flow chamber with channels or channel-like structures may be suitable. Such a flow chamber having the general architecture, albeit at smaller dimensions, of a device, which is used for the classical ECP procedure is depicted in Fig. 17.

However, other geometries such as those depicted in Fig. 18 a) to d) may also be used. Thus, the findings described herein allow to consider flow chambers of significantly simplified geometry, which also allows having better control over the process in terms of turbulences and shear stress occurring during the process.

A device having a multiplicity of flow chambers may be suitable. Such a flow chamber having the general architecture, albeit at smaller dimensions, of a device, which is used for the classical ECP procedure is depicted in Fig. 17.

Typically, a flow gradient will be created in the flow chamber such as channels as the monocytes-containing fraction is passed through. The monocytes will alternately bind to and disengage from the platelets and/or plasma components. Maturation of monocytes into dendritic cells is greatly enhanced by this interaction, with increased exposure to the platelets and/or plasma components thereby providing increased signaling of this maturational process.

In order to obtain a homogenous population of immuno-suppressive autologous and/or allogenic dendritic cells as possible, it is therefore desirable that the design and the dimensions of the flow chamber, such as channels is selected avoid different flow zones in the flow chamber.

The flow chamber such as channels may in principle have any cross-sectional shape suitable for the above described purposes. They thus may have a rectangular, round, elliptical, or other cross-sectional form. Even though the dimensions of such flow chamber will be discussed in the following mainly with respect to a rectangular cross-section, it can be preferred that flow chamber such as channels with an elliptical or round cross-section are used as such cross-sections should allow for e.g. more homogenous coating with plasma components and/or more continuous flow properties with less turbulences.

If having a rectangular cross-sections, flow chamber such as channels may have dimensions of about 5 μm to up to about 500 μm of height and of about 5 μm to up to about 500 μm of width. The channels or channel-like structures may also have dimensions of about 10 μm to up to and including about 400 μm of height and of about 10 μm to up to and including about 400 μm of width, of about 10 μm to up to and including about 300 μm of height and of about 10 μm to up to and including about 300 μm of width, of about 10 μm to up to and including about 250 μm of height and of about 10 μm to up to and including about 250 μm of width, of about 10 μm to up to and including about 100 μm of height and of about 10 μm to up to and including about 100 μm of width, or of about 10 μm to up to and including about 50 μm of height and of about 10 μm to up to and including about 50 μm of width.

If flow chambers such as channels of elliptical cross-section are used, the afore-mentioned dimensions of height and width would have to be adapted correspondingly to allow for a comparable volume.

If flow chambers such as channels of round cross-sections are used, the diameter may typically be in the range of about 5 μm to up to and including about 500 μm, of about 10 μm to up to and including about 400 μm, of about 10 μm to up to and including about 300 μm, of about 10 μm to up to and including about 250 μm, of about 10 μm to up to and including about 100 μm, or of about 10 μm to up to and including about 50 μm.

Smaller dimensions are generally preferred for the flow chambers with a particular preference for height, widths or diameters of below 100 μm such as 50 μm the reason being that it is assumed that for such smaller dimensions interaction of monocytes with platelets is more efficient and uniform and flow properties at the surfaces and in the center of the flow chamber are more comparable.

The length of the flow chamber such as channels channel-like structures is usually selected such that the flow chamber allows for passage of the volume of extracorporeal blood. For example the flow chamber and the device may be configured to allow for passing of an overall volume of between about 1 ml to about 50 ml, between about 1 ml to about 40 ml, or between about 1 ml to about 30 ml.

The flow chamber may have internal sub structures to increase the surface area or to make the flow conditions less heterogeneous.

The flow chamber may be filled with particles to increase the surface area or to make the flow conditions less heterogeneous.

The material of the flow chamber may be plastic or non-plastic.

If non-plastic materials are considered, one may use glass.

The surface of the chamber may be coated covalently or via adsorption.

Materials for auxiliary tubing, chambers, valves etc. may be selected to for having reduced interactions with blood components.

Surfaces of auxiliary tubing, chambers, valves etc. may be treated/coated for having reduced interactions with blood components.

If plastic materials are considered, one may use acrylics, polycarbonate, polyetherimide, polysulfone, polyphenylsulfone, styrenes, polyurethane, polyethylene, teflon or any other appropriate medical grade plastic. In a preferred embodiment of the present invention, the flow chamber is made from an acrylic plastic.

The flow chamber may be made of a material that provides a degree of transparency such that the sample within the flow chamber such as the monocytes-containing fractions can be irradiated with visible or UV light, preferably with UV-A. As is shown by the experiments, exposure to UV-A and 8-MOP leads to increased expression of GILZ and thus to activation and differentiation of monocytes into immuno-suppressive autologous and/or allogenic dendritic cells. Thus exposure to light such as UV-A and DNA-cross linking agents such as 8-MOP should be optimized when producing immuno-suppressive autologous and/or allogenic dendritic cells.

However, once monocytes have embarked on the maturation pathway long enough such that immuno-suppressive autologous and/or allogenic dendritic cells have formed as can be determined by the molecular markers mentioned above, one can envisage to stop adding DNA-cross linking agents such as 8-MOP and to stop exposing the immuno-suppressive autologous and/or allogenic dendritic cells to e.g. UV-A. As shown in Example 2, once the immuno-suppressive autologous and/or allogenic dendritic cells have formed as is determinable by increased expression of GILZ, they are resistant to e.g. LPS-induced maturation. However, by contacting autologous and/or allogenic leukocytes, which are contained within the extracorporeal amount of a mammalian's blood sample and which have become apoptotic by e.g. the UVA administration, GILZ expression of the immuno-suppressive autologous and/or allogenic dendritic cells can be further increased. Thus, one can e.g. incubate immuno-suppressive autologous and/or allogenic dendritic cells with such apoptotic leukocytes such that that the immuno-suppressive autologous and/or allogenic dendritic cells will take up and display antigens from the apoptotic leukocytes to obtain immuno-suppressive autologous and/or allogenic antigen-presenting cells. Given that the immuno-suppressive autologous and/or allogenic dendritic cells have already embarked on a tolerogenic state, such immuno-suppressive autologous and/or allogenic antigen-presenting cells can then be used for

treatment of autoimmune diseases, hypersensitivity diseases, rejection in solid-organ transplantation or treatment of Graft-versus-Host disease.

A typical flow chamber may have the geometry depicted in Fig. 19A). The flow path has dimensions of 20 mm by 80 mm. The chamber is made of polystyrene, PET (polyethylenteherephtalate), PMMA (poly (methyl mathacrylate)) and silicon. A blood sample may be spun at low speed through a Ficoll gradient to obtain e.g. 8 ml of sample with a concentration of white blood cells of e.g. 10^{10} cells/ml. The chamber may be pre-coated with platelets-rich plasma. The sample may be passed through the chamber at about 0.028 Pa for about … min. The chamber may then be washed with about 3 ml RPMI at 0.028 Pa. A second wash with 30-55 ml RPMI may be performed at about 1.2 Pa. The collected activated monocytes will then be combined and used for further analysis.

In this way immuno-suppressive autologous and/or allogenic dendritic cells and immuno-suppressive autologous and/or allogenic antigen-presenting cells can be obtained without the need for rather expensive cocktails of cytokines, which moreover use unphysiologically high concentrations and require cell incubation for a couple of days, for induction of monocyte to dendritic cell differentiation. Even though not necessary, it can be considered to cultivate such immuno-suppressive autologous and/or allogenic dendritic cells and immuno-suppressive autologous and/or allogenic antigen-presenting cells in a buffered culture medium with one or more cytokines and/or chemokines, such as IL-10, IL-4 or TGF-ß during the incubation period, to further enhance differentiation into the tolerogenic state. Cultivation, if considered, may be performed under standard conidtions, e.g. at 37° C and 5% CO_2 in standard mediums for culturing of human cells such as in RPMI-1640 medium (obtainable e.g. from GIBCO), supplemented with 15% AB serum (obtainable from e.g. Gemini Bio-Products).

Further, immuno-suppressive autologous and/or allogenic dendritic cells can then be manipulated ex vivo, prior to re-administration to the subject, in order to tailor them for the desired therapeutic purpose.

Thus, prior to re-administration to the subject, such immuno-suppressive autologous and/or allogenic dendritic cells can e.g. be processed ex vivo, such as by loading them with antigens, being derived from cells involved in autoimmune diseases, hypersensitivity diseases, solid-organ transplantation rejection and Graft-versus-Host disease. Such antigens may e.g. provided in the form of apoptotic cells comprising these antigens. This will lead to immuno-suppressive autologous and/or allogenic antigen-presenting cells. One of the advantages of the invention provided by the insight presented herein is that obtaining immuno-suppressive autologous and/or allogenic dendritic cells can be separated from loading with antigens, being derived from cells involved in autoimmune diseases, hypersensitivity diseases, solid-organ transplantation rejection and Graft-versus-Host disease. In this way, immuno-suppressive autologous and/or allogenic dendritic cells can be isolated and tailored for specific purposes.

The immuno-suppressive autologous and/or allogenic dendritic cells may in particular be loaded with disease antigens to produce antigen presenting dendritic cells which upon re-introduction into the subject will suppress an immune response against the antigens.

For treatment of autoimmune diseases the antigens may be selected for diseases such as wherein the autoimmune disease is selected from the group comprising rheumatoid arthritis, psoriasis, multiple sclerosis, type 1 diabetes and systemic lupus erythematosus.

The immuno-suppressive autologous and/or allogenic dendritic cells obtained in accordance with the invention and disease effector agents are incubated for a period

of time sufficient to maximize the number of functional antigen presenting dendritic cells in the incubated cell population. Typically, the treated blood cell concentrate and possibly antigens are incubated for a period of from about 1 to about 24 hours, with the preferred incubation time extending over a period of from about 12 to about 24 hours. Additional incubation time may be necessary to fully mature the loaded immuno-suppressive antigen-presenting cells prior to reintroduction to the subject. Preferably, the blood cell concentrate and disease effector agents are incubated at a temperature of between 35° C and 40° C. In a particularly preferred embodiment, the incubation is performed at about 37°C.

As mentioned above, by loading immune-suppressive dendritic cells obtainable by the methods described herein with antigens allows producing immuno-suppressive antigen-presenting cells.

In order to avoid e.g. protein degradation of delivered antigen and inefficient processing of soluble antigens by dendritic cells, it is contemplated to enhance formation of immuno-suppressive antigen-presenting cells by encapsulation of antigens in polymeric nanoparticles (NPs), which may be made from biodegradable polymers such as polylactic acid (Waeckerle-Men et al., Adv Drug Deliv Rev (2005), 57:475-82). Such NPs may be further modified with targeting moieties for DEC-205 such as an DEC-205 antibody to improve receptor-mediated endocytosis and antigen presentation.

Thus the invention contemplates to use encapsulation of antigens in polymeric nanoparticles to optionally further enhance formation of immuno-suppressive antigen-presenting cells.

Inducing monocyte differentiation according to the method described above provides immuno-suppressive autologous and/or allogenic dendritic cells which equal or exceed the numbers of dendritic cells that are obtained by expensive and laborious

culture of leukocytes in the presence of cytokines such as IL-10, IL4 or TGF-ß for a couple of days. The large numbers of functional dendritic cells generated by the method described above provide a ready means of presenting selected material, such as, for example, apoptotic cells, disease antigens, antigens, plasmids, DNA or a combination thereof, and are thereby conducive to efficient immunotherapy.

As mentioned above, immuno-suppressive autologous and/or allogenic dendritic cells can be preferably obtained by a method in accordance with the invention in the presence of a photoactivatable DNA-cross linking agent such as 8-MOP and by exposure to light such as UV-A.

The present invention thus aims at obtaining individual-specific functionally and maturationally synchronized immuno-suppressive autologous and/or allogenic dendritic cells.

In a second aspect, the present invention relates to immuno-suppressive autologous and/or allogenic dendritic cells obtainable by a method described herein, preferably for use in treating autoimmune diseases, hypersensitivity diseases, solid-organ transplantation rejection and Graft-versus-Host disease. The autoimmune disease may be selected from the group comprising rheumatoid arthritis, psoriasis, multiple sclerosis, type 1 diabetes and systemic lupus erythematosus.

In a third aspect, the present invention relates to a method of treating autoimmune diseases, hypersensitivity diseases, solid-organ transplantation rejection or Graft-versus-Host disease by administering to a patient in need thereof immuno-suppressive autologous and/or allogenic dendritic cells obtainable by a method described herein.

The invention is now described with respect to some specific examples which, however, are for illustrative purposes and not to be construed in a limiting manner.

Experiments

Experiment 1 – Shear stress and platelet activation for inducing monocyte activation

Materials and Methods

Procurement of leukocytes and platelets

All samples were acquired from young, healthy subjects not taking medications, including aspirin, known to influence platelet function. Samples were obtained under the guidelines of the Yale Human Investigational Review Board, and informed consent was provided according to the Declaration of Helsinki. Peripheral blood specimens were collected through a 19-gauge needle from the antecubital vein into syringes containing heparin, then layered on Ficoll-Hypaque (Gallard-Schlessinger, Carle Place, N.Y.). Following centrifugation at 180g, the interface containing the mononuclear leukocyte fraction was collected and washed twice in HBSS, then resuspended in RPMI-1640 medium (GIBCO) to a final concentration of 5×10^6 mononuclear cells/ml. Cells were utilized within one hour of being acquired.

Preparation of Platelet-rich-Plasma

Whole blood was centrifuged at 150 g for 15 min at room temperature. The platelet-rich-plasma (PRP) layer was collected and centrifuged at 900 g for 5 min, and the platelet pellet resuspended in RPMI 1640 to the desired concentration.

Preparation of Parallel-Plates

Two likes of parallel-plate flow chambers were used to model the flow dynamics of ECP. Experiments involving the assessment of cell phenotype post-flow were conducted using the larger Glycotech system (Glycotech, Rockville, MD). This system consisted of a volumetric flow path measuring 20000 x 10000 x 254 microns

(length x width x height). The bottom plate in this system was composed of a 15mm petri dish (BD Biosciences, Durham, NC) separated by a gasket and vacuum-connected to an acrylic flow deck, which formed the upper plate. For experiments requiring the plates to be pre-coated with platelets, prior to assembling the flow chamber, 20 drops of the desired concentration of PRP was placed in the center of the petri dish and platelets allowed to settle for 20 minutes at room temperature. The petri dish was washed twice with 2ml of RPMI, and the flow chamber then assembled.

For experiments not involving the collection and phenotyping of cells post-flow, Vena8 biochips (Cellix Ltd, Dublin, Ireland) were used to generate laminar flow. The volumetric flow path for a channel of the Vena8 biochips measured 20000 x 400 x 100 microns (length x width x height). Protein coating of these chips is described in the appropriate section below.

Experiments using Parallel-Plates

The parallel-plate flow chamber was mounted on the stage of a phase contrast optical microscope (CK40, Olympus, Japan) with a 10x objective. All runs were performed at room temperature. A uniform laminar flow field was simulated by use of a syringe pump (KD Scientific, New Hope, PA) capable of generating near-constant volumetric flow rates. The components of the configuration were devised to minimize tubing. Prior to infusing cell suspensions through the plates, the system was washed with 5 ml of RPMI at a flow rate producing a wall shear stress of approximately 1 dyne/cm^2. Cell suspensions of interest were then passed through the chamber at a fixed flow rate and wall shear stress.

All experiments were viewed in real time, recorded at 15.2 frames per second using a DP 200 digital camera and software (DeltaPix, Maalov, Denmark), and analyzed using Image J software (NIH).

Overnight culture

When overnight culture was required, cells were centrifuged and resuspended in RPMI-1640 medium (GIBCO), supplemented with 15% AB serum (Gemini Bio-Products) to a final concentration of 5 x 106 cells/ml. Cells were cultured overnight for 18 hours in 12-well polystyrene tissue culture plates (2 ml per well) at 37° C in 5% CO2.

Immunophenotyping

Monoclonal antibodies for immunophenotyping included CD14 (LPS receptor; monocytes), CD11c (integrin subunit; monocytes and DC), HLA- DR (class II MHC molecule), CD83 (DC marker), CD62p (P-selectin; activated platelets), and CD61 (integrin subunit; platelets). Antibodies were obtained from Beckman Coulter (CD14, CD11c, HLADR, CD83) or Sigma (CD62p, CD61) and used at their pre-determined optimal dilutions. Background staining was established with appropriate isotype controls, and immunofluorescence was analyzed using a FC500 flow cytometer (Beckman Coulter). Two-color membrane staining was performed by adding the pre-determined optimal concentrations of both antibodies directly conjugated to FITC or PE and incubating for 20 min at 4°C, followed by washing to remove unbound antibodies. Combined membrane and cytoplasmic staining was performed following manufacturer's instructions for cell fixation and permeabilization (Intraprep kit, Beckman Coulter).

Quantitative real-time PCR

Gene expression was compared between cells exposed during flow through the parallel plates to low (10 $\pm$ 5/low power field [lpf]) versus high (102 $\pm$ 32/lpf) levels of platelets, followed by overnight culture. Cell RNA was isolated using RNeasy Mini Kit columns with on-column DNase I treatment (QIAGEN). RNA yield and purity were measured using a NanoDrop ND-1000 Spectrophotometer and an Agilent 2100 Bioanalyzer. RNA was reverse transcribed to cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Reverse

transcription was carried out in a 96-well thermocycler (MJ Research PTC-200) in the following conditions: 25°C, 10 minutes, 37°C, 120 minutes, 85°C, 5 seconds. TaqMan real-time PCR was used to detect transcripts of DC-LAMP, CD40, ADAM Decysin, Lox1, CCR7, CD80, CD83, CD86, FPRL2, and GPNMB. Primers and probes for each sequence were obtained as inventoried Taqman Gene Expression Assays (Applied Biosystems). HPRT1 was used as a reference gene.

Co-cultures of Platelets with Monocytes

Experiments involving co-cultures of monocytes with additional platelets were performed as described in the Overnight Culture section, with a few necessary modifications. Following Ficoll-Hypaque separation, mononuclear cells were resuspended in 30% AB serum/RMPI to a final concentration of 10 x 106 cells/ml, of which 1 ml was allocated to each well of a 16- well plate. An additional 1ml of platelets (suspended in RPMI, at 2x the desired final concentration) or RPMI without platelets was then added to each well. To activate platelets, 500 µl containing 2 units of thrombin was added to half the wells, and 500 µl of RPMI was added to the others to balance the volume. Cells were then incubated as described previously.

Platelet Adhesion Studies

Platelet adhesion experiments were performed using the Vena8 flow chamber described above. Fibrinogen and fibronectin (Sigma) were dissolved in PBS to a final concentration of 200 mcg/ml. Channels of the Vena8 chips were incubated at room temperature in a humidified chamber for 2 hours with the protein solution, autologous plasma, or PBS alone. The channels were washed with 5x the volume RPMI. Platelet-rich-plasma was then perfused through the protein-coated channel at the indicated shear-stress, held constant. For each channel, still images were acquired exactly 90 seconds into the experiment at 4 pre-defined low power fields located along the flow path (fields were centered at 2500, 7500, 12500, and 17500 microns from the start point of infusion).

Some experiments involved pre-treating platelet-rich-plasma with protein fragments prior to infusion through the channels. Small RGD peptides, containing the amino-acid sequence Arg-Gly-Asp-Ser; DRG peptides, contain the amino-acid sequence Ser-Asp- Gly-Arg; or fragment 400-411 of fibrinogen, containing the amino-acid sequence His- His-Leu-Gly-Gly-Ala-Lys-Gln-Ala-Gly-Asp-Val, were incubated at a concentration of 2mM with PRP for 20 minutes at room temperature. The PRP was then perfused through the channels as previously described.

Receptor-Ligand Studies

Platelet-coated Vena8 channels were pre-treated with either 40 μg/ml anti-P-selectin (R&D Systems) or 40 μg/ml of an isotype control for 30 minutes at room temperature, then washed with 5x the volume RPMI. Mononuclear cell suspensions were pre-treated with either RGD or DGR peptides at a concentration of 2.5 mM. Video samples lasting 400 frames (26.3 seconds) were recorded 60 seconds after commencement of flow using a lower power field of view spanning 400 microns and centered at 7500 microns from the flow start point.

β-1 integrin conformation was assessed using the Glycotech flow chamber. 15mm platelet-coated petri dishes (described above) were pre-treated with 40 μg/ml anti-P-selectin or an isotype control for 20 minutes at room temperature, then washed with 5x the volume RPMI. Immediately following perfusion through the platelets, cells were immunophenotyped with anti-CD29 HUTS-21 (BD Biosciences), an antibody that specifically binds to the active (open) conformation of β1 integrins.

