Pharmacological manipulation of Dendritic Cells in vitro and in vivo

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1.   INTRODUCTION

1.1   Immunobiology of Dendritic Cells

1.1.1   Dendritic Cells in context of the immune system

Dendritic Cells (DC) were first literally mentioned by Paul Langerhans in 1868 [1, 2]. Langerhans studied the morphology of the human skin assuming that the observed dermal DC were neurons of the skin. It took more than 100 years until several research teams published studies revealing that DC are derived from bone marrow progenitors and present no neuronal cells but professional Antigen (Ag) presenting cells of the immune system [3, 4].

For an efficient host defence both Ag non-specific innate immunity and Ag specific adaptive immunity are needed [5-8]. The most important roles of the mammalian innate immune system include the ability to recognize rapidly pathogen and/or tissue injury and to signal allogeneic structures (i.e. pathogens) to cells of the adaptive immune system [9]. Important factors of the innate system are phagocytic cells, natural killer (NK) cells, complement and interferons (IFNs). Cells of the innate immune system use a variety of pattern recognition receptors to recognize patterns shared between pathogens for example bacterial lipopolysaccharide (LPS), carbohydrates and doublestranded viral RNA [10-12].

Key features of the adaptive immunity are the ability to rearrange genes of the immunoglobulin family, permitting creation of a large diversity of Ag-specific clones and immunological memory. This highly adaptive immune system is based on Ag Presenting Cells (APCs). DC belong to the class of APCs. They are unique APCs because they present the only cell type being capable to induce primary immune responses followed by immunological memory [13-16]. DC operate as characterized in the following: DC-progenitors in the bone marrow give rise to circulating precursors that home to tissues where they reside as immature cells with phagocytotic capacity. If a tissue is damaged or pathogens like bacteria are present, immature DC capture Ag and migrate to the lymphoid organs, where they select Ag-specific T-Cells, initiating immune responses by interacting with them. DC present the internalized Ag on their surface to $CD4^+$ T-helper cells. The $CD4^+$ T-helper cells regulate the immune effector cells

including Ag-specific CD8^{+} cytotoxic T-Cells and B-cells as well as non Ag-specific macrophages, eosinophils [17] and NK-cells.

1.1.2   Developmental steps of a Dendritic Cell

At a functional dimension it is possible to divide DC into at least 4 developmental stages that describe the life cycle of these cells in lymphatic tissues; including the bone marrow progenitors, circulating progenitors, tissue association, immature DC as well as mature DC [18].

Figure 1   Development stages of a Dendritic Cell
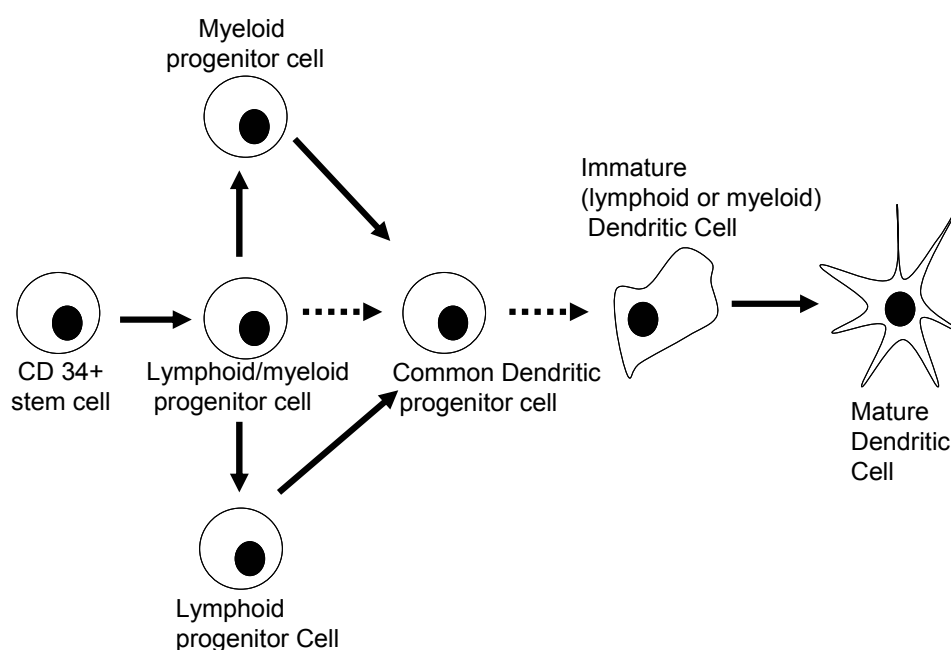
DC originally emerge from pluripotent CD 34^{+} stem cells. From either a myeloid or a lymphoid progenitor cell a common dendritic progenitor cell is developed. This cell is transformed to an immature (lymphoid-related or myeloid) DC. Via maturation stimuli this cell is finally transformed to a DC.