Results

Monocytes in flow transiently interact with immobilized platelets

ECP was initially developed as a means to enable extracorporeal chemotherapeutic exposure of pathogenic leukocytes to ultraviolet A (UVA)-activated 8-methoxypsoralen (8-MOP), a DNA-cross-linking drug. Therefore, ECP involves the

flow of leukapheresed blood between large transparent plastic parallel-plates separated by 1 mm. To permit detailed analysis of the flow dynamics involved during ECP, independent of UVA/8-MOP exposure, the flow conditions of ECP were reproduced using miniature parallel plates with surface area of only 0.8 mm^2, separated by 100 microns. This model permitted visualization using digital microscopy. Studies using the model revealed the following sequence (determined by video analysis): initial adherence of platelets from the flow stream to the plate, followed by transient binding of passaged monocytes to the immobilized platelets.

DC induction correlates with the number of monocyte-platelet interactions

Based on the initial qualitative observations described above, platelets were hypothesized to induce monocyte-to-DC differentiation under conditions of flow. To test the influence of platelets on monocyte-to-DC differentiation, monocytes were passed between parallel plates pre-coated with autologous platelets at low (10 $\pm$ 5/low power field [lpf]), medium (44 $\pm$ 20/lpf), and high (102 $\pm$ 32/lpf) densities. Cells were passed through the plates at a flow rate producing a wall shear stress of 0.5 dyne/cm^2, analogous to the wall shear stress in post-capillary venules. The number of monocyte-platelet interactions per unit time increased in proportion to augmented density of platelets (determined by video analysis). An average of 52.3 + 15 monocyte-platelet interactions per lpf per second were observed with the high-density plate, dropping to 18.3 $\pm$ 14 and 3.4 $\pm$ 1 interactions per second with the medium and low-density plates, respectively (Figure 1a).

Following overnight incubation, a correlation was found between the percentage of cells which developed a DC phenotype and the frequency of monocyte-platelet physical interactions observed the previous day (Figure 1b). An increasing number of monocyte-platelet interactions correlated with increasing proportion of cells expressing markers consistent with DC differentiation, membrane HLA-DR and CD83. An average of 14.2% of monocytes exposed to the high-density platelet-coated plate were HLA-DR+/ CD83+ after overnight incubation, compared to 4.9% and 0.8% of monocytes exposed to plates coated with medium and low levels of platelets, respectively.

Monocyte exposure to platelets results in changes in gene expression

To supplement the described changes in monocyte phenotype observed following platelet exposure, RT-PCR was performed to assess for changes in gene expression. Monocytes were passed through parallel plates coated with high or low densities of platelets as described in the previous section. Following overnight incubation, RNA was extracted and RT-PCR performed to determine level of expression for 10 genes

associated with DC (Figure 2). CD40, a costimulatory molecule with known expression on mature dendritic cells (Cella et al., 1996, see reference list), was found to be upregulated by over 567% in monocytes exposed to high densities of platelets relative to monocytes exposed to low levels. LAMP3, a marker specific to DC differentiation (de Saint-Vis at al., 1998, see reference list), was upregulated by 398%. CD80 is a costimulatory molecule known to be upregulated upon APC activation (Slavik et al., 1999, see reference list) upregulated by 220% in monocytes exposed to high levels of platelets. CCR7, a chemokine receptor known to play a role in DC migration to lymphoid organs, was upregulated by 376%. LOX1, CD83, CCR7, and ADAM Decysin, all genes associated with DC (Berger et al., 2010, see reference list), were also upregulated in the monocytes exposed to high levels of platelets. FPRL2, GPNMB, and CD86 were all downregulated in monocytes exposed to high levels of platelets. FPRL2 is a receptor that when activated is known to inhibit DC maturation (Kang et al., 2005, see reference list) GPNMB is a protein involved in decreasing cytokine production (Ripoll et al., 2007, see reference list); CD86 is a costimulatory molecule expressed by APCs.

DC induction in the presence of platelets does not occur under static conditions

Platelets could potentially influence monocytes through direct receptor-ligand interaction, or via cytokines and other secreted mediators. To determine whether the platelet induction of monocyte-to-DC differentiation requires flow dynamics, we tested the role of platelets under static conditions. Monocytes were co-cultured with low (<50,000/mm^3), medium (100-200,000/mm^3) and high (>400,000/mm^3) concentrations of platelets, with platelets in either an inactive or active state (induced by the addition of thrombin). After overnight incubation in static conditions (shear stress = 0), we found that neither activated nor non-activated platelets were capable of inducting DC differentiation of monocytes in the absence of flow (see Figure 3).

Platelets suspended in flow bind to serum proteins adsorbed onto the plate

Several proteins abundantly present in plasma, including fibronectin and fibrinogen, are well known adsorb onto glass and plastic surfaces; the contribution of adherent plasma proteins on platelet adhesion and activation was therefore assessed. Parallel plates were pre-coated with either fibrinogen, fibronectin, plasma, or saline. Unactivated platelets were then passed through at shear rates producing wall shear stresses ranging from 0.2 to 6.0 dyne/cm^2. The highest concentrations of platelets adhered to plates coated with fibrinogen (Figure 4). Adhesion to fibronectin-coated, plasma-coated, and uncoated plates was observed as well, but to a significantly lower extent ($p < 0.05$). In the absence of flow, platelet adherence was equivalent on all protein substrates.

Both fibrinogen and fibronectin contain segments with the amino acid sequence arginine(R)-gylcine(G)-aspartate(D), RGD. RGD segments are well-known to interact with many integrin receptors, particularly the I/A domain of beta subunits, which are exposed when the integrins are in the active conformation (Xiong et al., 2002, see references). In experiments using fibrinogen-coated plates, platelet adhesion was not significantly altered by pre- incubation of platelets with RGD peptides; however, adhesion was significantly decreased ($p < 0.05$) by pre-incubation of platelets with peptide fragments corresponding to amino acids 400-411 of fibrinogen, the gamma component of the protein (Figure 5 a). In experiments using fibronectin-coated plates, pre-incubating platelets with RGD peptides decreased adhesion significantly, while pre-incubating platelets with peptide fragments corresponding to amino acids 400-411 of fibrinogen had no effect, (Figure 5b). Interestingly, it should be noted that unlike the I/A domain of integrins, which is known to interact with RGD domains of proteins, the region of the integrin found to interact with the gamma component of fibrinogen is exposed in the integrin's inactive state (Weisel et al., 1992, see references). Therefore, this data suggests that unactivated platelets in flow bind to the gamma-component of fibrinogen-coated plates. The potential for platelets in the unactivated state to bind fibrinogen may

explain the greater level of platelet adhesion seen on fibrinogen-coated plates explained in the previous paragraph.

Platelets are activated by adhesion to the plate

Platelets physiologically circulate in an inactive state, with an array of proteins stored in intracellular granules. Upon encountering stimuli such as damaged endothelium or thrombin, platelets become activated and almost instantaneously translocate these intracellular proteins to the plasma membrane (Kaplan et al., 1979, see references). It was postulated that platelet adhesion to the plastic plate/absorbed proteins caused platelet activation similar to that caused by well-known stimuli. To test this hypothesis, surface expression of P-selectin, a well-known marker of platelet activation, was assessed before and after adhesion. Prior to adhesion, 6 $\pm$ 3% of platelets were found to express P-selectin, with a mean fluorescence intensity (MFI) of 12.4 $\pm$ 6.9; following adhesion, P-selectin positivity increased to 64 $\pm$ 13% (MFI 98.2 $\pm$ 14). The positive control, platelets activated with thrombin, was 71 $\pm$ 18 % P-selectin positive (MFI 108.3 $\pm$ 23). Expression of P-selectin was further assessed at 30, 60, and 90 minutes following platelet adhesion; P-selectin expression remained stable at all time points, with 72 $\pm$ 11% of platelets P-selectin positive 90 minutes after adhesion, indicating that platelets remain in an active state for the duration of the procedure. Similar trends were found in assessment of αIIb-β3, a fibrinogen-binding integrin, with surface expression of this protein increasing from 4 $\pm$ 3% prior to adhesion, to 49 $\pm$ 18% post-adhesion.

Monocytes interact with P-selectin and RGD-containing ligands expressed on activated platelets

The monocyte-platelet interactions observed on video were divided into two categories: (1) short-acting, arbitrarily defined as contact occurring for less than 3 seconds (46 frames), and (2) long-acting, defined as contact longer than 3 seconds, including stable binding. Since it had been previously determined that the platelets in the ECP system were in an activated state, and that activated platelets express an

array of proteins including P- selectin and RGD containing proteins (e.g. fibronectin, fibrinogen, and vitronectin), it was sought to determine the involvement, if any, of these proteins in either short or long- duration interactions. Plates were pre-coated with platelets, and four conditions tested: (1) platelets pre-treated with an irrelevant isotype control, and monocytes untreated (P+RGD+); (2) platelets pre-treated with an irrelevant isotype control, and monocytes pre-incubated with RGD peptides (P+RGD-); (3) platelets pre-treated with anti-P-selectin, and monocytes untreated (P-RGD+); (4) platelets pre-treated with anti- P-selectin, and monocytes pre-treated with RGD peptides (P-RGD-). It was assumed that pre-treating monocytes with RGD peptides should result in a decreased in the number of free RGD- recognizing receptors available to interact with RGD-containing proteins expressed by the platelets. Thus, the four conditions tested represent every permutation of potential interaction with two platelet ligands, P-selectin and RGD-containing-proteins. As shown by Figure 6, both short-acting and long-acting interactions were maximal when neither RGD nor P-selectin were blocked (P+RGD+); the level of interaction in all other conditions was expressed as a percentage of this maximum. Blocking with anti-P-selectin alone (P-RGD+) resulted in a decrease of both short and long monocyte- platelet interactions to almost zero ($p < 0.01$; Figure 6, also confirmed by video analysis). In contrast, blocking RGD alone (P+RGD-) did not significantly alter the number of short-duration interactions, but decreased the long-duration monocyte-platelet interactions by 44% ($p < 0.05$; Figure 6). Blocking both P-selectin and RGD simultaneously (P-RGD-) resulted in a pattern similar to that seen when only P-selectin was blocked, with both long and short duration interactions reduced to near zero. The most appropriate conclusions, based on the pattern of interactions observed in each of the four conditions, are as follows: (1) P-selectin is predominantly responsible for the short-duration interactions; (2) RGD-containing proteins expressed by the platelet are involved in long-duration interactions, but not short-duration interactions; (3) monocyte interaction with P-selectin must occur upstream of monocyte interaction with RGD-containing proteins expressed by platelets. This last conclusion is based on the observation that conditions of P-RGD+

decreased both short and long duration interactions to near zero, while P+RGD- conditions only decreased long-duration interactions. If the interactions were not sequential, conditions of P- RGD+ should have produced similar results to P+RGD+ in terms of long-duration interactions. Furthermore, the ordering of the interactions, i.e. that P-selectin acts upstream of RGD-interactions, is apparent by the finding that conditions of P+RGD- only influenced long duration interactions, while conditions of P-RGD+ produced similar results to those of P-RGD-.

Monocyte exposure to P-selectin results in downstream monocyte integrin-activation

Integrin receptors, in their open conformation, are known to interact with RGD-containing ligands (Ruoslathi et al., 1996, see references). Using an antibody that recognizes an epitope exposed only when the β1 integrin is in its open conformation, we assessed the conformation of monocyte integrins before and after flow through the model. Figure 7 shows that as the number of short-acting monocyte-platelet interactions increased, there was corresponding increase in the percentage of monocytes expressing integrins in their open conformation immediately post-flow. The black line shows that an average of 71% of monocytes which had received a high number of platelet-interactions (> 61± 19/lpf x sec) expressed β1 in the active form, compared to 9% of monocytes which had received a low number of platelet interactions (< 5.1 + 2 /lpf x sec). These results were not significantly affected by pre-treating the adherent platelets with an irrelevant isotype control (gray line). In contrast, pre-treating platelets with anti-P-selectin reduced the monocyte-platelet interactions to near zero, and monocytes emerging from flow in these conditions (dashed line) displayed low levels of active β1 integrins, irrespective of the density of platelets to which they were exposed. It is noteworthy that all cell populations prior to passage through the plates demonstrated similar low levels of baseline integrin activation (<10%); therefore, differences seen in short-duration monocyte-platelet interactions were not the result of differences in integrin conformation pre-flow.

Monocyte exposure to P-selectin is required for DC differentiation

Given the dependence of monocyte-platelet interactions on platelet P-selectin, we set out to determine if there was a relationship between monocyte exposure to P-selectin at time 0, and the phenotype later developed by the monocyte after overnight incubation, time 18-hours (Figure 8). Monocytes were passed though parallel plates coated with high densities (108 ± 36/lpf) of platelets that were either untreated (unblocked), or pretreated with either anti-P-selectin or an isotype control. 15.5 ± 4 % of monocytes exposed to unblocked platelets became membrane HLA-DR+/CD83+ (markers of maturing DC) after overnight incubation, and 13 ± 4 % of the those exposed to platelets blocked with the irrelevant isotype control. In contrast, only 3 ± 2% of the monocytes exposed to platelets blocked with anti-P-selectin became HLA-DR+/CD83+ after overnight incubation.

Experiment 2 – Identification of further molecular markers for immuno-suppressive dendritic cells

Materials and Methods

Sample Collection and Monocyte Enrichment

Peripheral blood specimens were acquired from healthy subjects under the guidelines of the Yale Human Investigational Review Board, and informed consent was provided according to the Declaration of Helsinki. PBMC were isolated by centrifugation over a Ficoll-Hypaque gradient (Isolymph, CTL Scientific). Monocytes were enriched from freshly isolated PBMC by: 1) plastic adherence for dexamethasone dose-titration experiments (purity: 71.6 ± 5.6% $CD14^+$); 2) CD14 magnetic bead positive selection (Miltenyi Biotec) for PUVA dose-titration experiments (purity: 88.1 ± 3.5% $CD14^+$), and; 3) Monocyte Isolation Kit II (Miltenyi Biotec) for LPS stimulation experiments (purity: 83.8 ± 3.8% $CD14^+$).

Generation of Monocyte-Derived Dendritic cells (MoDC)

Monocytes were cultured at a density of 5 x 10^6 cells/mL in 6- and 12-well polystyrene tissue culture plates at 37°C and 5% CO_2 in RPMI-1640 (Gibco) supplemented with heat-inactivated 15% AB serum (Gemini) and 1% penicillin/streptomycin (now referred to as complete media). 800 IU/mL recombinant human GM-CSF (R&D Systems) and 1000 IU/mL recombinant human IL-4 (R&D Systems) were added to cultures for 36 hr to induce monocyte to DC differentiation as described.

8-MOP and UVA Light Treatment

Cultures were incubated with 8-MOP (Uvadex, 20 μg/mL) for 30 min in the dark, and then irradiated with a desktop UVA light box containing a series of 12 linear fluorescent tubes. The tubes emitted UVA light ranging from 320 to 400 nm. The UVA irradiance (power, W/m^2) was measured using a photodiode. Given a measured irradiance and the absorption properties of the various components of the system, it was possible to determine the time (sec) needed to expose the cells to deliver a given dose of UVA radiation (J/cm^2).

MoDC/Lymphocyte Co-Cultures

Non-adherent cells (purity: 66.0 ± 4.5% $CD3^+$) removed during plastic adherence will now be generally referred to as lymphocytes. Lymphocytes were treated with 8-MOP (100 ng/mL) and UVA (1 J/cm^2), washed with PBS, and co-cultured in complete media at 37°C and 5% CO_2 with either PUVA-treated or untreated-MoDC in a ratio of 5 or 10 lymphocytes to 1 MoDC. MoDC treated for 24 hr with 100 nM dexamethasone (Sigma) served as the positive control group. After 24 hr, cells were harvested and MoDC were re-purified. To ensure that RNA was not isolated in significant amounts from lymphocytes, it was critical to re-purify MoDC from all cultures using CD11c magnetic bead (Miltenyi Biotec) positive selection (purity: 96.4 ± 1.0% $CD11c^+$). $CD11c^+$ MoDC were re-plated at 0.5-1.0 x 10^6 cells/mL in complete media and stimulated with 100 ng/mL LPS (Sigma). 24 hr after LPS

stimulation, cells were harvested for RNA isolation and immunophenotyping, and supernatants were collected for cytokine quantification. As negative controls, parallel groups did not receive LPS.

siRNA Experiments

Silencer select pre-designed and validated GILZ siRNA (Invitrogen), with off-target prediction algorithms, was used to knockdown GILZ expression. Mo-dendritic cells were transfected using Lipofectamine RNAiMAX Reagent (Invitrogen). RNA_i duplex and lipofectamine reagent were incubated together for 20 min, then added to MoDC cultures and incubated for 2 hr at 37°C and 5% CO_2. Transfected MoDC were treated in an identical fashion as described for the MoDC/lymphocyte co-cultures. MoDC were also transfected with scramble siRNA.

Immunophenotyping

Monoclonal antibodies included HLA-DR, CD80, CD83, CD3, CD86, CD14, CD11c and GILZ. Antibodies were obtained from Beckman-Coulter and eBioscience and were used at their pre-determined optimal dilutions. Apoptosis was assessed using the Annexin-V Apoptosis Detection Kit (eBioscience), with Annexin-V recognizing phosphatidylserine (PS) on the surface of apoptotic cells. 7-AAD substituted for PI as the cell viability dye. Cells displaying an Annexin-V^+/7-AAD^- phenotype were classified as early apoptotic cells, and cells displaying an Annexin-V^+/7-AAD^+ phenotype were classified as late apoptotic cells. Dual membrane and intracytoplasmic staining was performed using the IntraPrep fix and permeabilization kit (Beckman-Coulter). Background staining was established with appropriate isotype and fluorescence minus one controls. Immunofluorescence was analyzed using a FACSCalibur L (BD Biosciences) within 2 hr of fixation with 2% paraformaldehyde. A minimum of 10,000 events were collected for each group.

Quantitative Real-Time PCR

RNA was isolated from $CD11c^+$ MoDC using QIAShredder columns (QIAGEN) and the RNeasy Mini Kit (QIAGEN) with on-column DNase I treatment (QIAGEN). RNA yield and purity were assessed using a NanoDrop ND-1000 spectrophotometer. cDNA was obtained using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) in a 96-well thermocycler (MJ Research PTC-200). TaqMan real-time PCR was used to detect transcripts of GILZ, CD80, and CD86. Primers and probes were obtained as pre-designed and validated Taqman Gene Expression Assays (Applied Biosystems). SYBR green real-time PCR (Applied Biosystems) was used to detect transcripts of IL-12, IL-10, IL-6, TNF-alpha, and TGF-β. Primers were designed to span intron junctions using Primer3Plus. Primer melting curves were obtained to confirm a single product. HPRT-1 and GAPDH were used as reference genes. Samples were run in triplicate on a 7500 Real Time PCR System (Applied Biosystems). The delta-delta C(t) method was used to calculate the fold change.

Cytokine Quantification

Culture supernatants were analyzed in a multiplex format utilizing magnetic beads to IL-6, IL-8, IL-10, IL-12p70, IFN-γ, TNF-α, RANTES, MCP-1, and MIP-1β (BioRad Laboratories). For siRNA experiments, supernatants were analyzed with enzyme-linked immunosorbent assay (ELISA) kits for IL-10 (R&D Systems) and IL-12p70 (Enzo Life Science). All samples and standards were run in duplicate and analyzed using the LUMINEX 200 (LUMINEX), or the BioTek EL800 (BioTek).

Statistical Analysis

Student's *t*-tests were used for statistical comparisons between groups, with *p*-values < 0.05 considered statistically significant. Differential gene expression was considered statistically significant with a ≥ 2.5-fold change and a *p*-value < 0.05.

Results

Expression of GILZ is rapidly down-regulated as monocytes differentiate into immature MoDC

Freshly isolated $CD14^+$ monocytes express GILZ, but rapidly down-regulate GILZ by more than 99% as they differentiate into immature MoDC (Figure 10A). A reduction in GILZ mRNA was confirmed by a 61% decrease in GILZ protein levels (Figure 10B). GILZ down-regulation correlated with reduced expression of CD14 (monocyte-specific marker, see Zhou et al., references), and increased expression of cytoplasmic CD83, (immature MoDC marker, see Klein et al., references). Importantly, MoDC remained immature, expressing low membrane CD83 (mature DC marker, see Renzo et al., references, $p = 0.16$). MoDC up-regulate GILZ after treatment with dexamethasone (dex) in a dose-dependent manner (Figure 10C). Treatment with 100 nM dex for 24 hr was selected as the positive control for inducing GILZ expression in MoDC (Dex-dendritic cells) (Figure 10D).

8-MOP or UVA treatment alone did not effect GILZ expression (Figure 10E). However, when MoDC were treated with the combination of 8-MOP and UVA light (PUVA-dendritic cells), GILZ expression increased 5.5-fold. The induction of GILZ exhibited a slow time course, peaking 24 hr after treatment, and remaining significantly elevated for 72 hr (Figure 10F). In comparison, Dex-dendritic cells up-regulated GILZ as little as 2 hr after treatment.

Immature MoDC treated with the combination of 8-MOP and UVA light up-regulate GILZ and assume a tolerogenic, immuno-suppressive phenotype

It was next examined if there was a PUVA dose-dependent effect on GILZ expression. MoDC treated with 1 J/cm^2 UVA and 100 or 200 ng/mL 8-MOP up-regulated GILZ 2.9- and 4.4-fold respectively (Figure 11A). A similar dose-dependent phenomenon was observed with 2 J/cm^2, starting at an 8-MOP

concentration of 50 ng/mL. Treatment with 0.5 J/cm^2 had no effect on GILZ expression until the 8-MOP concentration reached 200 ng/mL, and treatment with 4 J/cm^2 resulted in high levels of non-specific cell death (data not shown). The number of photo-adducts formed per 10^6 base pairs is directly related to the product of the 8-MOP concentration and UVA dose, see Gasparro et al., references. As the product of 8-MOP and UVA reached 100, GILZ was up-regulated 3-fold, and as the product increased to 200 and 400, GILZ was up-regulated 4.8- and 8.6-fold respectively (Figure 11B).

The percentage of early apoptotic CD11c$^+$ cells was minimally ($p > 0.05$) higher at 2 J/cm^2 as compared to 1 J/cm^2 for all doses of 8-MOP tested (Figure 11C). At 2 J/cm^2 and 200 ng/mL, there was an increase in the percentage of early apoptotic CD11c$^+$ cells as compared to untreated MoDC (Figure 11C). The percentage of late apoptotic CD11c$^+$ cells remained less than 13% at 1 J/cm^2, and less than 16% at 2 J/cm^2 for all doses of 8-MOP tested (Figure 11D). Moreover, dot plots highlight the relative resistance of MoDC to the pro-apoptotic effect of escalating doses of PUVA (Figure 11E). The number of cells recovered from cultures did not statistically differ in any group treated with 1 or 2 J/cm^2 (data not shown), and greater than 90% CD11c$^+$ cells (range 91.0-97.5%) were harvested after treatment.

In contrast, lymphocytes display Annexin-V as early as 2 hr after treatment with 1 J/cm^2 and 100 ng/mL (data not shown). In contrast to MoDC treated with 100 ng/mL and 1 J/cm^2 (Figure 11F), 24 hr after treatment with the same dose of PUVA, the percentage of early apoptotic lymphocytes increased from 6.6% in untreated MoDC to 44.3% in PUVA-dendritic cells, and the percentage of late apoptotic lymphocytes increased from 4.5% to 33.7% (Figure 11G). Given that 64.3 ± 3.2% of lymphocytes were Annexin-V$^+$ 24 hr after treatment, PUVA-treated lymphocytes are subsequently referred to as apoptotic lymphocytes (ApoL).

The PUVA dose-dependent induction of GILZ correlated with a decrease in cell surface expression of CD80, CD86, and CD83 (Figure 12A, 3B). Down-regulation of these markers paralleled the induction of GILZ (see Figure 11B), beginning at 8-MOP concentrations of 100 ng/mL for both 1 and 2 J/cm^2. As the product of 8-MOP and UVA exceeded 100, CD83, CD80 and CD86 expression were reduced by 31%, 30% and 54% respectively, and HLA-DR expression increased by 38%.

MoDC exposed to apoptotic lymphocytes up-regulate GILZ and are resistant to LPS-induced full maturation

To dissect the individual contributions of PUVA and exposure to apoptotic cells, MoDC were first co-cultured with varying ratios of ApoL. GILZ was up-regulated in an ApoL dose-dependent fashion (Figure 13A). When PUVA-dendritic cells were exposed to ApoL, GILZ was expressed at higher levels than in PUVA-dendritic cells cultured alone (Figure 13B). PUVA-dendritic cells exposed to ApoL also expressed GILZ at higher levels than in untreated MoDC exposed to ApoL (6.7-fold and 3.6-fold higher, respectively). There was a corresponding 1.5-fold increase in the GILZ protein level in all groups in which GILZ mRNA was up-regulated (Figure 13C). Induction of GILZ was not related to an increase in the number of early or late apoptotic CD11c$^+$ cells, as there were < 12% early apoptotic (range 3.8-11.4%) and late apoptotic (range 6.3-11.5%) CD11c$^+$ cells in all groups demonstrating up-regulation of GILZ.

MoDC expressing GILZ greater than 2.5-fold above untreated MoDC were resistant to full maturation by LPS and exhibited a semi-mature, tolerogenic phenotype. LPS stimulation increased CD80 expression in MoDC up-regulating GILZ to only 50% of the levels seen after LPS stimulation in untreated MoDC (Figure 13D, range 0.48-0.57%), and increased CD86 expression to only 45% of untreated MoDC (Figure 13D, range 0.42-0.47%). Similar results were obtained for HLA-DR and CD83 (Figure 14E, range 47-65% and 23-57% of untreated MoDC after LPS respectively). In addition, MoDC up-regulating GILZ expressed 6% of the CD80 mRNA of

untreated MoDC (range 4.5-7.5%), and expressed 50% of the CD86 mRNA of untreated MoDC (range 12.4-85.1%), as assessed by qRT-PCR.

MoDC expressing GILZ display a tolerogenic cytokine profile, and knockdown of GILZ reduces the IL-10 to IL-12p70 ratio

Supernatants were harvested from co-cultures as described in Figure 13B. Dex-dendritic cells up-regulated GILZ 4.29-fold (see Figure 13B), increased production of IL-10 (Figure 14A), and decreased production of all pro-inflammatory cytokines (Figure 14B, 14C) and chemokines (Figure 14D, 14E) tested. In comparison, PUVA-dendritic cells up-regulated GILZ 2.78-fold (see Figure 13B), increased production of IL-10, and decreased production of all pro-inflammatory cytokines and chemokines tested, except TNF-α and IFN-γ. PUVA-dendritic cells or untreated MoDC, exposed to ApoL expressed GILZ at higher levels that PUVA-dendritic cells cultured alone (3.6- and 6.7-fold higher, respectively; see Figure 13B). These two groups increased production of IL-10, and decreased production of all pro-inflammatory cytokines and chemokines tested. Cytokine levels were confirmed at the RNA level, with MoDC that up-regulated GILZ also demonstrating up-regulation of IL-10 mRNA 8-fold above untreated MoDC (range 5.5-11.8, $p < 0.01$). Reductions in IL-12, TNF-α, and IL-6 were also confirmed at the RNA level (data not shown). TGF-β was up-regulated 2.5-fold in MoDC up-regulating GILZ (data not shown). TGF-β was not included in the multiplex analysis and therefore was only analyzed at the mRNA level.

The IL-10 to IL-12p70 ratio is a useful indicator of tolerogenicity, since tolerogenic dendritic cells are characterized by an increased IL-10 to IL-12p70 ratio, see Steinman et al., references). The ratio of IL-10 to IL-12p70 increased from 6.7 in untreated MoDC to 67.7 in Dex-dendritic cells. Similarly, the IL-10 to IL-12p70 ratio increased to 38.7 in PUVA-dendritic cells, and to 89.4 and 114.9 in untreated MoDC and PUVA-dendritic cells exposed to ApoL, respectively ($p < 0.05$).

To assess whether induction of GILZ was mediating the tolerogenic cytokine profile, MoDC were transfected with siRNA to knockdown GILZ expression. Transfection with GILZ siRNA reduced GILZ expression in Mo-dendritic cells by 68% (Figure 15A, range 59-79%). Transfection with scramble siRNA did not significantly change GILZ expression. There was also no significant difference in the number of cells recovered from any groups transfected with siRNA as compared to non-transfected groups (data not shown).

Treated MoDC up-regulating GILZ 2.5-fold higher than untreated MoDC produced higher levels of IL-10 (Figure 15B), and knockdown of GILZ reduced IL-10 production by 39% (range 34-48%, $p < 0.05$). Treated MoDC up-regulating GILZ 2.5-fold higher than untreated MoDC also produced lower amounts of IL-12p70 (Figure 15C), and knockdown of GILZ increased IL-12p70 production by 188% (range 149-214%, $p < 0.05$). Treatment with scramble siRNA had no appreciable effect on the production of IL-10 or IL-12p70. Knockdown of GILZ reduced the IL-10 to IL-12p70 ratio that had been elevated after GILZ induction. Dex-dendritic cells treated with GILZ siRNA demonstrated a reduction in the IL-10 to IL-12p70 ratio from 15.3 in non-transfected MoDC to 3.9 in transfected Dex-dendritic cells. In PUVA-dendritic cells the ratio decreased from 8.4 in non-transfected MoDC to 2.9 in PUVA-dendritic cells, and in untreated MoDC and PUVA-dendritic cells exposed to ApoL, reductions in the ratio from 18.1 to 7.8 and 28.4 to 8.3, respectively, were observed.

These results demonstrates that like other immunosuppressive mediators, PUVA induces the expression of GILZ and generates tolerogenic immuno-suppressive dendritic cells, characterized by low expression of the co-stimulatory molecules CD80 and CD86, and the maturation marker CD83. GILZ induction is necessary for the polarization towards a tolerogenic cytokine profile, characterized by increased IL-10 production, and decreased pro-inflammatory cytokine and chemokine

production, including IL-12p70. These results further implicate GILZ as the molecular switch mediating the immunosuppressive effects of apoptotic cells.

Experiment 3 – Identification of further molecular markers for immunostimulatory dendritic cells

Patient samples

Leukocytes from patients undergoing ECP using the UVAR XTS Photopheresis System (Therakos) were obtained under the guidelines of the Yale Human Investigational Review Board. Informed consent was provided according to the Declaration of Helsinki. Aliquots were procured at 3 time points: before treatment (Pre ECP), immediately after 8-MOP/ultraviolet A (UVA) exposure (ECP Day 0) or after 18-hour incubation of treated blood mononuclear leukocytes (ECP Day 1) in a 1-L platelet storage bag (PL-2410; Baxter).

Normal subjects

To determine whether ECP induces monocytes from healthy subjects to convert to DC, mononuclear leukocytes from normal subjects were examined in 2 ways. Leukapheresed leukocytes from normal subjects (N = 3) were studied pretreatment (pre-ECP), immediately after ECP (ECP Day 0), and 18 hours after ECP (ECP Day 1). A desktop apparatus, incorporating a UVA light source and a plastic exposure plate, enabled laboratory reproduction of the clinical ECP system and sample access for parallel RNA isolation, immunophenotyping, and functional studies. Alternatively, a unit of blood from normal subjects was drawn into a transfer bag and passed through the ECP treatment apparatus in an identical fashion to that of treated patients (N = 3). The cells obtained from the unit of normal blood were used for microarrays and antigen presentation assays.

Psoralen addition

As is routinely done during ECP, the standard 8-MOP concentrated solution (Therakos) was added directly to the clinical ECP apparatus and to the laboratory model system. That mode of introduction enabled precise 100-200 ng/mL concentrations throughout the clinical procedures and experimentation.

Overnight culture

In ECP, it is not possible to examine phenotypic and functional changes in treated monocytes, because those cells are immediately reinfused into patients. Therefore, after ECP, cells were cultured for 18 hours (RPMI 1640/15% autologous serum) to study induced monocyte gene activation, maturation and function. Prior to (pre-ECP) and immediately after ECP (ECP Day 0), patient and normal subject samples were isolated by centrifugation over a Ficoll-Hypaque gradient. The cells were resuspended in RPMI-1640 medium (Gibco), supplemented with 7.5% AB serum, 7.5% autologous serum (Gemini Bio-Products) and cultured (for patients) in 6-well polystyrene tissue-culture plates at a density of $5*10^6$ cells/mL and in Baxter platelet storage bags (for normal subjects 37°C, 5% CO2). After overnight culture (ECP Day 1), cells were harvested before undergoing monocyte enrichment. To generate DC for comparative phenotypic analysis, cells were cultured in RPMI 1640 15% serum in the presence of 1 mL of GMCSF and IL4 (25 ng/mL; R&D Systems) for 6 days.

Magnetic bead enrichment of the monocyte population

To enable determination of whether ECP activates genes directing monocytes into the dendritic cell maturational pathway, it was necessary to develop a gentle negative monocyte enrichment method that eliminates contribution of lymphocytes to the transcriptome analysis while minimizing monocyte physical or cell membrane perturbation. Monocytes were enriched from the mononuclear cell pool by single passage through affinity columns. This negative selection method limited physical perturbation, whereas lymphocytes adherent to magnetic microbeads (Miltenyi Biotec), conjugated to relevant monoclonal antibodies (anti-CD4, CD8, CD19), were

depleted. However, enrichment of ECP Day 1 monocytes beyond 60%-80% proved challenging, because diminished surface display of lymphocyte markers by ECP-damaged lymphocytes permitted a fraction of T and B cells to escape retention in the columns. Repetitive passes through the affinity column, to further enhance monocyte purity, was not an option because that approach compounds the physical perturbation of passively filtered monocytes. Fortuitously, a series of analyses revealed that ECP's preferential damage of lymphocytes precluded the necessity of full purification of monocytes for accurate assessment of level of DC gene activation. Due to their extreme sensitivity to UVA-activated 8-MOP, 99% of ECP-processed lymphocytes were apoptotic after overnight incubation (as determined by staining with APO2-PE, Trypan blue, and/or annexin–fluorescein isothiocynate FITC/ propidium idodide). Because ECP causes global lymphocyte apoptosis, 90%-95% of viable mononuclear leukocytes in the ECP day 1 fraction were monocytes. This phenomenon accounts for the observation that multiple step magnetic bead removal of apoptotic lymphocytes, performed as follows and yielding monocyte purity of greater than 95%, does not alter levels of observed gene expression in the studied cell populations. To accomplish that comparison we modified the monocyte purification procedure by adapting a negative selection protocol using magnetic beads and the EasySep magnet. Peripheral blood mononuclear cells were centrifuged at low speed (120g for 10 minutes) to remove platelets. Cells were then labeled using the Monocyte Isolation Kit II (Miltenyi Biotec) following the manufacturers procedure with the follow- ing modifications: (1) buffer consisted of ice-cold phosphate-buffered saline containing 2% autologous serum and 1mM EDTA (ethylenediaminetetraacetic acid); (2) blocking time was increased to 10 minutes; (3) labeling with the Biotin-Antibody Cocktail was increased to 20 minutes; and (4) cells were washed once between labeling with the Biotin-Antibody Cocktail and the Anti-Biotin Microbeads. To avoid stimulating the monocytes by passing them over a column, the magnetically labeled cells were instead separated from the unlabeled monocytes using the EasySep magnet (StemCell Technologies). Cells, in 2 mL of buffer in a 5-mL polystyrene tube, were placed in the magnet for 10 minutes, and then the

unlabeled cells were carefully poured off into a new tube. This procedure was repeated 2x, to maximally enhance monocyte purity. At this point, because the purity was still insufficient, cells were relabeled with the Monocyte Isolation Kit II reagents and placed in the EasySep magnet for an additional 10 minutes, and the unlabeled monocytes were eluted. Final purity (X=96%+4.5) was assessed by flow cytometric analysis of CD14 staining.

Immunophenotyping

Monoclonal antibodies specific for monocytes and dendritic cells, included: CD14 (lipopolysaccharide [LPS] receptor, monocytes); CD36 (receptor for apoptotic cells, monocytes); human leukocyte antigen DR-1 (HLA-DR; class II major histocompatibility complex [MHC] molecule); CD83 (dendritic cell marker); cytoplasmic dendritic cell-lysosome–associated membrane protein (DC-LAMP; dendritic cell marker); and CD80 and CD86 (B7.1 and B7.2 costimulatory molecules). Antibodies were obtained from Beckman Coulter and used at their predetermined optimal dilutions. Background staining was established with appropriate isotype controls, and immunofluorescence was analyzed using a FC500 flow cytometer (Beckman Coulter). Combined membrane and cytoplasmic staining was performed following manufacturer's instruc- tions for cell fixation and permeabilization (Intraprep kit; Beckman Coulter).

Antigen presentation assay

Volunteer freshly isolated, magnetic bead-enriched, antigen-experienced $CD4^+$ populations ($2*10^6$/mL, 50 µL/well) were added to monocytes ($2*10^6$/mL, 50 µL/well) in the presence of tetanus toxoid (10 µg/mL, 100 µL/well) and RPMI medium 1640/15% autologous serum. After 5 days of culture, the cells received 1 µCi of [^{3}H]-thymidine and were incubated overnight, harvested, and counted in a Beta liquid scintillation counter (PerkinElmer). Results are presented as the mean and standard deviation of 5 replicate cultures.

MLR/CML assay

To assess whether ECP-processed monocytes are functionally capable of stimulating MHC class I–restricted cytotoxicity by CD8 T cells, mononuclear leukocytes from 3 normal subjects were studied. One unit of anti-coagulated blood, freshly procured from each of 3 HLA-A2–positive volunteers, served as sources of stimulator monocyte/dendritic cells, before and after being processed through the clinical ECP apparatus in a manner identical to the actual ECP procedure. Mononuclear fractions were isolated from the blood immediately prior to ECP processing (pre-ECP) and immediately after ECP (ECP D0). After gamma irradiation (3000 rad, Cesium source) to ensure unidirectional T-cell stimulation, the Pre ECP fraction was serially diluted in RPMI 1640/15% autologous serum, and 100 μL containing from 25 000 to 250 cells was plated in round-bottom microtiter plate wells, in 5 replicates. The ECP D0 fraction was incubated for 18 hours in large well plates and harvested by scraping the wells to free adherent cells. The re-suspended cells were then serially diluted and plated as above. An A-2–negative normal donor served as the source of responder CD4 and CD8 T cells, purified by positive selection on Miltenyi magnetic bead columns (average purity 98%). Responder T cells (50 000/well in 100 μL) were then added to the wells containing either Pre-ECP or ECP-D0 stimulators, and the plates were cultured for 7 days at 37°C in a CO2 incubator. For target cells, the A-2–positive T-B hybridoma lymphoblast line, 174 x CWM.T1, was labeled with ^{51}Cr and added to the MLR cultures at 10^4 cells/well. After 4-hour incubation, plates were centrifuged, and 100 μL of supernatant was removed from each well for counting in a gamma counter. “Percent-specific lysis” was defined as 100 times the following fraction:

$$\frac{\text{Mean cpm (sample) - Mean cpm (T cell only)}}{\text{Mean cpm (detergent maximum release) - mean cpm (T cells only)}}$$

RNA isolation and microarray hybridization

Total RNA was isolated using RNeasy Mini Kit columns with on-column DNase I treatment (QIAGEN). RNA yield and purity were measured using the NanoDrop ND-1000 Spectrophotometer and the Agilent 2100 Bioana- lyzer. Fragmented cRNAs were hybridized on Affymetrix HG U133 Plus 2.0 human chips, and screening for approximately 47 400 human genes and ESTs was performed by the Yale University W. M. Keck Resource Laboratory. The microarray results are available on Gene Expression Omnibus under accession number GSE23604.

Data analysis

Raw data without normalization generated from Affymetrix GeneChip Operating Software Version 1.2 (GCOS 1.2; Affymetrix) were analyzed using GeneSpring software 7.2 (Agilent Technologies-Silicon Genetics). Data were normalized using Robust Multi-Array. Only probe sets with a minimal fold change of >2.0 combined with an average signal intensity of 500 or higher in either apheresis such as leukaphereses or treated samples were included in the analysis. Differential gene expression was considered as a $\geq$2-fold change and $P \leq .05$. Principal component analysis (PCA) of the induced transcriptomes was performed by standard methodology.Signal transduction pathway involvement was identified with MetaCore Software Version 1.0 (GeneGo).

Quantitative real-time PCR

Microarray expression of selected genes was confirmed in aliquots of the same RNA samples, using quantitative real-time polymerase chain reaction (PCR). RNA was reverse transcribed to cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Reverse transcription was carried out in a 96-well thermocycler (MJ Research PTC-200) in the following conditions: 25°C, 10 minutes, 37°C, 120 minutes, 85°C, 5 seconds. TaqMan real-time PCR was used to detect transcripts of DC-LAMP, CCR7, CD80, CD86, and CD14. Primers and probes for

each sequence were obtained as inventoried Taqman Gene Expression Assays (Applied Biosystems). HPRT1 was used as a reference gene.

Results

Large ECP-induced changes in individual gene expressions

The stimulation by ECP of individual gene activation in monocytes was expressed as the ratio of ECP Day 1 to pre-ECP expression for the relevant gene. To preclude inadvertent gene induction during monocyte enrichment, a negative column purification method was used, whereby lymphocytes were retained, and monocytes were passively filtered. The results revealed that the ECP-processed monocytes from both patients and normal subjects remain sufficiently viable to reproducibly express a shared transcriptome signature.

Genes were considered significantly up- or down-regulated by ECP if fold change was ≥ 2 and significance was $P \leq .05$ compared with pre ECP. Levels of RNA transcripts from approximately 3000 genes were significantly changed in each patient group and in normal subjects (Table 2). Overall, 1129 genes were up- or down-regulated in common by ECP-processed monocytes from both CTCL and GVHD patients and from normal subjects, indicating commonality in ECP-induced gene activation.

Table 2: Number of Monocyte Genes with Altered Expression after ECP.