CD 34[+] haemapoietic stem cells differentiate to various DC subsets. Different hypotheses for the differentiation to DC are discussed. The conservative/classical hypothesis suggests two separated differentiation lines including the myeloid and lymphoid-related Dendritic Cell line, whereas recently a common dendritic progenitor for myeloid and lymphoid DC was proposed [19]. The latter alternative hypothesis is supported by experimental data, identifying myeloid and lymphoid DC, differentiated from both lymphoid and myeloid progenitors [20, 21]. Therefore it remains still unclear if

DC either develop from a line (myeloid/lymphoid) progenitor or from a common dendritic progenitor (Figure 1).

1.1.3   Heterogeneity of Dendritic Cell subsets in Mice

Flt3 ligand (Flt3L) is able to expand mature DC in mice. Flt3L targets primitive haemotopeietic progenitors in the bone marrow inducing their expansion and differentiation [22] and DC increase dramatically upon Flt3L injection from less than one to over 30%[23-25].With the discovery of the impact of Flt3L on DC expansion in mice, it became easier to distinguish them into at least three different subtypes: the myeloid, the lymphoid and the plasmacytoid DC [26-33].

Lymphoid, myeloid and plasmacytoid DC differ in phenotype, localisation and function. The subsets are all CD11c positive: myeloid DC are $CD11c^{bright}$ $CD11b^{bright}$ $CD45b220^{neg}$, lymphoid DC are $CD11c^{bright}CD11b^{dim}CD45b220^{neg}$ and plasmacytoid DC are $CD11c^{bright}CD45b220^{bright}CD11b^{neg}$ [33].

Lymphoid DC are localized in the T-cell rich areas of the periarteriolar lymphatic sheets in the spleen and lymph nodes [24, 32, 34, 35]. Myeloid DC are mainly found in the marginal zone bridging channels of the spleen [24, 32, 34, 35] whereas plasmacytoid cells (pDC) are a cell population with a characteristic plasma cell-like morphology found in many tissues of the mouse, including blood, thymus, bone marrow, liver, and the T-cell areas of lymphoid organs [36]. The lymphoid-related DC produce higher levels of interleukin (IL) 12 [24, 37-39] and are less phagocytic than myeloid DC [24, 34, 35]. Plasmacytoid DC are unsurpassed in producing IFNs, i.e. IFN-α [40].

1.1.4   Heterogeneity of Dendritic Cell subsets in Humans

In Humans it is important to divide DC into at least three subsets:

(1) $CD11c^{-}$ $CD123^{bright}$ DC have a plasmacytoid morphology. They depend on IL-3 for their survival and differentiation into mature DC with typical dendritic morphology and potent T cell stimulatory function. As they have some features like the expression of pre-TCR α-chain, they may arise from lymphoid precursors [41, 42].

(2) $CD11c^{+}$ $CD11c^{bright}$ $CD123^{dim}$ have a monocyte appearance, express CD45R and can mature in vitro without the influence of exogenous cytokines. Myeloid DC have an

unsurpassed capacity to produce IL-12 and were used in many experiments to show pharmacological effects of SFA.

The third group of recently discovered subsets **(3)** CD123^{-} CD11c^{+} share many immunophenotypic features with classical CD11c^{+} CD11c^{bright} CD123^{dim} DC, but they lack expression of CD1c, CD2, and several of the FC receptors [43].