Monocyte Source	Total	Up-regulated	Down-regulated
Normal Subjects (alone): N=6	3,666	1494 (41%)	2172 (59%)
CTCL (alone): N=3	4,315	2613 (61%)	1702 (38%)
GVHD (alone): N=3	4,350	2658 (61%)	1692 (39%)

Number of genes significantly induced or suppressed by ECP.

Increased expression of numerous genes associated with dendritic cell differentiation, adhesion, and function (Table 3) further support ECP stimulation of entry of monocytes into that pathway.

Table 3: : ECP-Enhanced Expression of DC Marker Genes, Ratio* of Post-ECP/Pre-ECP Levels

Gene	Attributes	CTCL and GVHD (N-6) Induced Expression Ratio	Normal Subjects (N-6) Induced Expression Ratio
DC-LAMP	DC Lysomal Protein	27.6 $p=1.2x10^{-09}$	17.2 $p=1.4\ x10^{-07}$
GPNMB	Transmembrane glycoprotein	205.7 $p=9.6x10^{-15}$	123.3 $p=2.8x10^{-14}$
CD80	Co-stimulatory molecule, B7.1	13.4 $p=2.3x10^{-13}$	NC
CD86	Co-stimulatory molecule, $B7.2^{8}$	NC	5.0 $p=1.4x10^{-05}$
CD40	Involved in DC survival	2.3 $p=5.7^{-04}$	NC
Decysin	ADAM-like, Expressed in LPS matured DC	26.5 $p=1.0x10^{-09}$	7.1 $p=5.6x10^{-04}$
CCR7	Lymph node homing molecule	2.6 $p=7.0x10^{-03}$	NC
CD83	DC maturation molecule	NC	2.3 p=0.03
OLR1	Lox1, lectin-like receptor	13.6 $p=3.3x10^{-05}$	100.1 $p=8.3x10^{-08}$
CLEC5A	MDL-1	10.9 $p=9.5x10^{-07}$	45.5 $p=1.6x10^{-08}$
FPRL2	Formyl peptide receptor-like-2	33.9 $p=2.1x10^{-08}$	43.2 $p=1.9x10^{-08}$
SDC2	Syndecan, cell surface proteoglycan	21.7 $p=9.3x10^{-08}$	98.9 $p=3.3x10^{-09}$
THBS1	Thrombospondin 1	6.2 $p=7.8x10^{-08}$	10.4 $p=4.7x10^{-09}$

*Ratio = (Pre-ECP Gene Expression) to (Post-ECP Gene Expression), Fold increase in expression of multiple genes involved in DC maturation and function induced by ECP. Impact of treatment on gene expression is displayed as an *Induced Expression Ratio* (ratio of post-ECP to pre-ECP expression for the relevant gene). RNA was isolated from 3 CTCL patients and 3 GVHD patients and 6 normal subjects at the relevant time points.

Further genes, the expression of which was found to be increased and which can be considered to be molecular markers of immune-stimulatory dendritic cells are depicted in Table 1.

As would be expected during monocyte-to-dendritic cell maturation, CD14 (monocyte marker) expression was diminished, as assessed by measuring the mean fluorescence intensity on the monocyte populations of all patients and normal subjects, after overnight culture of ECP-processed monocytes. This result was confirmed in RT-PCR studies of the patients' post-ECP cells (results not shown). Further factors, the expression of which was reduced indicating monocyte-to-dendritic cell maturation are shown in Table 4.

Table 4: ECP-Reduced Expression of Monocyte Marker Genes , Ratio* of Post-ECP/Pre-ECP Levels

Gene	Attributes	CTCL and GVHD (N=6) Induced Expression Ratio	Normal Subjects (N=6) Induced Expression Ratio
CD33	Cell surface protein expressed on monocytes	-2.2 $p=4.5x10^{-04}$	NC
CD36	Receptor for apoptotic cells	-7.4 $p=7.9x10^{-05}$	NC
FCGR1A	Receptor for IgGFc fragment 1A	-6.9 $p=6.6x10^{-05}$	-4.4 $p=2.1x10^{-03}$

*Ratio = (Pre-ECP Gene Expression) to (Post-ECP Gene Expression), Fold decrease in expression of genes distinctive of monocytes induced by ECP, as the monocytes differentiate into DC. Impact of treatment on gene expression is displayed as an *Induced Expression Ratio* (ratio of post-ECP to pre-ECP expression for the relevant gene). RNA was isolated from 3 CTCL patients and 3 GVHD patients and 6 normal subjects at the relevant time points.

Further factors, the expression of which was reduced and thus indicating monocyte-to-immuno suppressive dendritic cell maturation are shown in Table 5.

Table 5: ECP-Enhanced Expression of Immunosuppression-Associated Genes, Ratio* of Post-ECP/Pre-ECP Levels.

Gene	Attributes Normal	CTCL and GVHD (N-6) Induced Expression Ratio	Normal Subjects (N-6) Induced Expression Ratio
IDO	Indoleamine	27.8 $p=4.0x10^{-10}$	9.4 $p=1.1x10^{-06}$
KMO	kynurenine 3-hydroxylase	6.0 $p=2.5x10^{-06}$	NC
IL10	Interleukin 10	6.3 $p=9.2x10^{-06}$	8.6 $p=5.7x10^{-06}$

*Ratio = (Pre-ECP Gene Expression) to (Post-ECP Gene Expression), ECP-induced fold increase in expression of genes which contribute to DC capacity suppress T cell-mediated immunologic reactions. Impact of treatment on gene expression is displayed as an *Induced Expression Ratio* (ratio of post-ECP to pre-ECP expression for the relevant gene). RNA was isolated from 3 CTCL patients and 3 GVHD patients and 6 normal subjects at the relevant time points.

Experiment 4 - Surface molecule markers and functional mediators of immuno-stimulatory DC.

Further analysis of the ECP-induced dendritic cells transcriptome was performed to identify a subset of surface molecule gene products as markers and functional mediators of immuno-stimulatory dendritic cells. Of 466 genes upregulated in ECP-induced dendritic cells were cross referenced to approximately 2000 known or presumed full-length human transmembrane genes to identify 87 shared surface proteins.

Materials and Methods

Procurement of leukocytes and platelets

All samples were acquired from young, healthy subjects not taking medications, including aspirin, known to influence platelet function. Samples were obtained under the guidelines of the Yale Human Investigational Review Board, and informed consent was provided according to the Declaration of Helsinki. Peripheral blood specimens were collected through a 19-gauge needle from the antecubital vein into syringes containing heparin, then layered on Ficoll-Hypaque (Gallard-Schlessinger, Carle Place, N.Y.). Following centrifugation at 180g, the interface containing the mononuclear leukocyte fraction was collected and washed twice in HBSS, then resuspended in RPMI-1640 medium (GIBCO) to a final concentration of 5×10^6 mononuclear cells/ml. Cells were utilized within one hour of being acquired.

Preparation of Platelet-rich-Plasma

Whole blood was centrifuged at 150 g for 15 min at room temperature. The platelet-rich-plasma (PRP) layer was collected and centrifuged at 900 g for 5 min, and the platelet pellet resuspended in RPMI 1640 to the desired concentration.

Preparation of Plates

Plate passage was conducted using a Glycotech system (Glycotech, Rockville, MD). This system consisted of a volumetric flow path measuring 20000 x 10000 x 254 microns (length x width x height). The bottom plate in this system was composed of a 15mm petri dish (BD Biosciences, Durham, NC) separated by a gasket and vacuum-connected to an acrylic flow deck, which formed the upper plate. For pre-coating with platelets, prior to assembling the flow chamber, 20 drops of the desired concentration of PRP was placed in the center of the petri dish and platelets allowed to settle for 20 minutes at room temperature. The petri dish was washed twice with 2ml of RPMI, and the flow chamber then assembled.

Overnight culture

When overnight culture was required, cells were centrifuged and resuspended in RPMI-1640 medium (GIBCO), supplemented with 15% AB serum (Gemini Bio-Products) to a final concentration of 5 x 106 cells/ml. Cells were cultured overnight for 18 hours in 12-well polystyrene tissue culture plates (2 ml per well) at 37° C in 5% CO2.

Immunophenotyping

Monoclonal antibodies for immunophenotyping included CD14 (LPS receptor; monocytes), CD11c (integrin subunit; monocytes and DC), HLA-DR (class II MHC molecule), CD83 (DC marker), CD62p (P-selectin; activated platelets), and CD61 (integrin subunit; platelets). Antibodies were obtained from Beckman Coulter (CD14, CD11c, HLADR, CD83) or Sigma (CD62p, CD61) and used at their pre-determined optimal dilutions. Background staining was established with appropriate

isotype controls, and immunofluorescence was analyzed using a FC500 flow cytometer (Beckman Coulter). Two-color membrane staining was performed by adding the pre-determined optimal concentrations of both antibodies directly conjugated to FITC or PE and incubating for 20 min at 4°C, followed by washing to remove unbound antibodies. Combined membrane and cytoplasmic staining was performed following manufacturer's instructions for cell fixation and permeabilization (Intraprep kit, Beckman Coulter).

Results

Plate-passed and/or PBMC D1 populations showed significant upregulation of analyzed surface expression of SIRPa, ICAM1, CXCL16, LIGHT, PLAUR (CD87, plasminogen activator, urokinase receptor), MSR1, Neu1 (sialidase), CD137L, and CATB (CTSB, cathepsin B).

Experiment 5 – Determining expression of molecular markers and FSC/SSC complexity after passing monocytes through flow chamber

Materials and Methods

Monocytes were passed through a device depicted in Fig. 19. In brief, a blood sample was spun at low speed through a Ficoll gradient to obtain e.g. 8 ml of sample with a concentration of peripheral blood mononuclear cells (PBMC) of e.g. 10^{10} cells/ml. The chamber was pre-coated with platelets. The sample was passed through the chamber at about 0.028 Pa. The chamber and then washed with about 3 ml RPMI at 0.028 Pa. A second wash with 30-55 ml RPMI was performed at about 1.2 Pa. The collected activated monocytes were combined, incubated for a day and used for further analysis (PP D1 PBMC). As a control PBMCs were not passed through the device and incubated for a day (D1 PBMC). As another control immature fast DC were obtained by directly cultivating PBMC in the presence of GM-CSF and IL-4

(immature Fast DC). Further, PBMC were analyzed directly after harvest through a Ficoll gradient (Fresh (Ficoll) PBMC).

The cells and controls were then analyzed for expression of HLA-DR, CD86, ICAM-1, and PLAUR. They were further analyzed for FSC/SSC complexity. The results are depicted for HLA-DR in Figure 20 and for FSC/SSC complexity in Figure 21 and 22. A summary is shown in Figure 23.

Results

The results show that cells subjected to centrifugation through a Ficoll gradient alreads seem to experience enough physical forces to start differentiating as becomes apparent from incubating these cells for one day (D1 PBMC). However, activation and differentiation is more pronounced upon plate passage through the device (PP D1 PDMC). The dendritic cells obtained by methods in accordance with the invention in the absence of e.g. 8-MOP and UV-A moreover have a more complex and distinct pattern than immature Fast DC obtained with cytokine cocktails.

Experiment 6 – Determining inlfuence of 8-MOP and UVA on activated monocytes

Materials and Methods

Monocytes were passed through a device derived from the Therakos system. The dimensions of the flow chamber were 30 mm width, 1600 mm length, and 1 mm height. In brief, a blood sample was spun at low speed through a Ficoll gradient to obtain e.g. 80 ml of sample with a concentration of peripheral blood mononuclear cells (PBMC) of e.g. $1.5*10^6$ cells/ml. The chamber was pre-coated with platelets. The sample was passed through the chamber for 60 min at a flow rate of about 24

ml/min corresponding to a shear stress of about 0.13 Pa. The collected activated monocytes were spun at low speed and resuspended in a small volume of medium comprising 8-MOP and irradiated with combined, incubated for a day and used for further analysis (Day 1PBMC + PP). As a control PBMCs were not passed through the device and incubated for a day (Day 1 PBMC). Further, PBMC were analyzed directly after harvest through a Ficoll gradient (Day0 PBMC). Day 1 PBMC + PP were then further treated with 8-MOP and UVA (PUVA).

The cells and controls were then analyzed for expression of GILZ. The results are depicted for GILZ in Figure 24.

Results

The results show that cells that activated monocytes that would differentiate into immuno-stimulatory dendritic cells can be channeled into immuno-suppressive dendritic cells by application of 8-MOP and UVA.

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CLAIMS

1. A method for inducing differentiation of monocytes contained in an extracorporeal quantity of a mammalian subject's blood sample into immuno-suppressive antigen-presenting cells, said method comprising at least the steps of:
    a) subjecting said extracorporeal quantity of said mammalian subject's blood sample to a physical force such that said monocytes are activated and induced to differentiate into immuno-suppressive antigen-presenting cells, which are identifiable by at least one molecular marker, wherein said at least one molecular marker is indicative of immuno-suppressive antigen-presenting cells.

2. Method according to claim 1,
wherein said immuno-suppressive antigen-presenting cells are immuno-suppressive dendritic cells which are identifiable by an increased expression of GILZ IDO, KMO, and/or IL-10.

3. Method according to any of claims 1 or 2,
wherein said immuno-suppressive antigen-presenting or dendritic cells are identifiable by a GILZ induced increased IL-10 to IL-12p70 ratio.

4. Method according to any of claims 1, 2, or 3,
wherein said immuno-suppressive antigen-presenting or dendritic cells are identifiable by determining expression of at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20 , 25, 30, 35, 40, 45, 50, 55, or 60 molecular markers, which are indicative of immuno-stimulatory antigen-presenting or dendritic cells

5. Method according to any of claims 1, 2, 3, or 4,

wherein said at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20 , 25, 30, 35, 40, 45, 50, 55, or 60 molecular markers are selectable from table 1 do not show an increased expression.

6. Method according to claim 5,
wherein said at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20 , 25, 30, 35, 40, 45, 50, 55, or 60 molecular markers include PLAUR, NEU1, CTSB, CXCL16, ICAM1, MSR1, OLR1, SIRPA, TNFRSF1A, TNFSF14, TNFSF9, PMB22, CD40, LAMP3, CD80, CCR7, LOX1, CD83, ADAM Decysin, FPRL2, GPNMB and/or CD86.

7. Method according to any of claims 1, 2, 3, 4, 5, or 6,
wherein said monocytes are activated and induced to differentiate into said immuno-suppressive antigen-presenting or dendritic cells without the need for addition of a molecular cocktail comprising cytokines and/or chemokines.

8. Method according to any of claims 1, 2, 3, 4, 5, 6, or 7,
wherein said extracorporeal quantity of said mammalian subject's blood sample is subjected to a physical force by passing said extracorporeal quantity of said mammalian subject's blood sample through a flow chamber of a device, which allows for fixed or tunable adjustment of the flow rate of said extracorporeal quantity of said mammalian subject's blood sample through said flow chamber of said device such that a shear force is applied to said monocytes contained within said mammalian subject's blood sample.

9. Method according to any of claims 1, 2, 3, 4, 5, 6, 7, or 8,
wherein said extracorporeal quantity of said mammalian subject's blood sample is subjected to a physical force by passing said extracorporeal quantity of said mammalian subject's blood sample through a flow chamber of a device, which allows adjustment of the flow rate of said extracorporeal quantity of said

mammalian subject's blood sample through said flow chamber of said device such that a shear force is applied to said monocytes contained within said mammalian subject's blood sample, and
wherein said device additionally allows for adjustment of at least one parameter selected from the group comprising temperature, and light exposure.

10. Method according to any of claims 1, 2, 3, 4, 5, 6, 7, 8, or 9, wherein said monocytes are activated and induced to differentiate into immuno-suppressive antigen-presenting or dendritic cells through interaction with activated platelets and/or plasma components.

11. Method according to any of claims 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10, wherein activation of said monocytes and differentiation into immuno-suppressive antigen-presenting or dendritic cells can be influenced by the design and dimensions of the flow chamber, the flow rate at which the monocytes are passed through the flow chamber, the light to which the monocytes are exposed to in the presence or absence of DNA cross-linking agents such as 8-MOP, the temperature at which the monocytes, platelets, platelets-derived factors and/or plasma components are passed through the flow chamber, the order by which the monocytes, platelets, platelets-derived factors and/or plasma components are passed through the flow chamber, the density by which plasma components are coated to the surfaces of the flow chamber, the density by which platelets and/or platelets derived factors adhere to the surfaces and or to the plasma components of the flow chamber, and/or the density by which monocytes adhere to the platelets and/or platelets derived factors and or plasma components adhered to the surfaces of the flow chamber.

12. Method according to any of claims 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or 11, wherein said method comprises at least the steps of:

a) applying said extracorporeal quantity of said mammalian subject's blood sample comprising at least monocytes to a device, which is configured to provide for a flow chamber through which said extracorporeal quantity of said mammalian subject's blood sample can be passed,

b) activating platelets, which may be comprised within said extracorporeal quantity of said mammalian subject's blood or which may be provided separate from said mammalian subject's blood sample comprising at least monocytes,

c) treating said extracorporeal quantity of said mammalian subject's blood sample comprising at least monocytes in said device by applying a physical force to the monocytes contained within said extracorporeal quantity of said mammalian subject's blood sample such that said monocytes are activated and induced to differentiate into immuno-suppressive antigen-presenting or dendritic cells by binding to said activated platelets obtained in step b).

13. Method according to any of claims 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or 11, wherein said method comprises at least the steps of:

a) applying said extracorporeal quantity of said mammalian subject's blood sample comprising at least monocytes to a device, which is configured to provide for a flow chamber through which said extracorporeal quantity of said mammalian subject's blood sample can be passed,

b) passing plasma components, which may be comprised within said extracorporeal quantity of said mammalian subject's blood sample or which may be provided separate from said mammalian subject's blood sample,

c) treating said extracorporeal quantity of said mammalian subject's blood sample comprising at least monocytes in said device by applying a physical force to the monocytes contained within said extracorporeal quantity of said mammalian subject's blood sample such that said

monocytes are activated and induced to differentiate into immuno-suppressive antigen-presenting or dendritic cells by binding to said plasma components obtained in step b).

14. Method according to any of claims 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or 11, wherein said method comprises at least the steps of:

a) applying said extracorporeal quantity of said mammalian subject's blood sample comprising at least monocytes to a device, which is configured to provide for a flow chamber through which said extracorporeal quantity of said mammalian subject's blood sample can be passed,

b) passing plasma components, which may be comprised within said extracorporeal quantity of said mammalian subject's blood or which may be provided separate from said mammalian subject's blood sample,

c) activating platelets, which may be comprised within said extracorporeal quantity of said mammalian subject's blood sample or which may be provided separate from said mammalian subject's blood sample comprising at least monocytes,

d) treating said extracorporeal quantity of said mammalian subject's blood comprising at least monocytes in said device by applying a physical force to the monocytes contained within said extracorporeal quantity of said mammalian subject's blood sample such that said monocytes are activated and induced to differentiate into immuno-suppressive antigen-presenting or dendritic cells by binding to said activated platelets and/or plasma components obtained in steps b) and c).

15. Method of any of claims 12, 13, or 14, wherein said extracorporeal quantity of said mammalian subject's blood sample is not obtained by .

16. Method of claim 15,

wherein said extracorporeal quantity of said mammalian subject's blood sample is between about 10 ml to about 500 ml of extracorporeal whole blood of said mammalian subject.

17. Method of any of claims 15 to 16,
wherein said extracorporeal quantity of said mammalian subject's blood sample is obtained by isolating leukocytes from about 10 ml to about 500 ml of extracorporeal whole blood of said mammalian subject.

18. Method of any of claims 15, 16, or 17,
wherein said extracorporeal quantity of said mammalian subject's blood sample is obtained by isolating buffy coats from about 10 ml to about 500 ml of extracorporeal whole blood of said mammalian subject.

19. Method of any of claims 15, 16, 17, or 18,
wherein said extracorporeal quantity of said mammalian subject's blood sample does not comprise plasma components.

20. Method of any of claims 15, 16, 17, 18, or 19,
wherein said extracorporeal quantity of said mammalian subject's blood sample does not comprise platelets.

21. Method of claim 20,
wherein said platelets have been separated from said extracorporeal quantity of said mammalian subject's blood before said extracorporeal quantity of said mammalian subject's blood said is applied to said device.

22. Method of any of claims 12, 13, or 14,
wherein said extracorporeal quantity of said mammalian subject's blood is obtained by .

23. Method of claim 22,
wherein said extracorporeal quantity of said mammalian subject's blood is obtained by isolating leukocytes by .

24. Method of any of claims 22 to 23,
wherein said extracorporeal quantity of said mammalian subject's blood is obtained by isolating buffy coats by .

25. Method of any of claims 22, 23, to 24,
wherein said extracorporeal quantity of said mammalian subject's blood does not comprise plasma components.

26. Method of any of claims 22, 23, 24, or 25,
wherein said extracorporeal quantity of said mammalian subject's blood does not comprise platelets.

27. Method of claim 26,
wherein said platelets have been separated from said extracorporeal quantity of said mammalian subject's blood before said extracorporeal quantity of said mammalian subject's blood said is applied to said device.

28. Method of any of claims 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, or 27, wherein said flow chamber has dimensions of about 1 μm to up to about 400 μm of height and of about 1 μm to up to about 400 μm of width.

29. Method of claim 28, wherein said flow chamber has dimensions of about 5 μm to up to and including about 300 μm of height and of about 5 μm to up to and including about 300 μm of width.

30. Method of claim 29, wherein said flow chamber has dimensions of about 10 μm to up to and including about 250 μm of height and of about 10 μm to up to and including about 250 μm of width.

31. Method of claim 30, wherein said flow chamber has dimensions of about 50 μm to up to and including about 200 μm of height and of about 50 μm to up to and including about 200 μm of width.

32. Method of claim 31, wherein said flow chamber has dimensions of about 50 μm to up to and including about 100 μm of height and of about 50 μm to up to and including about 100 μm of width.

33. Method of any of claims 28, 29, 30, 31, or 32, wherein said flow chamber is configured to take up a volume of between about 1 ml to about 50 ml of said extracorporeal amount of said mammalian subject's blood sample.

34. Method of any of claims 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, or 33, wherein the material of said flow chamber is not plastic.

35. Method of claim 34, wherein said non-plastic material is selected from the group consisting of glass.

36. Method of any of claims 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, or 33, wherein the material of said flow chamber is plastic.

37. Method of claim 36, wherein said plastic material is selected from the group consisting of acrylics, polycarbonate, polyetherimide, polysulfone,

polyphenylsulfone, styrenes, polyurethane, polyethylene, teflon or any other appropriate medical grade plastic.

38. Method of any of claims 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, or 37, wherein said flow chamber is configured to allow for transmittance of light.

39. Method of claim 38, wherein said flow chamber is configured to allow for transmittance of UV light.

40. Method of any of claims 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, or 39, wherein activation of said platelets is achieved by disposing plasma components, which are comprised within said extracorporeal quantity of said mammalian subject's blood sample, on the surface of said flow chamber such that at least some of said platelets can interact with said plasma components and are immobilized on the surface of said flow chamber.

41. Method of any of claims 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, or 40, wherein activation of said platelets is achieved by disposing proteins selected from the group comprising fibrinogen, fibronectin, and the gamma component of fibrinogen on the surface of said flow chamber such that at least some of said platelets can interact with said proteins and are immobilized on the surface of said flow chamber.

42. Method of claim 41, wherein activation of said platelets is achieved by disposing fibronectin on the surface of said flow chamber such that at least some of said platelets can interact with said fibronectin and are immobilized on the surface of said flow chamber.

43. Method of any of claims 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, or 42, wherein said platelets are passed through said flow chamber under a shear force of about 0.1 to about 10.0 dynes/cm^2, preferably in a range of about 0.1 to about 2.0 dynes/cm^2

44. Method of any of claims 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42 or 43, wherein said monocytes are passed through said flow chamber with a flow rate of about 10 ml/minute to about 200 ml/minute to produce a shear force of about 0.1 to about 20.0 dynes/cm^2.

45. Method of any of claims 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, or 44, wherein activation of platelets can be monitored by expression of P-selectin and/or αIIb-β3 integrin.

46. Method of any of claims 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, or 45, wherein said monocytes are activated and induced to differentiate into immuno-suppressive antigen-presenting or dendritic cells by passing said monocytes through said flow chamber under a shear force of about 0.1 to about 10.0 dynes/cm^2, preferably force of about 0.1 to about 1.0 dynes/cm^2 such that said monocytes can bind to said activated platelets.

47. Method of any of claims 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45 or 46, further comprising the step of incubating the activated monocytes to allow the formation of immuno-suppressive dendritic cells.

48. Method of any of claims 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, or 47 for obtaining individual-specific functionally and maturationally synchronized immuno-suppressive antigen-presenting or dendritic cells.

49. Method of any of claims 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, or 48,
wherein said monocytes are exposed to UV light in the presence of a DNA-cross linking agent to effect increased expression of GILZ.

50. Method of claim 49,
wherein said monocytes are exposed to UVA light in the presence of 8-MOP to effect increased expression of GILZ.

51. Method of any of claims 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50, for obtaining immuno-suppressive autologous antigen-presenting or dendritic cells.

52. Method of any of claims 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50, for obtaining immuno-suppressive allogenic antigen-presenting or dendritic cells.

53. Immuno-suppressive autologous dendritic cells obtainable by a method in accordance with any of claims 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38,

39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, or 51 for use in treating autoimmune diseases.

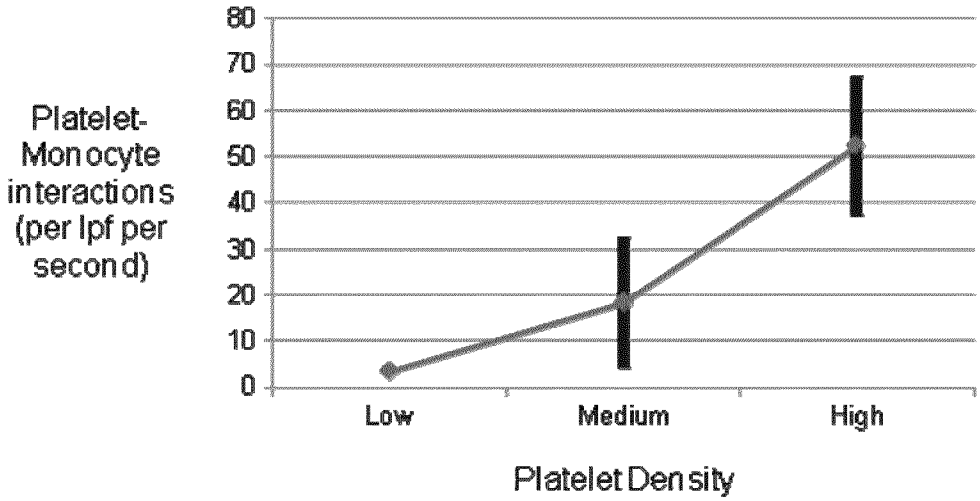
54. Immuno-suppressive autologous dendritic cells for use of claim 53, wherein the autoimmune disease is selected from the group comprising rheumatoid arthritis, psoriasis, multiple sclerosis, type 1 diabetes and systemic lupus erythematosus.

55. Immuno-suppressive autologous dendritic cells obtainable by a method in accordance with any of claims 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, or 51 for use in treating an hypersensitivity diseases.

56. Immuno-suppressive allogenic dendritic cells obtainable by a method in accordance with any of claims 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, or 52 for use in treating rejection in solid-organ transplantation and/or Graft-versus-Host disease.

Figure 1

a)


Platelet-Monocyte interactions (per lpf per second)
80
70
60
50
40
30
20
10
0
Low
Medium
High
Platelet Density

b)

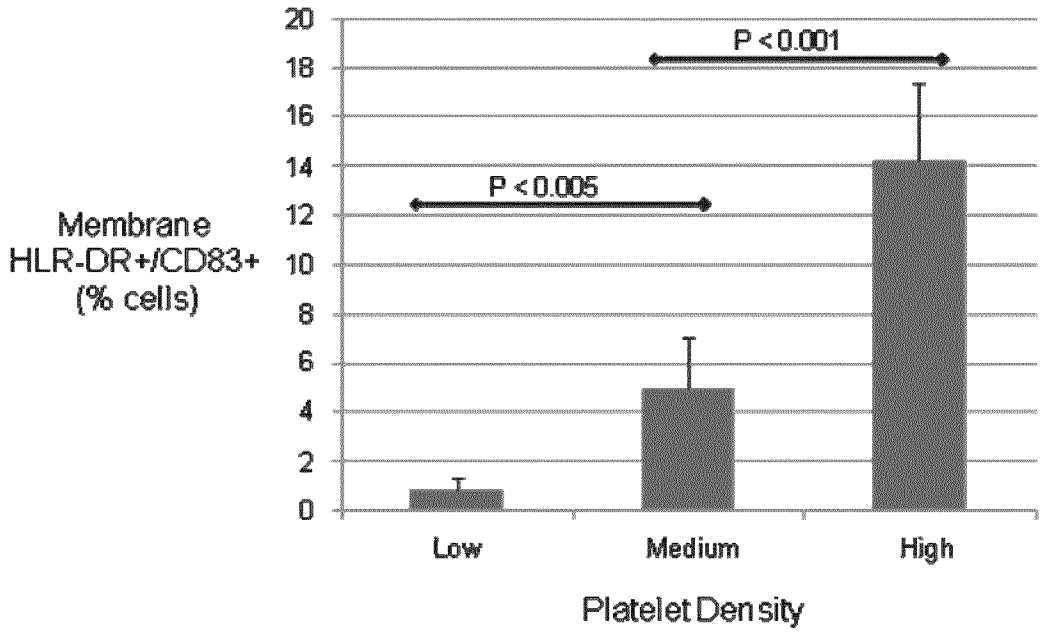

Membrane HLR-DR+/CD83+ (% cells)
20
18
16
14
12
10
8
6
4
2
0
P < 0.001
P < 0.005
Low
Medium
High
Platelet Density

Figure 2

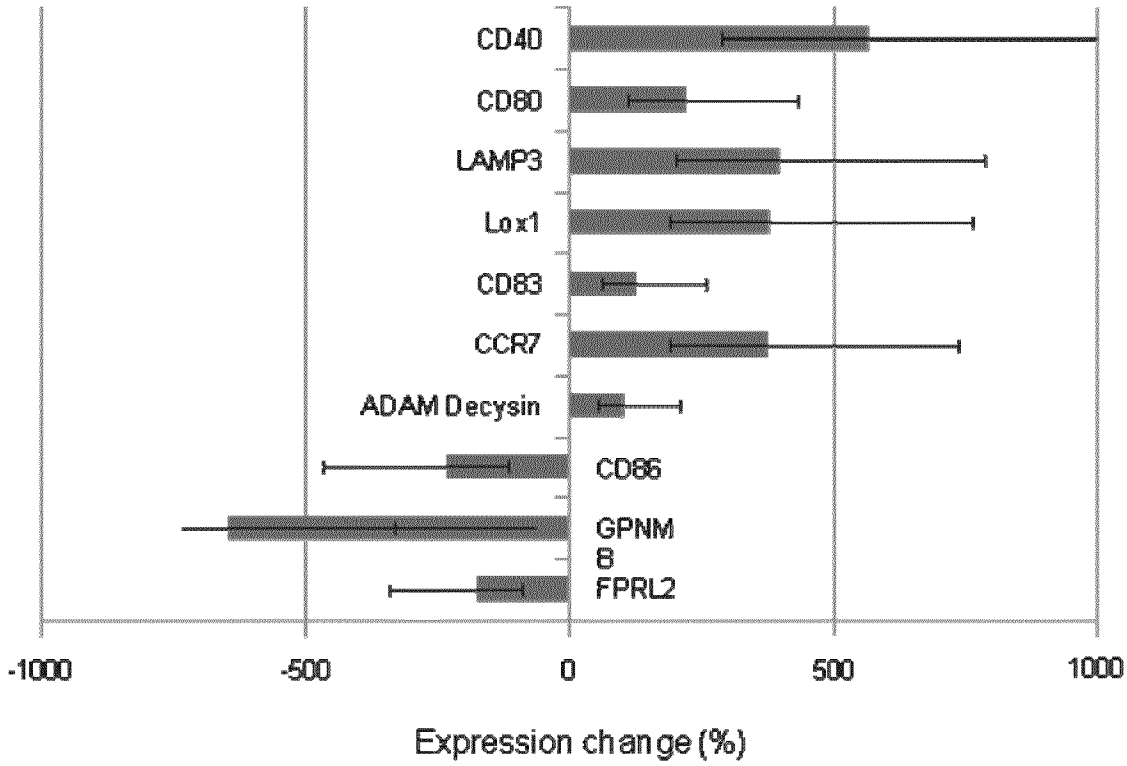

CD40
CD80
LAMP3
Lox1
CD83
CCR7
ADAM Decysin
CD86
GPNMB
FPRL2
-1000
-500
0
500
1000
Expression change (%)

Figure 3

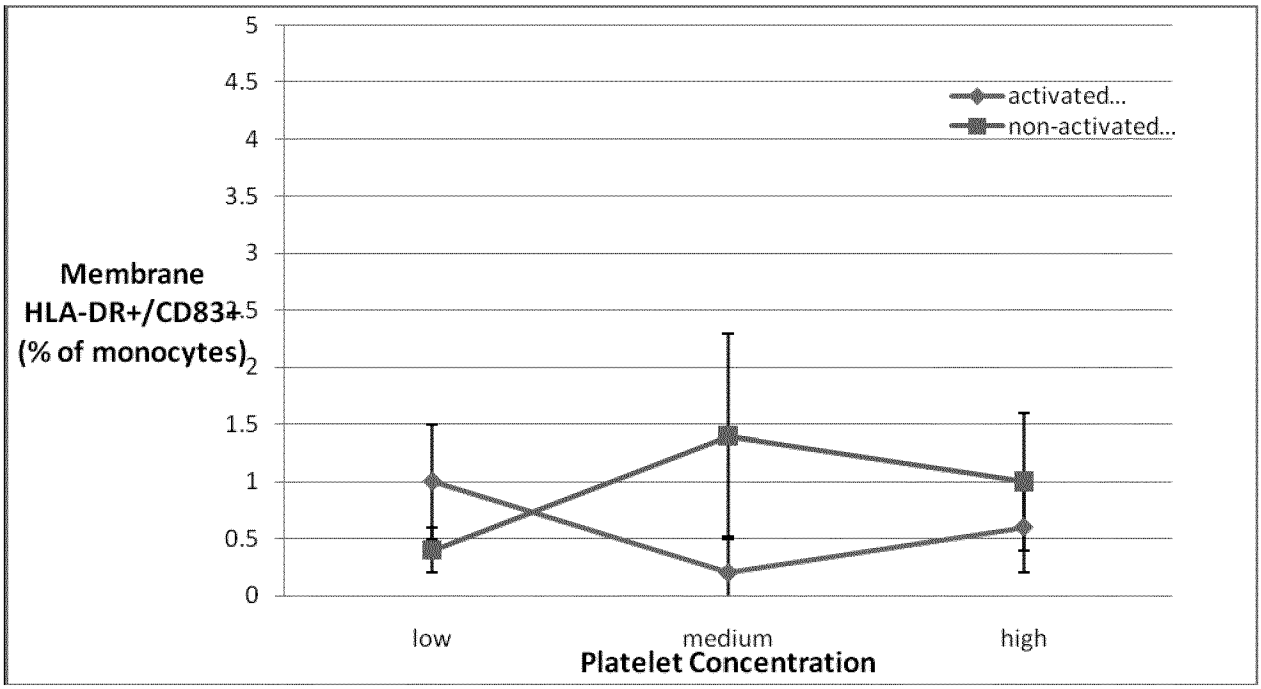

5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
Membrane HLA-DR+/CD83+ (% of monocytes)
activated...
non-activated...
low
medium
high
Platelet Concentration

Figure 4

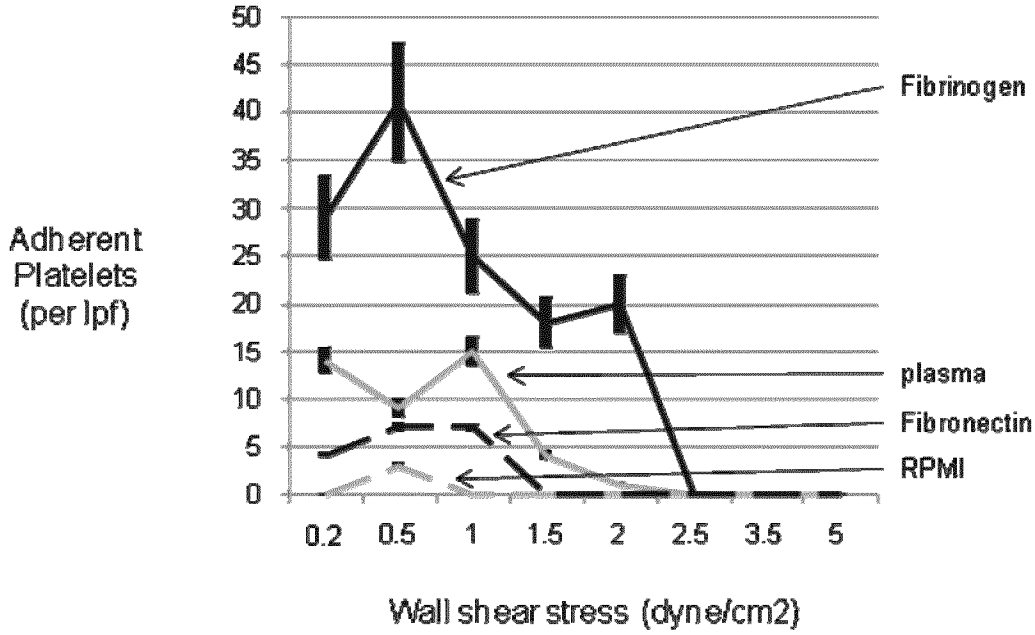
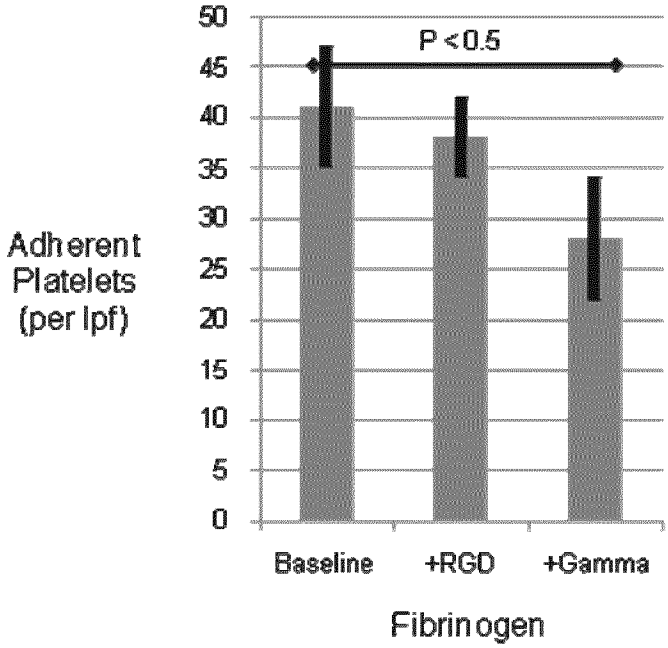

Adherent Platelets (per lpf)
50
45
40
35
30
25
20
15
10
5
0
0.2
0.5
1
1.5
2
2.5
3.5
5
Wall shear stress (dyne/cm2)
Fibrinogen
plasma
Fibronectin
RPMI

Figure 5

a)


P < 0.5
Adherent Platelets (per lpf)
50
45
40
35
30
25
20
15
10
5
0
Baseline
+RGD
+Gamma
Fibrinogen

b)

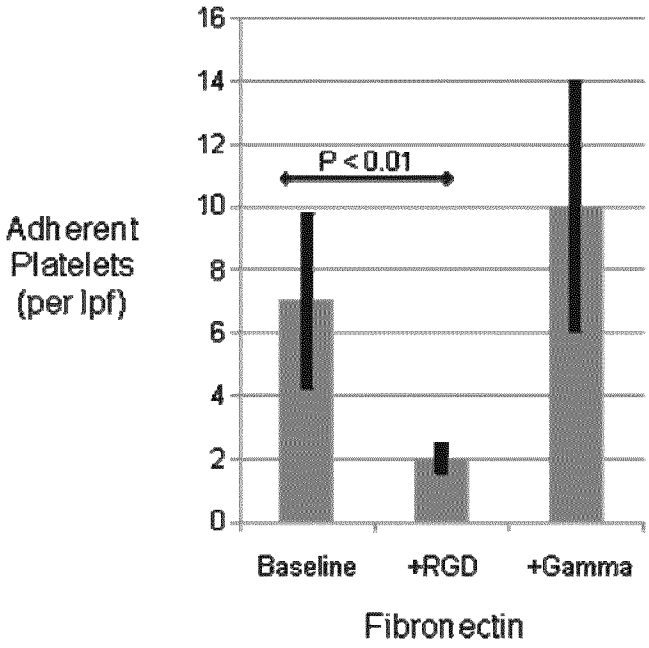

P < 0.01
Adherent Platelets (per lpf)
16
14
12
10
8
6
4
2
0
Baseline
+RGD
+Gamma
Fibronectin