1.1.5   Antigen capture, migration and maturation

Immature DC are very efficient in Ag capture using several pathways, such as (a) macropinocytosis; (b) receptor mediated endocytosis via C-type lectin receptors (mannose receptor, DEC-205) [44-49] or Fcγ receptor types I (CD64) and II (CD32) [50]; and (c) phagocytosis of particles such as latex beads [51], apoptotic and necrotic cell fragments (involving CD36 and $\alpha v\beta 3$ or $\alpha v\beta 5$ integrins) [52-54] viruses, and bacteria including mycobacteria [55, 56] as well as particular parasites such as Leishmania major [57]. DC can also internalize the peptide loaded heat shock proteins gp96 and Hsp70 through presently unknown mechanisms [58, 59] (d). The Ag/pathogen induces the immature DC to undergo phenotypic and functional changes that lead to a complete transition from Ag capturing cell to an Ag presenting cell. DC maturation is linked with their migration from the peripheral tissue to the draining lymphoid organs.

Numerous factors induce and/or regulate DC maturation, including (a) pathogen-related molecules such as LPS [56], bacterial DNA [60-62] and double stranded RNA [63]; (b) the balance between proinflammatory and antinflammatory signals in the local microenvironment, including TNF, IL-1; IL-6, IL-10; transforming growth factor-β (TGF-β) as well as prostaglandins; and (c) T-cell derived signals. The maturation process is associated with (a) loss of endocytotic/phagocytic receptors, (b) upregulation of costimulatory molecules CD40, CD58, CD80, and CD86, (c) changes in morphology, (d) shift in lysosomal compartments and (e) change in class II MHC compartments.

Morphological changes accompanying DC maturation include a loss of adhesive structures, cytoskeleton reorganization, and acquisition of high cellular motility [64].

1.1.6   Antigen processing and presentation

DC are well equipped to capture and process Ag, and a number of molecules involved in this process have been identified: Soluble and particulate Ag are efficiently captured

by immature DC and targeted to MHC class II compartments [44, 48, 65-67]. Immature DC constantly accumulate MHC class II molecules in lysosome-related intracellular compartments identified as MHC class II-rich compartments (MIICs), with multivesicular and multilamelar structure. The captured Ag is directed towards MIICs containing human leucocyte antigen (HLA)-DR that promotes the catalytic removal of class II-associated invariant chain peptide and enhances peptide binding to MHC class II molecules [68, 69].

In immature DC, class II molecules have a short half-life, maturation and inflammatory stimuli lead to a strong increase of class II synthesis and translocation of the MHC II-peptide complexes to the cell surface where they remain stable for days and are available for recognition by $CD4^+$ T-cells [64, 65, 70, 71].

MHC class I molecules are used to generate $CD8^+$ cytotoxic T-cells, which are loaded exogenously or endogenously.

1.1.7   Induction of adaptive immune responses via Dendritic Cells

DC induce Ag specific immunological answers by their ability to let naïve T- and B-lymphocytes proliferate. DC control both parts of the adaptive immune system: the cellular and the humoral immunity pointing out the key role in the regulation of adaptive immunity [13, 72].

The induction and regulation of cellular immune responses via DC is possible on two functional levels: Activation (1) and polarization (2) of $CD3^+$ cytotoxic T-cells. According to the current knowledge the activation requires at least three signals: (I) the MHC associated Ag presentation, (II) the expression of costimulatory molecules (e.g. CD80, CD86) and (III) the production of cytokines [13, 73]. The polarisation of the T-cell answer is mainly influenced via cytokine production, and both the cytokines produced by DC themselves as well the cytokines in the tissue area of the cell to cell interaction are important. Especially for IL-12 the link between cytokine production and polarisation of the immunological answer has been demonstrated by different studies [74, 75]. A high production of IL-12 via Ag presenting DC is one of the strongest stimuli for the generation of T-helper 1 effector cells, which on their behalf produce large amounts of INF-γ and enhance the cytotoxic T-cell answer [73, 74]. The absence of IL-12 during

Ag presentation leads to the generation of T-helper 2 effector cells that produce IL-4, IL-5 and IL-10 and stimulate a humoral immune answer [42].

DC regulate the humoral immune answers indirectly via the polarization of T-helper cells and directly via the interaction with B-Lympocytes [76]. DC directly stimulate the proliferation of naïve B-cells and memory B-cells [77-79]. They induce surface expression of ImmunoglobulinA (IgA) on B-cells and IgA secretion in the absence of T-helper 2 cells [80].

CD40 activated DC secrete IL-12 and soluble IL-6 receptor which differentiates naïve B-cells in IgM producing plasma cells [81]. DC in the follicular lymph nodes activate the proliferation and the production of IgG by B-cells [82].

1.1.8   Induction of innate immune responses via Dendritic Cells

DC recognize and eliminate pathogens via expression of a variety of receptors (for example Toll-like receptors), that bind bacterial and viral components [40, 83-86]. The activation of DC does not only induce an adaptive immunological answer but also stimulates unspecific defence mechanisms [18]. An important part within this unspecific defence beside phagocytosis of the alloAg is the ability of DC to produce high amounts of IL-12 and IFN-α [74, 87]. Both, IL-12 and IFN-α activate the proliferation of NK-cells, that lysate virus infected cells or tumor cells without Ag recognition and enhance their production of IFN-γ [18, 88]. DC enhance the development of a local inflammatory tissue reaction, leading to phagocytosis of the lysed cells and a maturation process of the DC.

1.1.9   The importance of Dendritic Cells for tolerance against self tissue

DC have two important functions: (I) the immune stimulatory activation of B- and T-cells and (II) the immunoregulatory inhibition of inflammatory immune reactions. In general the research focused on maximally stimulating B- and T-cells.

No other Ag presenting cell has a higher capacity to process Ag MHC complexes and to present them in a high density on the surface [65, 89].

The importance of DC for tolerance induction against self tissue was just recently recognized. The immune system achieves tolerance against auto Ag via deletion of autoreactive T-cell clones in the thymus (central tolerance) [90, 91] and via regulation

(anergy, suppression, apoptosis) of peripheral T-cells in the lymphatic tissue (peripheral or extrathymic tolerance). Under normal conditions the deletion of autoreactive T-cell clones in the thymus is incomplete, but the uptake of dead self tissue does not lead to an inflammatory answer against self tissue. The prevention of this inflammatory answer was the objective of different studies [92]. A recent study showed that immature DC continuously transport Ag of dead epithelial cells to the T-cell region of mesenterial lymph nodes without inducing an inflammatory response [93]. At least two reasons are responsible: (I) the kind of Ag (apoptotic cells) and (II) the maturation status of DC (immature DC) are crucial for tolerance induction [91, 94, 95]. The importance of the maturation status of DC could be shown experimentally via the use of a CD40 activating antibody in a T-cell receptor transgene mouse model [96]. The animals developed an immune tolerance against the model peptide ovalbumin, if DC processed Ag of ovalbumin pulsed apoptotic cells. The tolerance could not be reversed with injections of Freuds adjuvans. A strong adaptive T-cell answer against the model peptide was shown, when the maturation of DC during the Ag pulsing was induced with CD40 antibodies.

In their immature stage DC cannot effectively activate T-cells due to the low expression of costimulatory and MHC class II molecules [91, 97]. Recent studies have shown, that DC in an immature state are not inert cells that are ignorated by T-cells. Immature DC induce in vitro and in vivo anergic and immunoregulatory T-cells, that can effectively suppress an immune response [98-101].

1.1.10   The maturation status and the induction of tolerance or immunity

With regard to the dual role of DC in immunity and tolerance more information about the decisive signals that transform tolerogenic into immunogenic DC is presently available. The "simple" concept that immature DC possess tolerogenic and mature DC immunogenic properties has been revised by several reports demonstrating that phenotypically mature myeloid DC [102] as well as CD40L-matured plasmacytoid DC [103] can induce regulatory T-cell responses. This is supported by studies demonstrating that phenotypically mature DC constitutively take up and subsequently present self Ag in the draining lymphoid tissue without inducing autoimmunity [93, 104]. Moreover, CD86+ mature myeloid DC can expand CD25+ and CD4+ T-regulatory cells

in vivo while retaining their suppressive capacities [105] and CD8^{+} mature murine donor DC have been found to prolong heart allograft survival [106]. The systematic evaluation of these studies revealed in most cases, that tolerogenic DC were characterized as phenotypically mature and functionally immature [107].

1.2 Mechanism of action of immunosuppressive drugs and their clinical relevance

Immunosuppressive drugs can be classified into five groups:
- glucocorticoids
- cytostatics
- antibodies
- immunophilin-binding agents
- miscellaneous drugs

1.2.1 Glucocorticoids

In pharmacological (supraphysiological) doses, glucocorticoids are used to suppress various allergic, inflammatory and autoimmune disorders. In addition they are administered to prevent acute transplant rejection and graft versus host disease. An early discovery was that glucocorticoids suppress cell-mediated immunity [108].