Figure 6

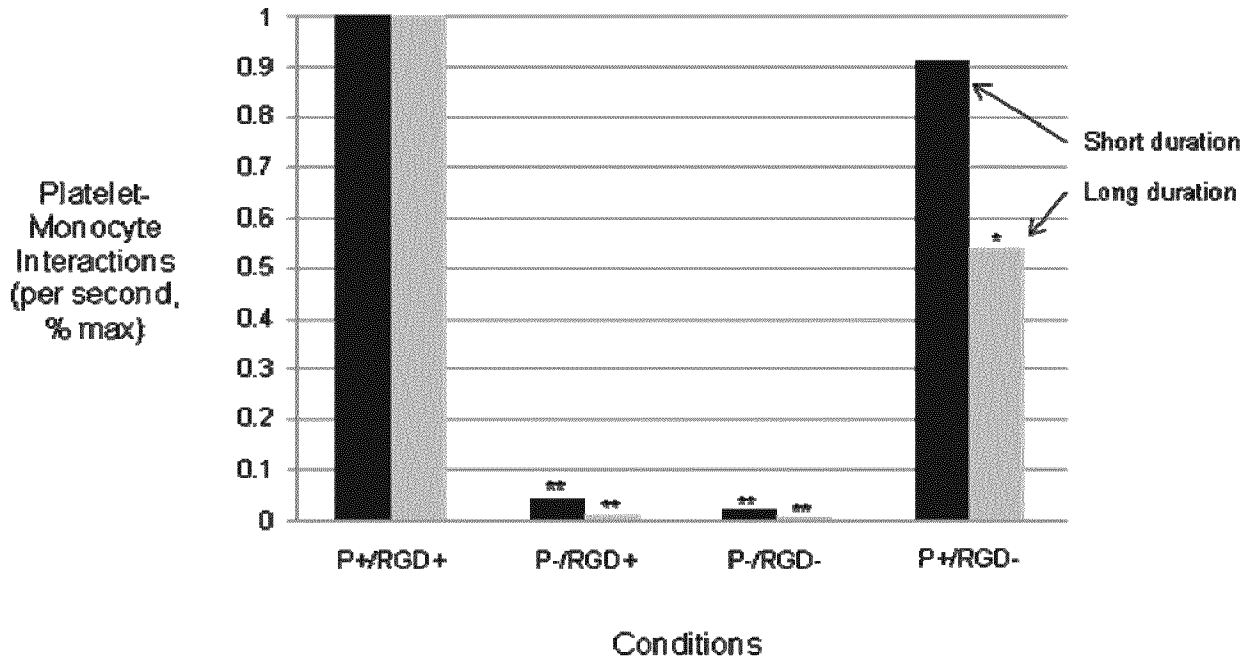

Platelet-Monocyte Interactions (per second, % max)
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
**
**
**
**
*
Short duration
Long duration
P+/RGD+
P-/RGD+
P-/RGD-
P+/RGD-
Conditions

Figure 7

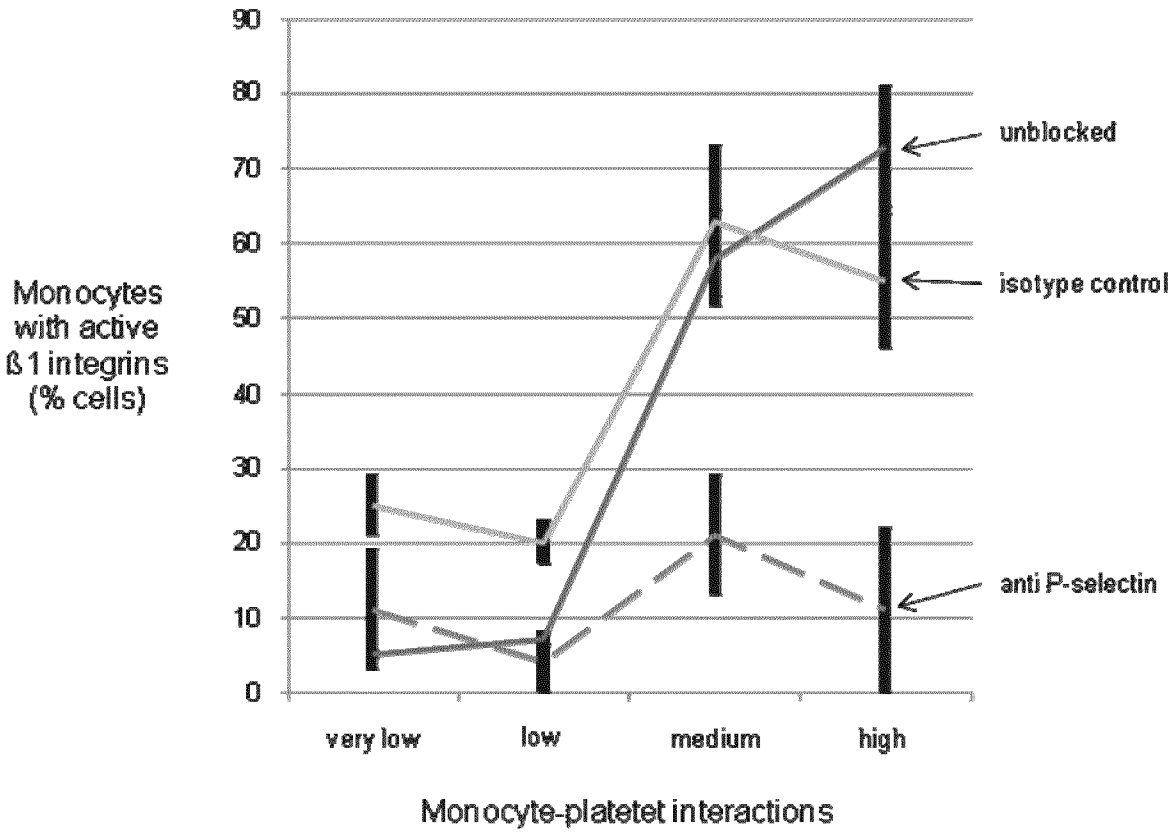

Monocytes with active ß1 integrins (% cells)
90
80
70
60
50
40
30
20
10
0
very low
low
medium
high
Monocyte-platetet interactions
unblocked
isotype control
anti P-selectin

Figure 8

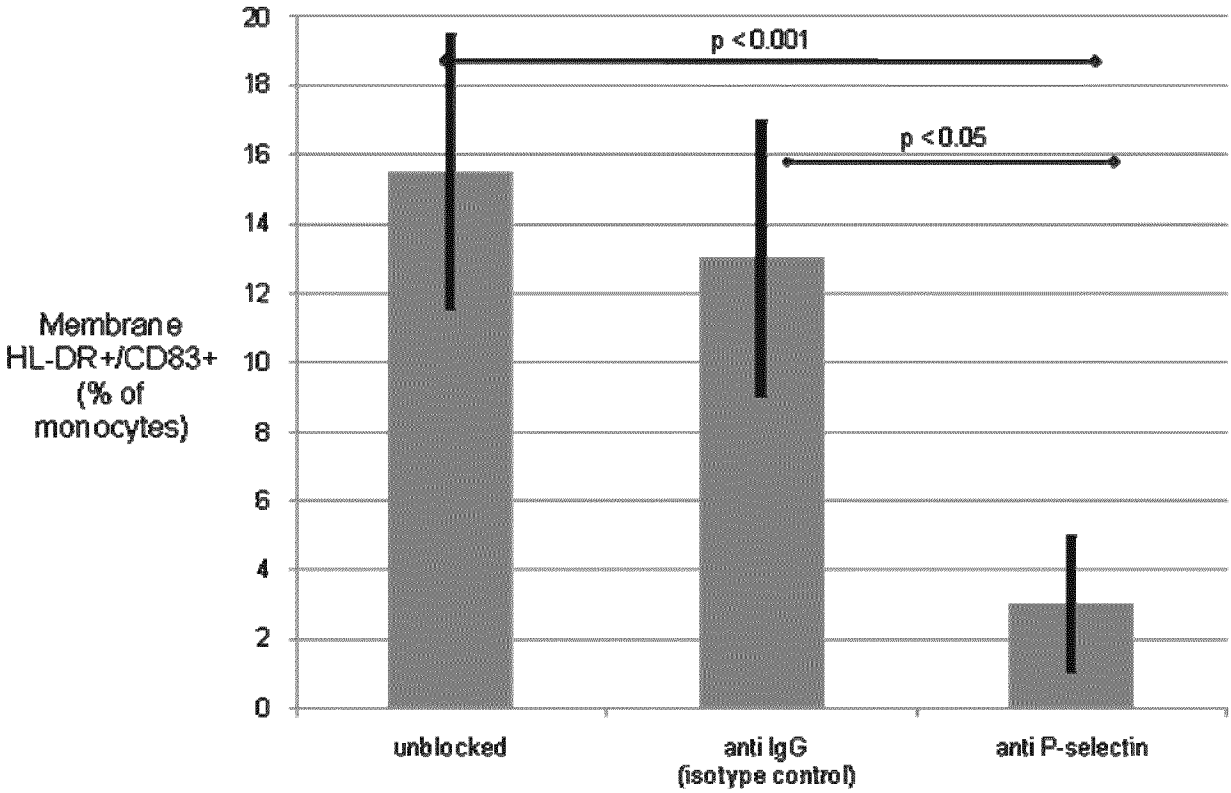

Membrane HL-DR+/CD83+ (% of monocytes)
20
18
16
14
12
10
8
6
4
2
0
p < 0.001
p < 0.05
unblocked
anti IgG (isotype control)
anti P-selectin

Figure 9

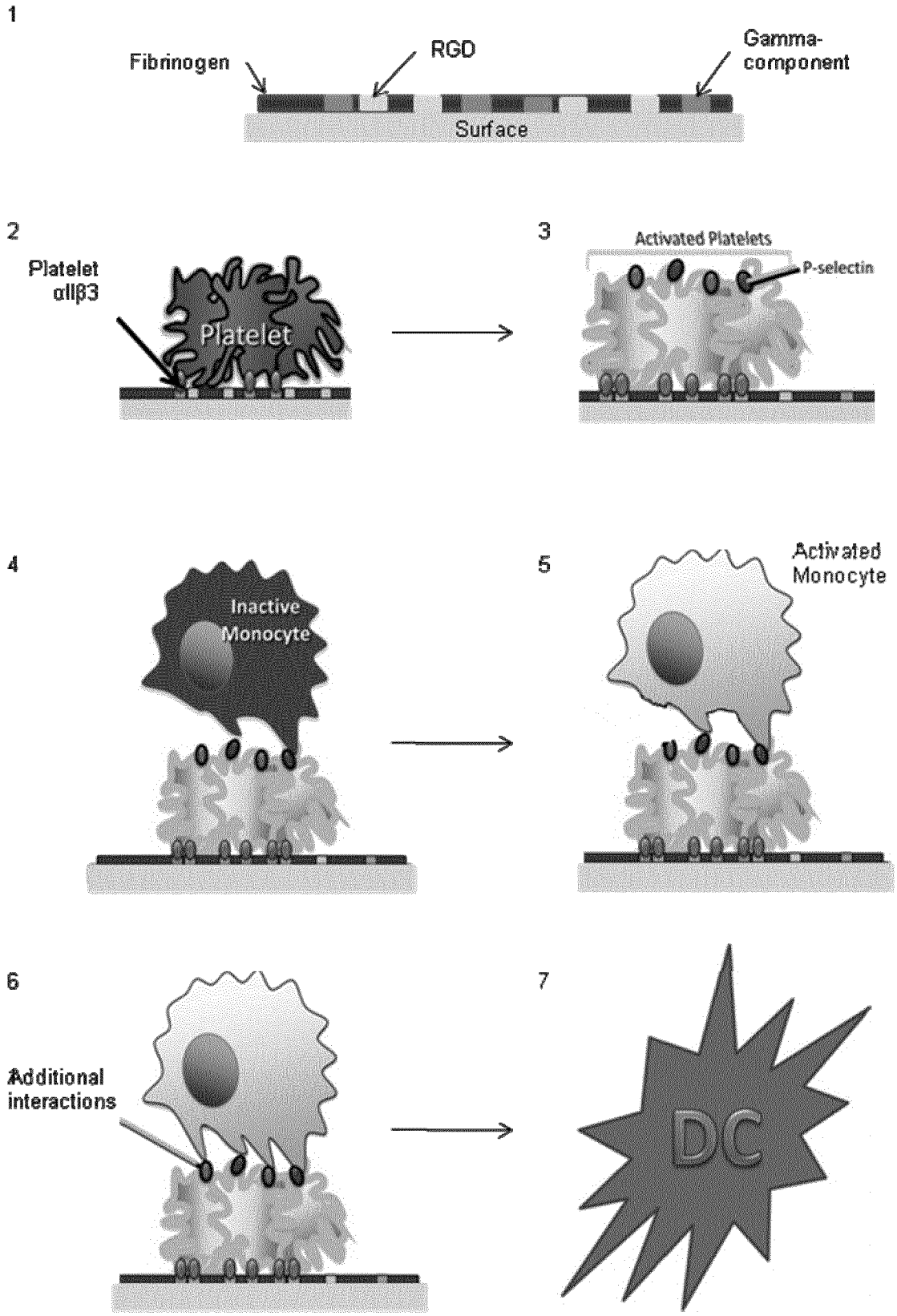

1
Fibrinogen
RGD
Gamma-component
Surface
2
Platelet αIIβ3
Platelet
3
Activated Platelets
P-selectin
4
Inactive Monocyte
5
Activated Monocyte
6
Additional interactions
7
DC

Figure 10

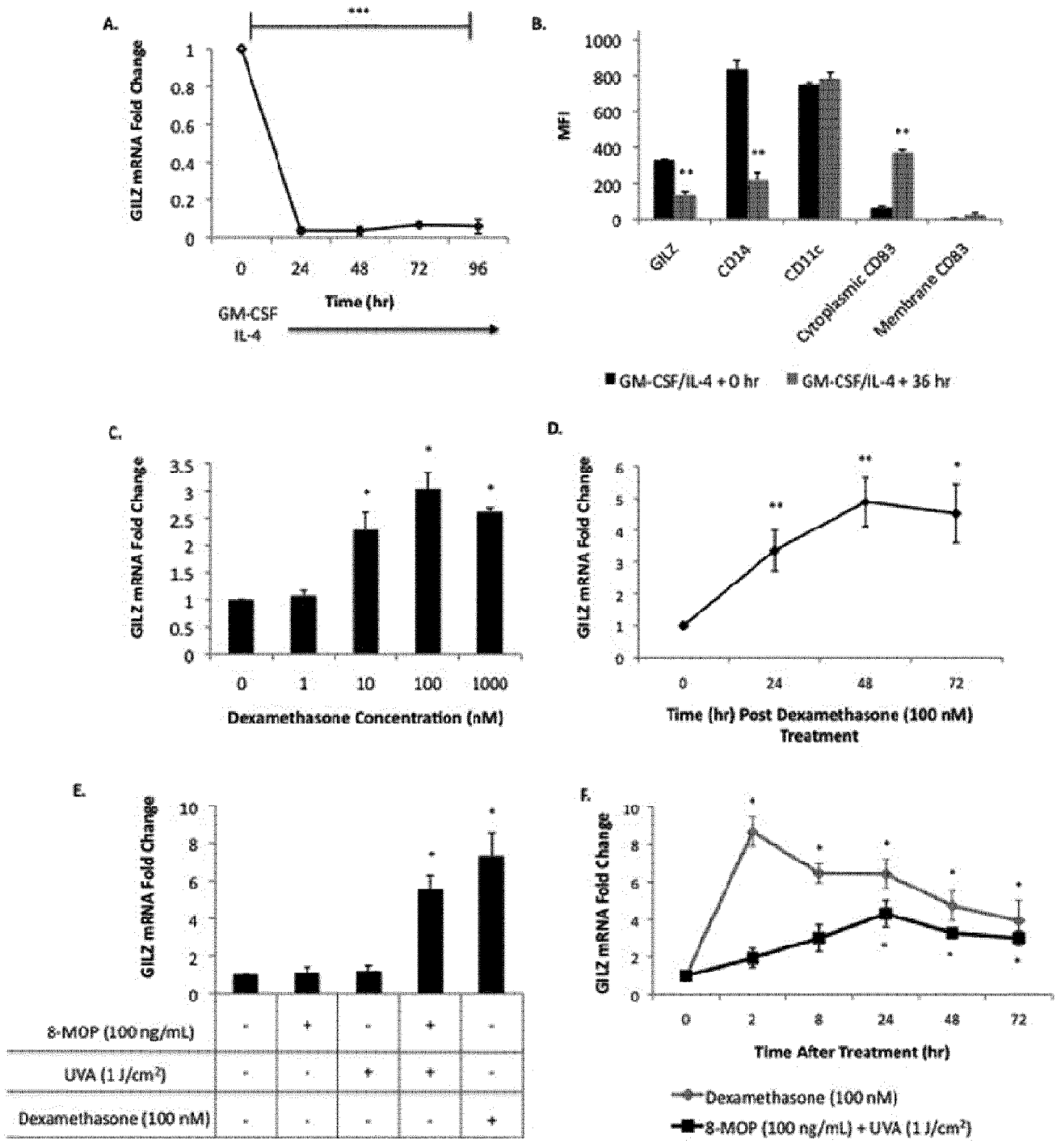

A.
GILZ mRNA Fold Change
Time (hr)
GM-CSF
IL-4
B.
MFI
GILZ
CD14
CD11c
Cytoplasmic CD83
Membrane CD83
GM-CSF/IL-4 + 0 hr
GM-CSF/IL-4 + 36 hr
C.
GILZ mRNA Fold Change
Dexamethasone Concentration (nM)
D.
GILZ mRNA Fold Change
Time (hr) Post Dexamethasone (100 nM) Treatment
E.
GILZ mRNA Fold Change
8-MOP (100 ng/mL)
UVA (1 J/cm²)
Dexamethasone (100 nM)
F.
GILZ mRNA Fold Change
Time After Treatment (hr)
Dexamethasone (100 nM)
8-MOP (100 ng/mL) + UVA (1 J/cm²)

Figure 11

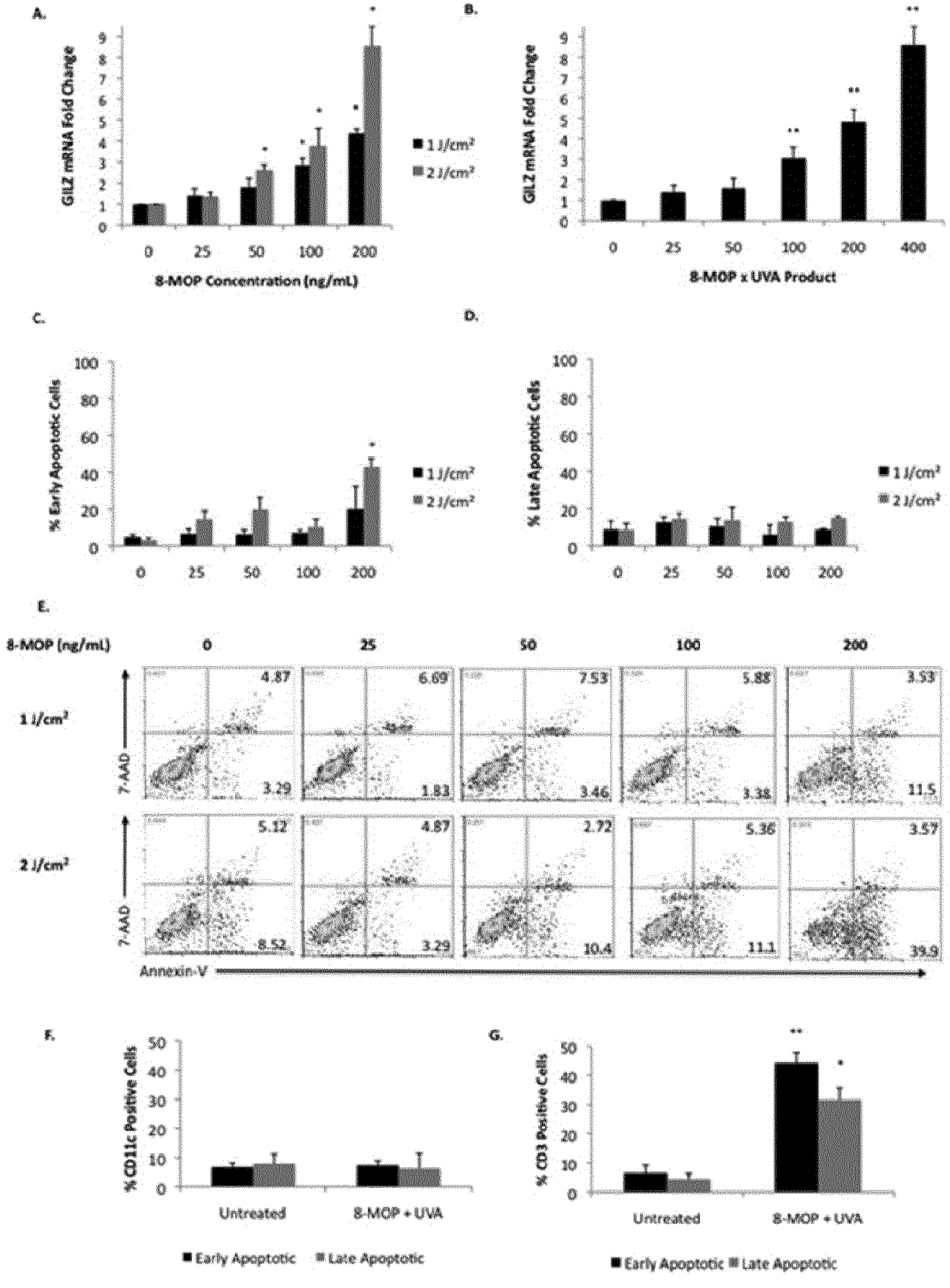

A.
GILZ mRNA Fold Change
8-MOP Concentration (ng/mL)
1 J/cm²
2 J/cm²
B.
GILZ mRNA Fold Change
8-MOP x UVA Product
C.
% Early Apoptotic Cells
D.
% Late Apoptotic Cells
E.
8-MOP (ng/mL)
1 J/cm²
2 J/cm²
7-AAD
Annexin-V
F.
% CD11c Positive Cells
Untreated
8-MOP + UVA
Early Apoptotic
Late Apoptotic
G.
% CD3 Positive Cells