Glucocorticoids stimulate the lipocortin-1 [110] to bind to the leukocyte membrane receptors followed by the inhibition of various inflammatory events: epithelial adhesion, emigration, chemotaxis, phagocytosis, respiratory burst and the release of various inflammatory mediators (lysosomal enzymes, cytokines, tissue plasminogen activator, chemokines etc.) from neutrophils, macrophages, mastocytes and DC. Main disadvantages of glucocorticoids are the overall suppression accompanied with high risk of infection, their inhibition of reparative processes and their risk for malignancies [111-113].

1.2.2   Cytostatics

Cytostatics inhibit cell division. Within systemic immunosuppressive therapy they are applied in smaller doses for the treatment of malignant diseases. They affect the proliferation of both T-cells and B-cells. Most frequently, due to their effectiveness purine analogs are administered.

Alkylating agents applied in system immunosuppressive therapy are nitrogen mustards (cyclophosphamide), nitrosoureas, platinum compounds and others. Cyclophosphamide and related agents are among the most potent compounds. In small doses, it is very efficient in the therapy of systemic lupus erythematosus, autoimmune haemolytic anaemias, Wegener`s granulomatosis and other immune diseases. High doses cause pancytopenia and hemorrhagic cystitis.

Antimetabolites interfere with the synthesis of nucleic acids. These include:

-folic acid analogues such as methotrexate

-purine analogues such as azathioprin

-pyrimidine analogues

-protein synthesis inhibitors

Methotrexate is a folic acid analogue. It binds dihydrofolate reductase and prevents synthesis of tetrahydrofolate. It is used in the treatment of autoimmune diseases (e.g. rheumatoid arthritis) and in transplantation.

Azathioprin is the most commonly used immunosuppressive cytotoxic substance. It is extensively used to control transplant rejection reactions. After the nonenzymatically cleavage to mercaptopurine it acts as a purine analogue and an inhibitor of DNA synthesis. Mercaptopurine itself can also be administered directly. By preventing the clonal expansion of lymphocytes in the induction phase of the immune response, it affects both the cell and the humoral immunity. It is also efficient in the treatment of autoimmune diseases.

1.2.3   Antibodies

Antibodies can be divided into polyclonal (produced by a mixture of various B-lymphocyte clones) and monoclonal (secreted by a single clone of B-lymphocytes) types that are used as quick and potent immunosuppressives in order to prevent the acute rejection reaction.

Heterologous polyclonal antibodies are obtained from the serum of animals (e.g. rabbit or horse), that were injected with the human's thymocytes or lymphocytes. The antilymphocyte and antithymocyte Ag are used to prevent allograft rejection. They are added to other immunosuppressants particularly with regard to reduce their dosage and toxicity. Polyclonal antibodies inhibit T-Lymphocytes by causing their lysis, which includes both complement mediated cytolysis and cell mediated opsonization. In this way, polyclonal antibodies inhibit cell mediated immune reactions, graft rejection, delayed hypersensitivity (i.e. tuberculin skin reaction) and the graft versus host disease (GVHD), but influence thymus dependent antibody production. Currently the two drug products Atgam$^{®}$ and Thymglobuline$^{®}$ obtained from horse and rabbit serum are approved. Polyclonal antibodies affect all lymphocytes and cause general immunosuppression possibly promoting post-transplant lymphoproliferative disorders or serious infections, especially by Cytomegalovirus. Polyclonal antibodies are immunogenic, so that patients may have an acute reaction to the treatment, i.e. fever rigor, even anaphylaxis and sometimes later develop a serum sickness or immune complex nephritis.

Monoclonal antibodies act towards exactly defined Ag. Therefore, they cause fewer side effects compared to polyclonal antibodies. Antibodies directed towards the IL-2 receptor (CD25) and CD3 are most frequently used. OKT3 is the first approved anti CD3 monoclonal antibody. It is a mouse anti-CD3 monoclonal antibody of the IgG$_2$a type that prevents T-cell activation. Based on the mechanism, OKT3 is one of the most potent available immunosuppressive substances that is clinically used to control the steroid and/or polyclonal antibodies resistant acute rejection episodes. CD3 blocks the TCR-Ag binding and causes conformation change or the removal of the entire TCR3/CD3 from the T-cell surface. This lowers the number of T-cells, perhaps by sensitizing them for the uptake by the reticular epithelial cells. The cross binding of

CD3 molecules also activates an intracellular signal, causing T-cells anergy or apoptosis. Although CD3 antibodies act more selective than polyclonal antibodies, they impair the cell mediated immunity significantly, predisposing the patient to opportunistic infections and malignancies (anaphylactic reactions, cytokin-release-syndrom, Lymphomas etc.). In 1998 two chimeric mouse/human antibodies, basiliximab (Simulect®) and daclizumab (Zenapax®) were introduced into the market. These drugs act by binding the IL-2a receptor's α-chain, preventing the IL-2 induced clonal expansion of activated T-lymphocytes and shortening their survival. They are used in the prophylaxis of acute organ rejection (e.g. kidney transplantation), both being similarly effective with few side effects.

1.2.4   Drugs acting on immunophilins

The most important Immunophilin-binding immunosuppressants are Cyclosporine A (CsA), FK506 (Tacrolimus) and Rapamycin (RAPA). A novel immunophilin binding immunosuppressant still under investigation is Sanglifehrin A (SFA).

Cyclosporin A

Since the discovery of its anti-lymphocytic activity [114], CsA presented the most important immunosuppressive substance in solid organ transplantation [115]. CsA and FK506 bind to the intracellular receptors immunophilins cyclophilin and FKBP12, followed by the inhibition of calcineurin [116]. Calcineurin inhibition results in the suppression of gene transcription regulatory proteins, called NFAT (Nuclear factor of activated T-cells) and other factors [116-118]. NFAT regulates the expression of a large number of immune response genes, including interleukins and their receptors.

CsA is used in the treatment of acute rejection reactions. However, the nephrotoxic CsA is increasingly replaced by newer immunosuppressants with lower side-effects.

Tacrolimus (FK 506)

Tacrolimus has the same mechanism of action like CsA, but was detected to be about 100-fold more potent compared to CsA [119]. In 1989 FK506 was introduced successfully into clinical trials in organ transplantation [120]. The drug substance is

used particularly within liver and kidney transplantations, sometimes for heart or lung and combined heart/lung transplants.

Sirolimus (RAPA)

RAPA is a bacterial macrolide antibiotic substance from Streptomyces hygroscopicus with potent immunosuppressive action introduced in recent years as anti-rejection therapy in organ transplantation [121, 122]. In addition, RAPA is a potent anti-proliferative agent and may therefore reduce the risk of cancer development in transplant patients [123]. RAPA binds intracellularly to FK506 binding proteins and inhibits the function of serine/threonine kinase target of RAPA: the mammalian TOR (MTOR) [124]. MTOR is a common effector protein shared by many signalling pathways. Inhibition of MTOR results in suppression of cytokine-driven cell proliferation, ribosomal protein synthesis, translation initiation and cell cycle arrest. It also possesses an inhibitory effect on B-cell differentiation and plasma cells, which lowers the produced quantity of IgM, IgG and IgA antibodies.

SFA, a novel immunophilin-binding immunosuppressive agent

SFA originally described by Sanglier and Fehr [125, 126], is produced by the Actinomycetes strain Streptomyces A92-308110. As SFA belongs to the novel family of immunophilin-binding agents like CsA, it binds with high affinity to cyclophilin, but does not inhibit the phosphatase activity of calcineurin [127, 128]. Studies involving a non immunosuppressive cyclophilin-binding derivative of CsA have revealed that the immunosuppressive activity of SFA is not dependent on cyclophilin binding [129]. In addition, SFA does not bind to FK506 binding protein12 and does not inhibit enzymatic activity of p70^{s6k}kinase, a major downstream target of MTOR [127, 128]. The evaluation of these results indicate that SFA presents a novel class of immunophilin binding immunosuppressants with a new undefined mechanism. Studies concerning the immunosuppressive impact of SFA have been performed in T and B-lymphocytes. SFA has been reported to exhibit a lower immunosuppressive activity in the mixed lymphocyte reaction (MLR) when compared to CsA [129]. Concerning T-cells, SFA does not inhibit IL-2 transcription, but impairs IL-2-induced T-cell proliferation at the G1 phase of the cell cycle [129, 130]. SFA also inhibits proliferation of LPS stimulated B-cells, but