Figure 12

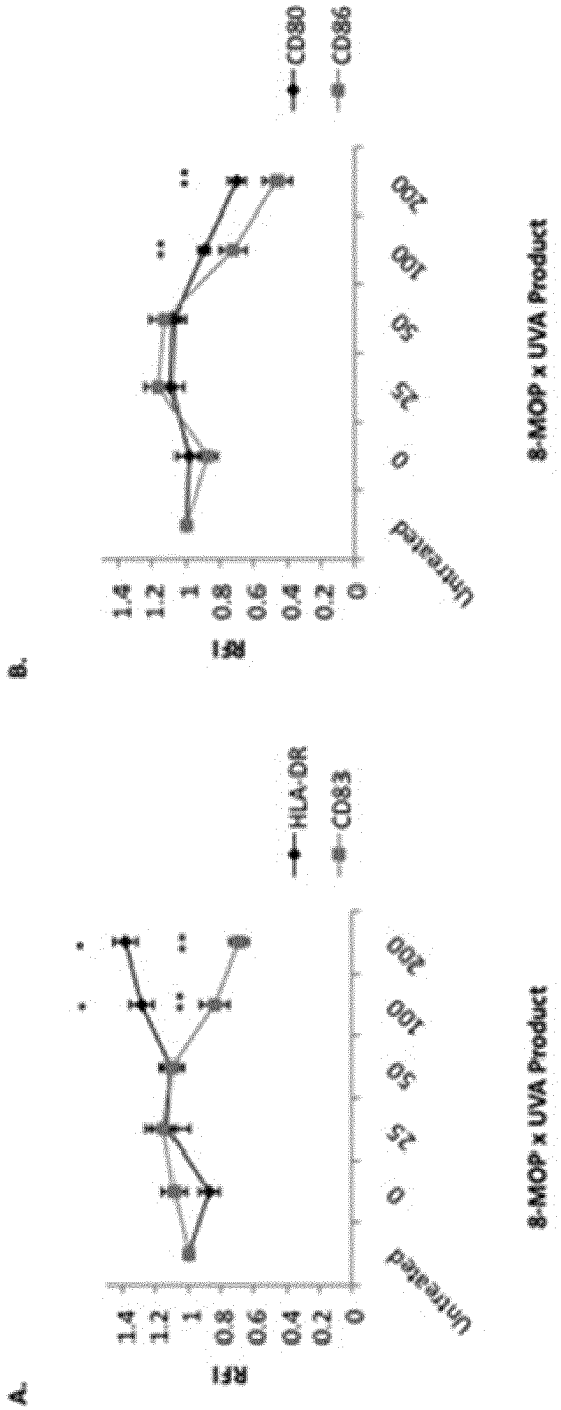

A.
HLA-DR
CD83
RFI
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Untreated
0
25
50
100
200
8-MOP x UVA Product
B.
CD80
CD86
RFI
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Untreated
0
25
50
100
200
8-MOP x UVA Product

Figure 13

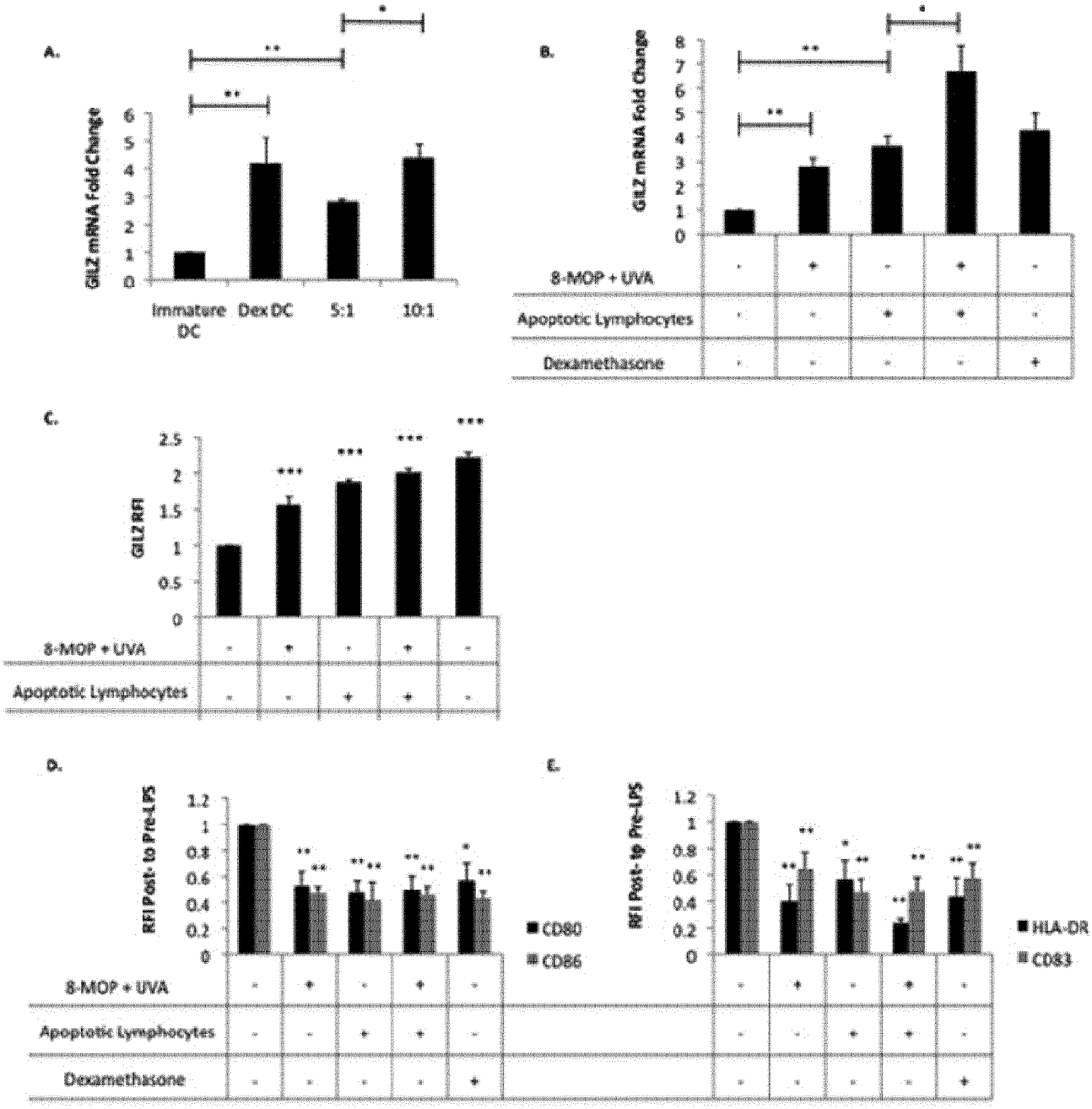

A.
GILZ mRNA Fold Change
Immature DC
Dex DC
5:1
10:1
B.
GILZ mRNA Fold Change
8-MOP + UVA
Apoptotic Lymphocytes
Dexamethasone
C.
GILZ RFI
8-MOP + UVA
Apoptotic Lymphocytes
D.
RFI Post- to Pre-LPS
CD80
CD86
8-MOP + UVA
Apoptotic Lymphocytes
Dexamethasone
E.
RFI Post- tp Pre-LPS
HLA-DR
CD83

Figure 14

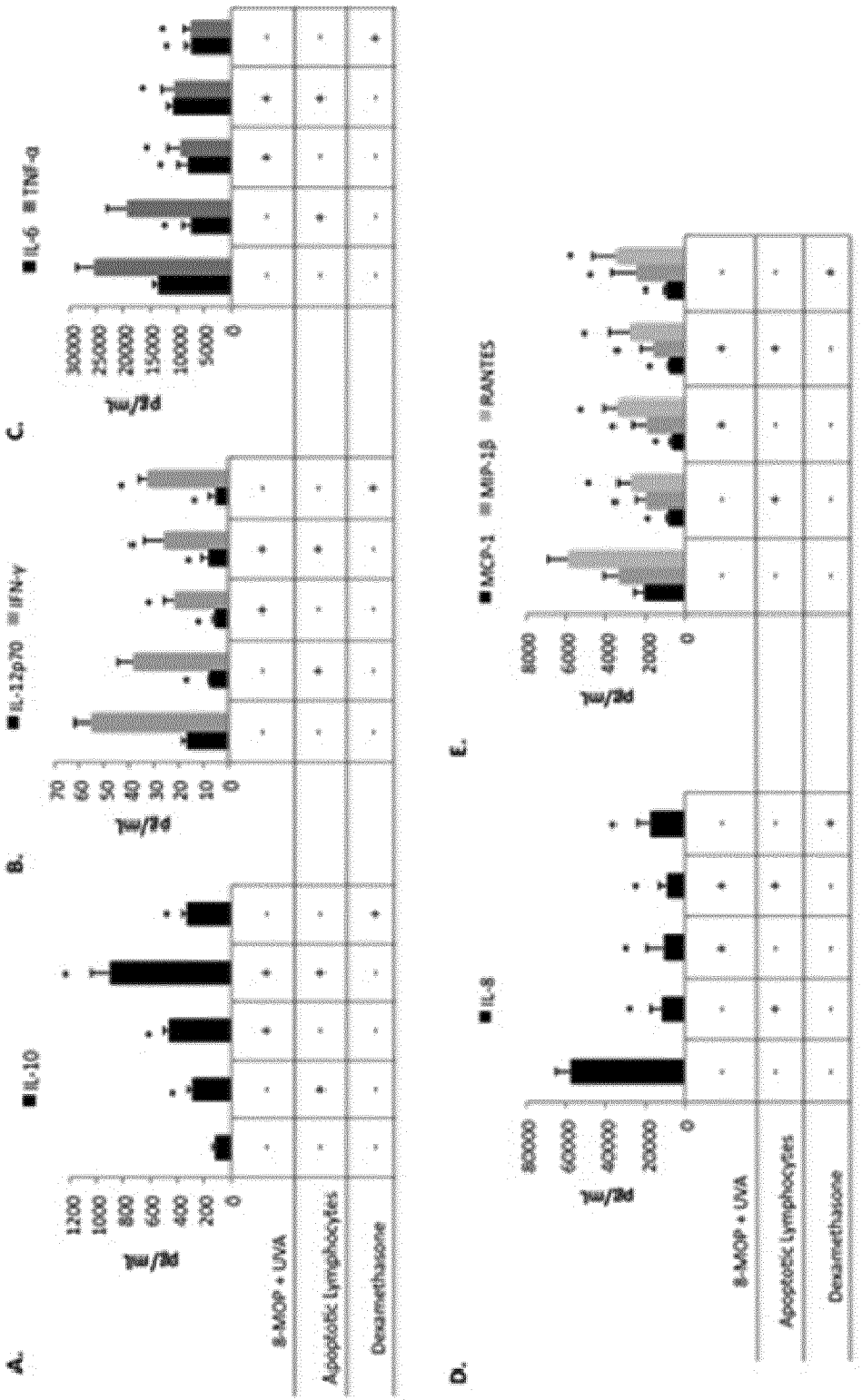

A.
IL-10
pg/mL
1200
1000
800
600
400
200
0
B.
IL-12p70
IFN-γ
pg/mL
70
60
50
40
30
20
10
0
C.
IL-6
TNF-α
pg/mL
30000
25000
20000
15000
10000
5000
0
D.
IL-8
pg/mL
80000
60000
40000
20000
0
E.
MCP-1
MIP-1β
RANTES
pg/mL
8000
6000
4000
2000
0
8-MOP + UVA
Apoptotic Lymphocytes
Dexamethasone

Figure 15

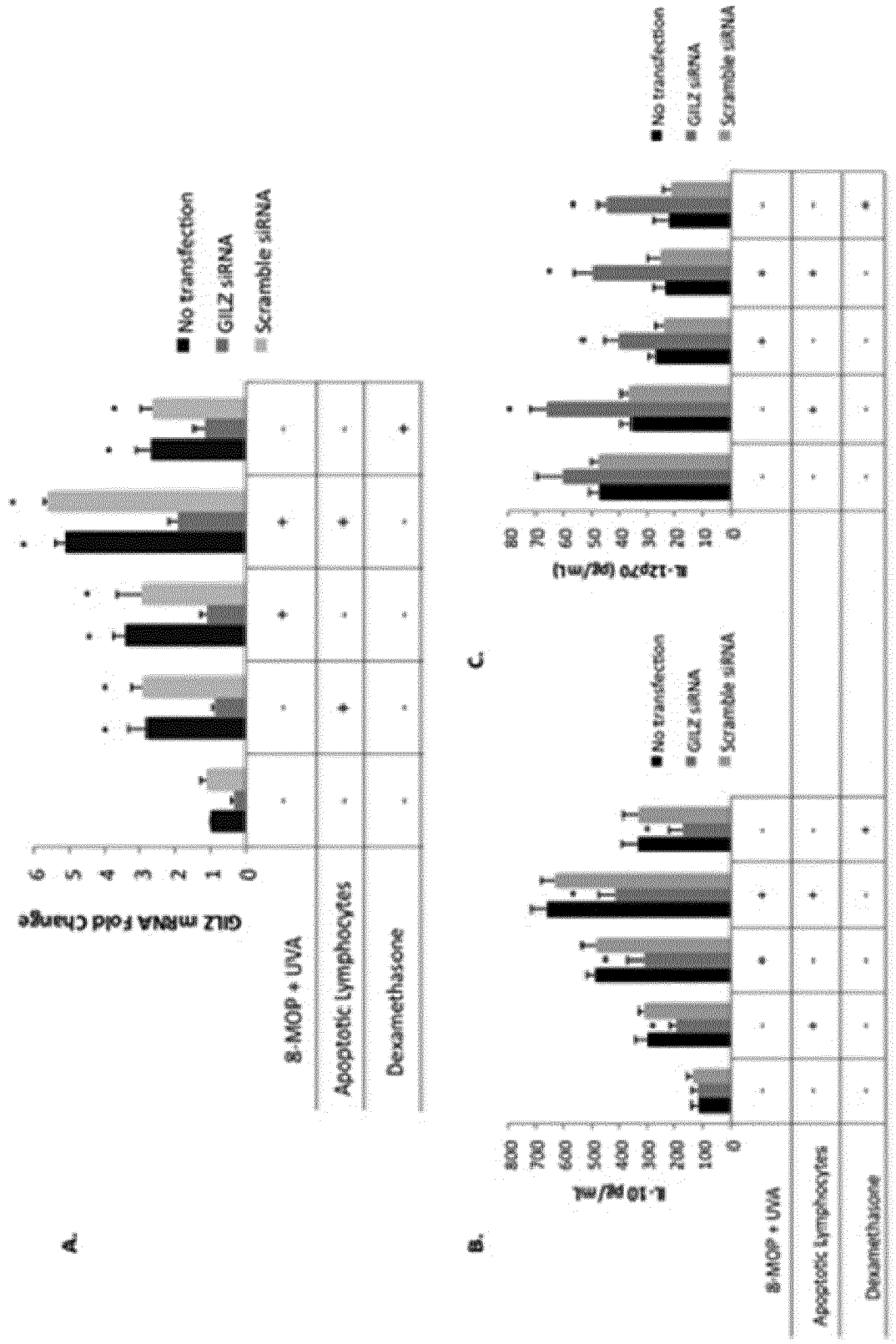

A.
No transfection
GILZ siRNA
Scramble siRNA
GILZ mRNA fold Change
8-MOP + UVA
Apoptotic Lymphocytes
Dexamethasone
B.
IL-10 pg/mL
C.
IL-12p70 (pg/mL)

Figure 16

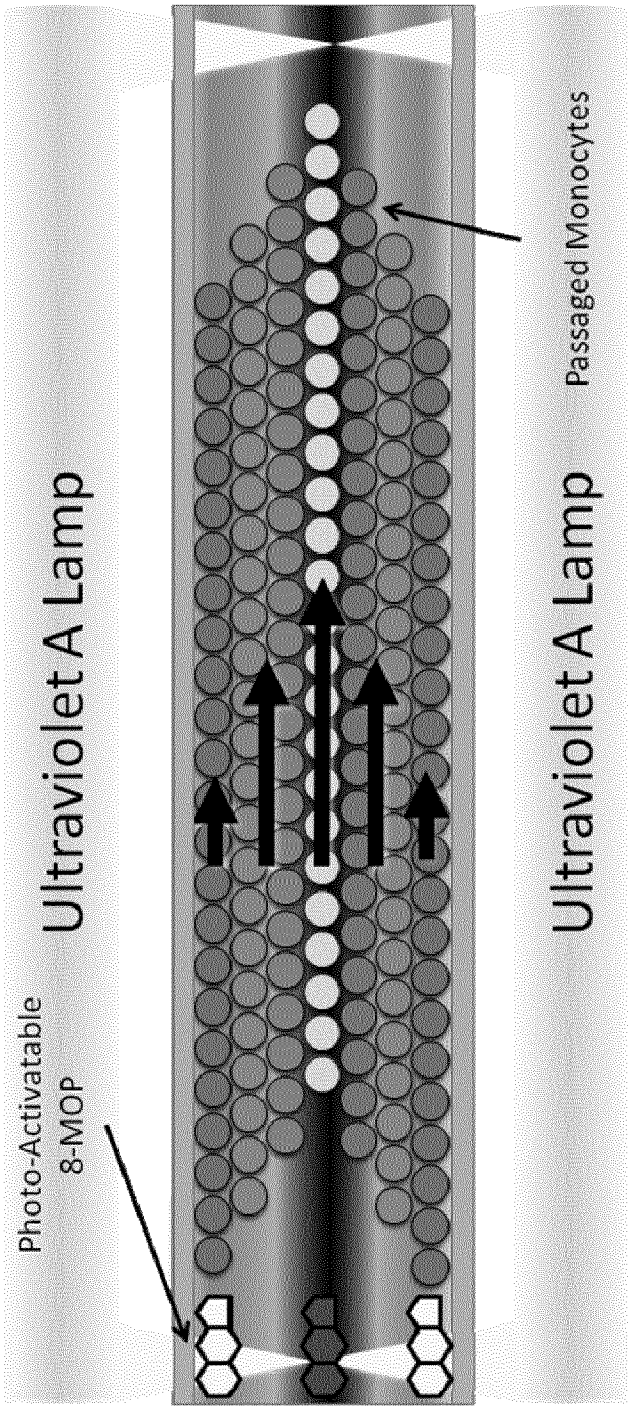

Ultraviolet A Lamp
Ultraviolet A Lamp
Passaged Monocytes
Photo-Activatable
8-MOP

Figure 17

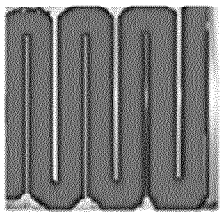
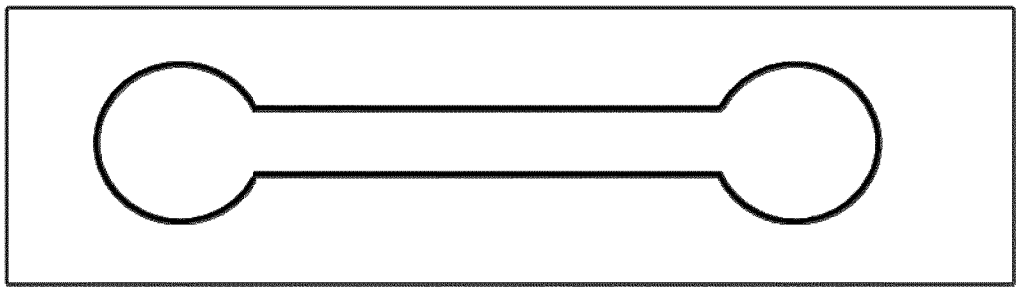


Figure 18

a)



b)

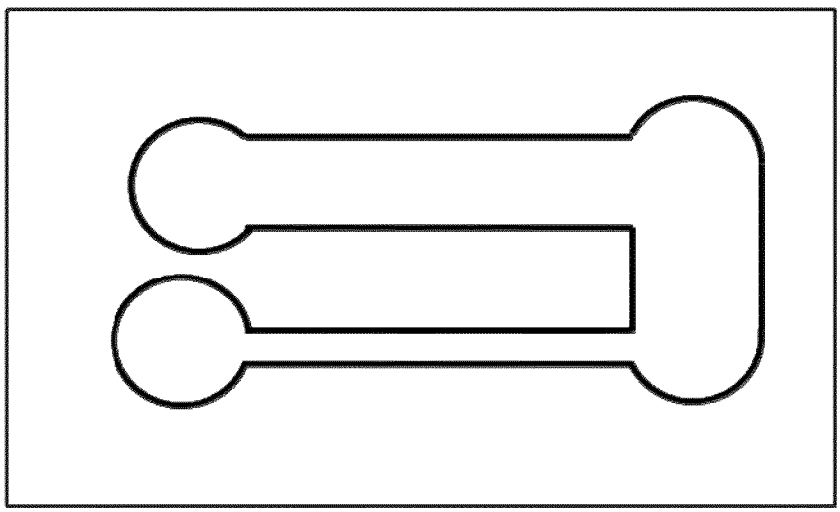
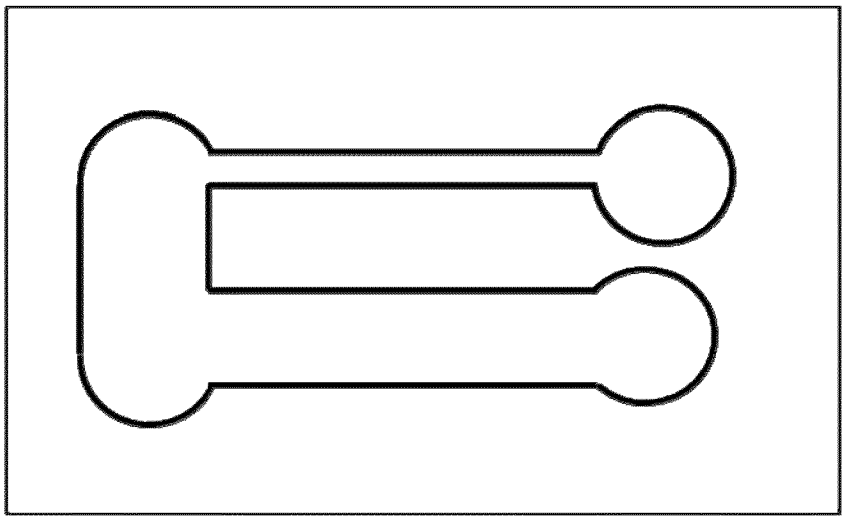


Figure 18 cont.

c)



d)

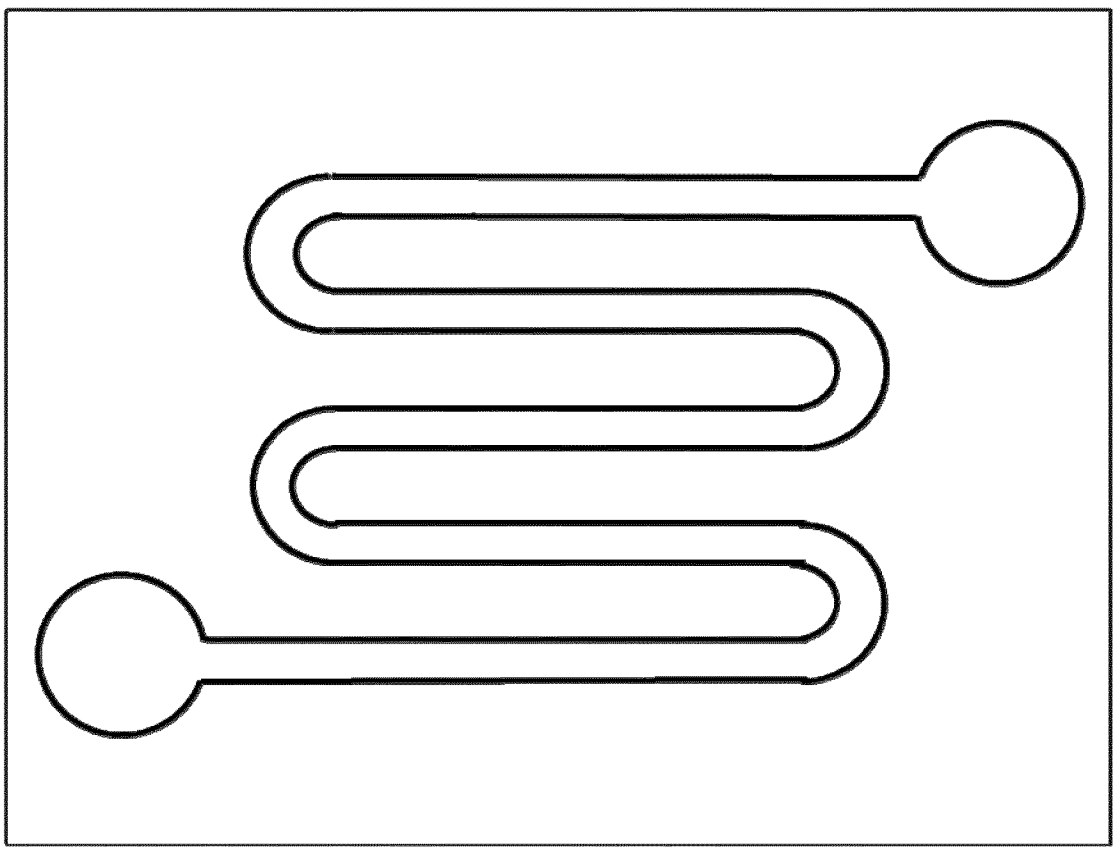
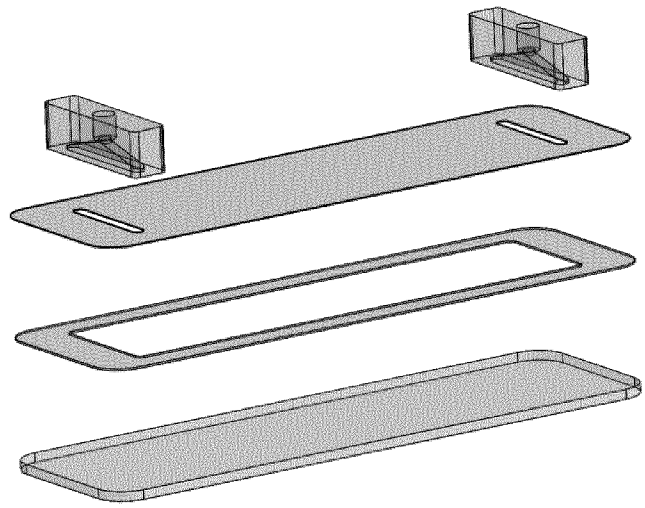


Figure 19

A)



B)

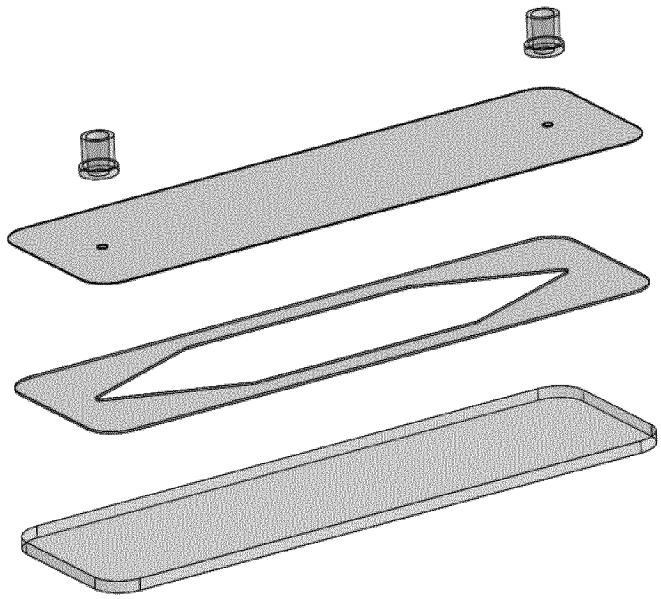


Figure 20

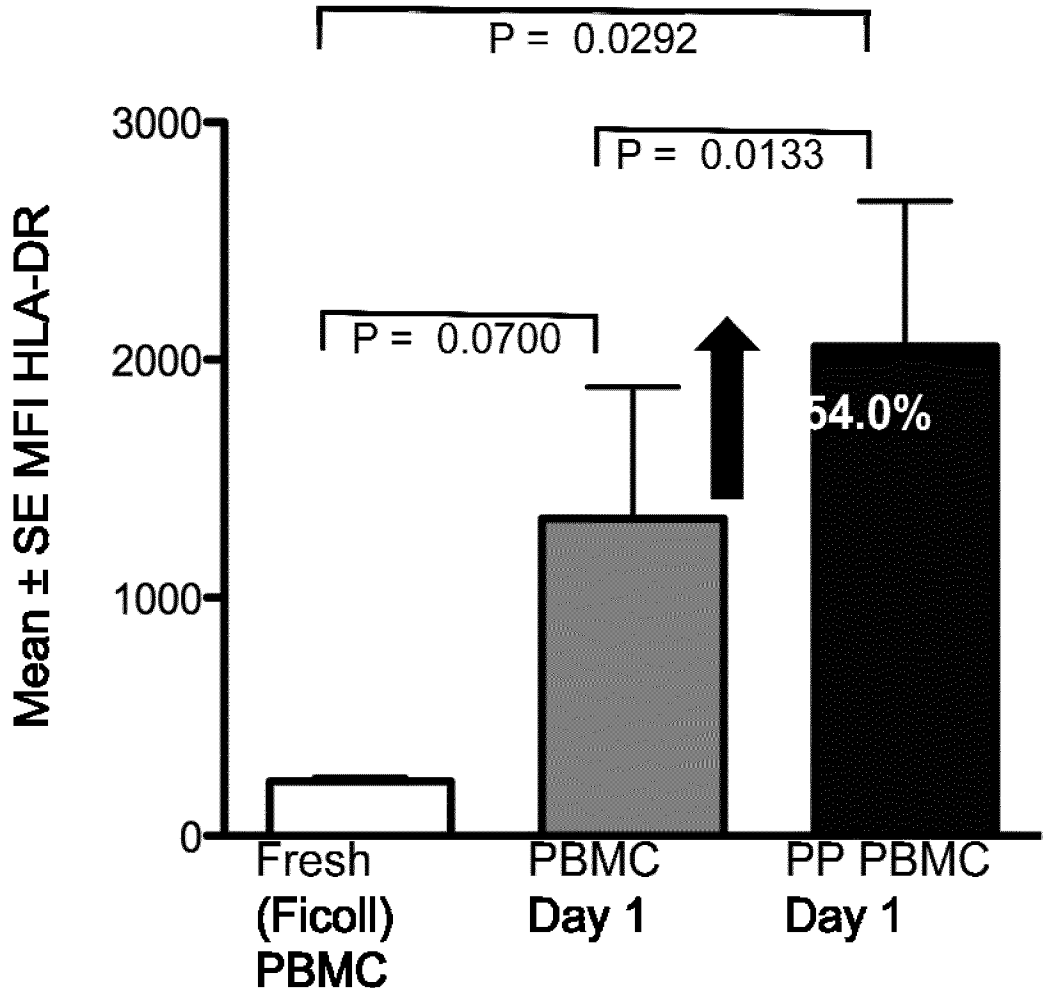

P = 0.0292
P = 0.0133
P = 0.0700
3000
2000
1000
0
Mean ± SE MFI HLA-DR
54.0%
Fresh (Ficoll) PBMC
PBMC Day 1
PP PBMC Day 1

Figure 21

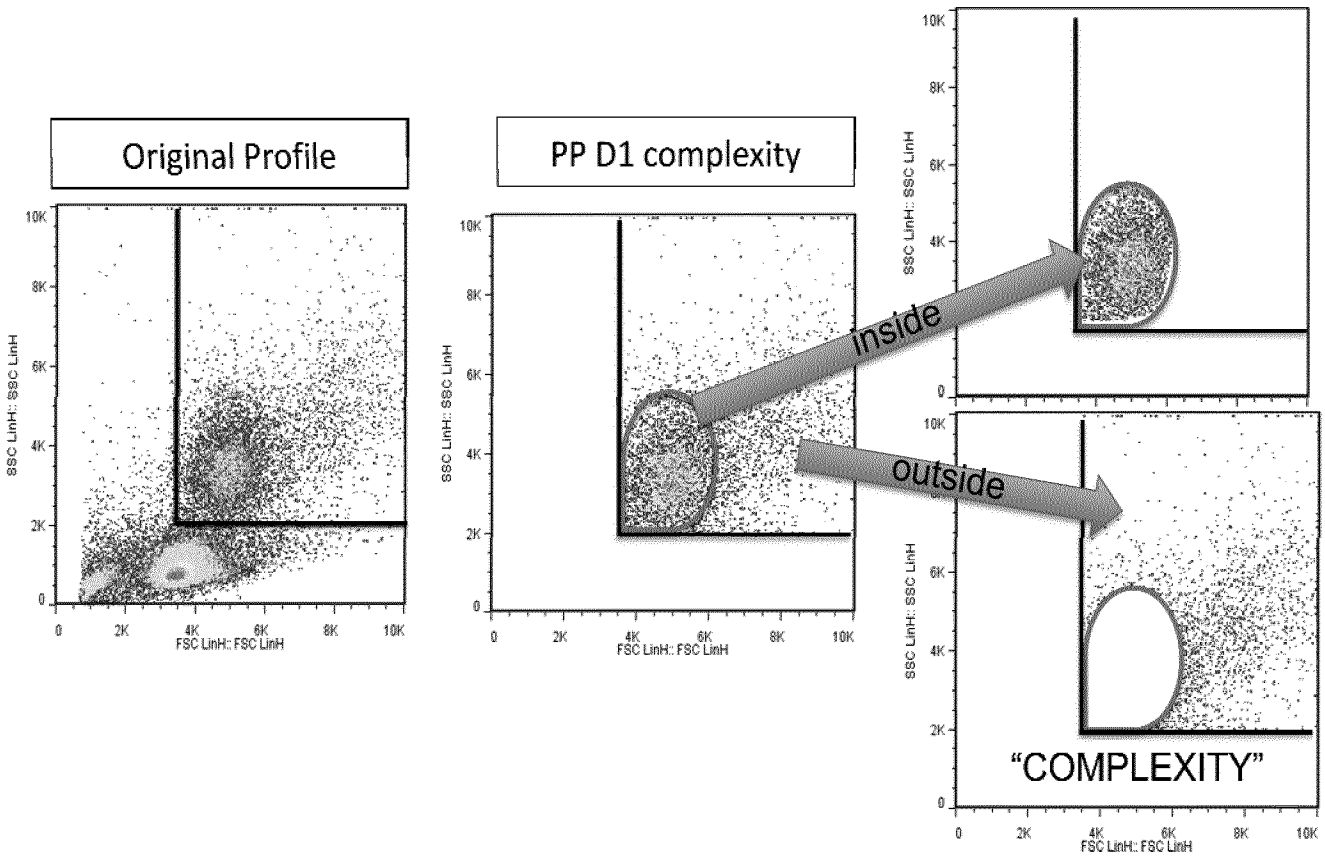

Original Profile
PP D1 complexity
inside
outside
"COMPLEXITY"
SSC LinH:: SSC LinH
FSC LinH:: FSC LinH
0
2K
4K
6K
8K
10K

Figure 22

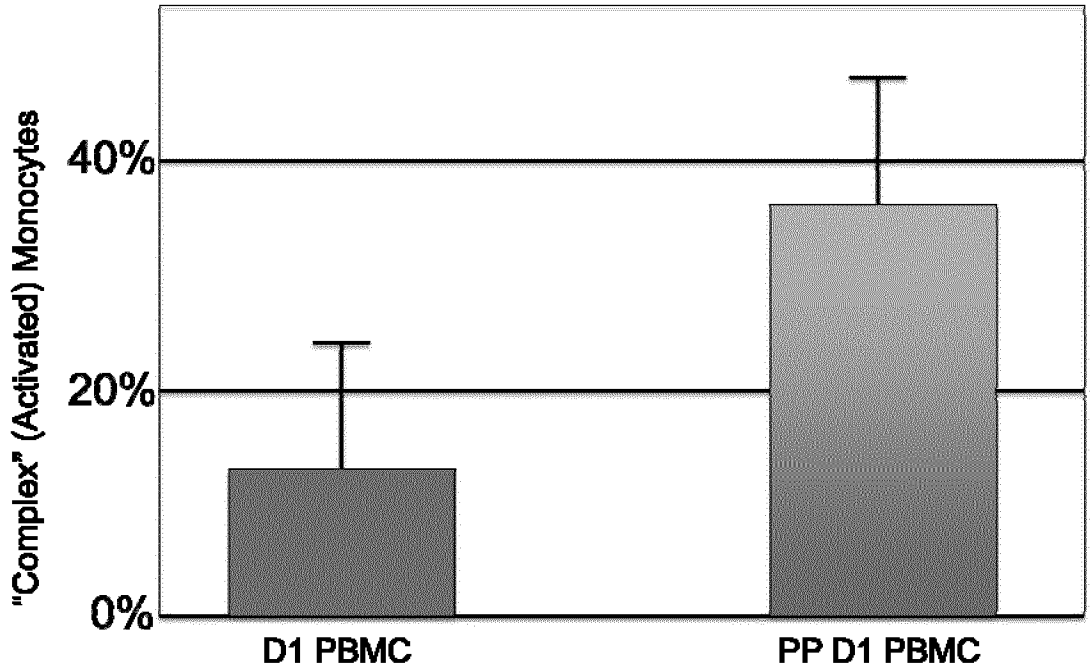


Figure 23

	HLA-DR	CD86	ICAM-1	PLAUR	FSC/SSC Complexity
Fresh (Ficoll) PBMC	0	0	0	0	0
D1 PBMC	++	0	++	++	+
PP D1 PBMC	+++	+	++++	++++	+++
Immature Fast DC	++++	+++	++	–	?

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2014/050012

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N5/0784 A61K35/15
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, WPI Data, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2011/137365 A1 (UNIV YALE [US]; EDELSON RICHARD LESLIE [US]) 3 November 2011 (2011-11-03)	1-54,56
Y	the whole document	55

X	SPISEK RADEK ET AL: "Maturation state of dendritic cells during the extracorporeal photopheresis and its relevance for the treatment of chronic graft-versus-host disease.", TRANSFUSION JAN 2006, vol. 46, no. 1, January 2006 (2006-01), pages 55-65, XP55112614, ISSN: 0041-1132	1-54,56
Y	the whole document	55

	-/--	

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
2 May 2014	09/05/2014

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Manu, Dominique

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Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BAIRD A H ET AL: "Generation of immature dendritic cells by modified Extracorporeal photophoresis", JOURNAL OF INVESTIGATIVE DERMATOLOGY, vol. 130, no. Suppl. 1, April 2010 (2010-04), page S136, XP55112893, & ANNUAL MEETING OF THE SOCIETY-FOR-INVESTIGATIVE-DERMATOLOGY; ATLANTA, GA, USA; MAY 05 -08, 2010 ISSN: 0022-202X	1-52,56
Y	abstract	55

A	BERGER CAROLE ET AL: "Rapid generation of maturationally synchronized human dendritic cells: contribution to the clinical efficacy of extracorporeal photochemotherapy.", BLOOD 2 DEC 2010, vol. 116, no. 23, 2 December 2010 (2010-12-02), pages 4838-4847, XP55112768, ISSN: 1528-0020 the whole document	1-14, 28-56

A	KHALIL D ET AL: "Conversion of monocytes to dendritic cells by platelet signaling", JOURNAL OF INVESTIGATIVE DERMATOLOGY, vol. 132, no. Suppl. 1, May 2012 (2012-05) , page S101, XP55113116, & 75TH ANNUAL MEETING OF THE SOCIETY-FOR-INVESTIGATIVE-DERMATOLOGY; RALEIGH, NC, USA; MAY 09 -12, 2012 abstract	1-14, 28-56

A	DURAZZO T S ET AL: "Platelet induction of monocyle-to-dendritic cell maturation", JOURNAL OF INVESTIGATIVE DERMATOLOGY, vol. 130, no. Suppl. 1, April 2010 (2010-04), page S121, XP55113123, & ANNUAL MEETING OF THE SOCIETY-FOR-INVESTIGATIVE-DERMATOLOGY; ATLANTA, GA, USA; MAY 05 -08, 2010 ISSN: 0022-202X abstract	1-14, 28-56

Y	HU JIM ET AL: "Tolerogenic dendritic cells and their potential applications.", IMMUNOLOGY MAR 2011, vol. 132, no. 3, March 2011 (2011-03), pages 307-314, XP55112839, ISSN: 1365-2567 cited in the application the whole document	55

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2014/050012

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		CA 2797965 A1	03-11-2011
		EP 2563425 A1	06-03-2013
		US 2010267137 A1	21-10-2010
		US 2013323710 A1	05-12-2013
		WO 2011137365 A1	03-11-2011