does not affect IgG production by CD154/IL-4 activated B-cells [129]. With regard to monocytes, a moderate suppressive effect on TNF-α production was detected [129]. However, these studies investigated the in vitro effects of SFA and reliable in vivo data with respect to the efficacy and toxicity profile are still not available. In parallel to the immunosuppressive effect, SFA has been reported to inhibit opening of mitochondrial permeability transition pore (MPTP) through cyclophilin D binding [131, 132]. MPTP opening is a critical event in cell death and reperfusion injury indicating alternative potential for SFA as a cell death inhibitor in vivo. In the following Figure 2 the molecule model of SFA is presented.

Figure 2   Molecule Model of Sanglifehrin A
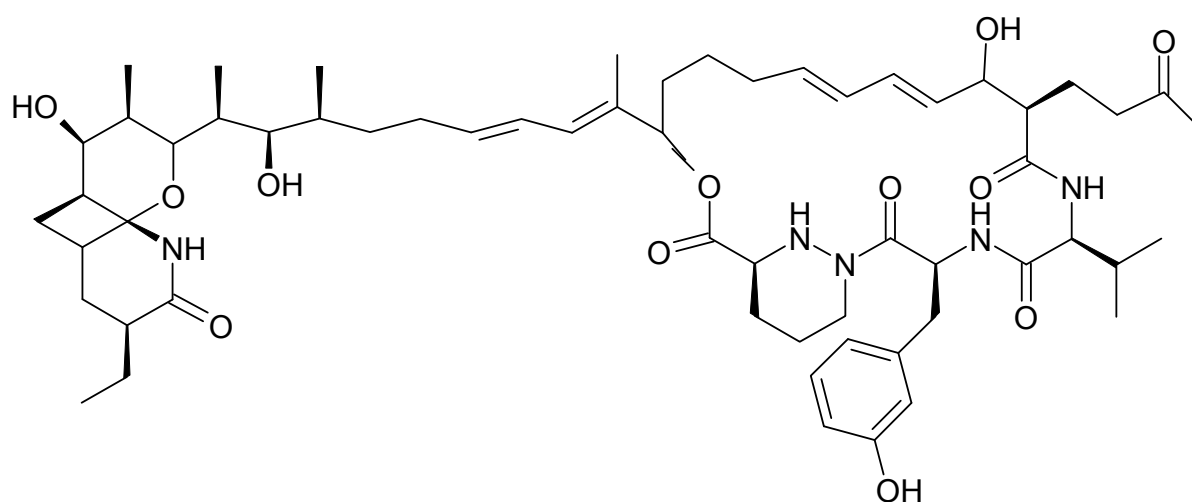
Molecular Weight =1088,45
Exact Mass =1087
Molecular Formula =C61H93N5O12
Molecular Composition =C   67.31%   H   8.61%   N   6.43%   O   17.64%

1.2.5   Miscellaneous Drugs

TNF binding Proteins

A TNF-α binding protein is a monoclonal antibody or a circulating receptor such as infliximab (Remicade$^®$), etanercept (Enbrel$^®$), or adalidumab (Humira$^®$) that binds to TNF-α preventing the synthesis of IL-1, IL-6 and the adhesion of lymphocyte activating molecules. They are used in the treatment of rheumatoid arthritis, ankyloing spondylitis, Crohn`s disease and psoriasis.

Mycophenolate mofetil (MMF)

Mycophenolate mofetil acts as a non-competitive, selective and reversible inhibitor of iosine monophosphate dehydrogenase which presents a key enzyme in the de novo guanosine nucleotide synthesis. In contrast to other human cell types, lymphocytes, B- and T-cells are highly dependent on this process.

1.3   Immunosuppressive drugs target Dendritic Cells

The traditional focus for the development of immunosuppressive drugs has been to target lymphocytes. However, in recent years several established immunosuppressive agents have been shown to target DC in addition to lymphocytes [133, 134]. In particular, DC differentiation, DC expansion, DC migration, DC Ag uptake, DC maturation and DC survival have been the objective of these studies [135] (Figure 3).

Figure 3   Immunosuppressive and anti-inflammatory drugs target Dendritic Cells
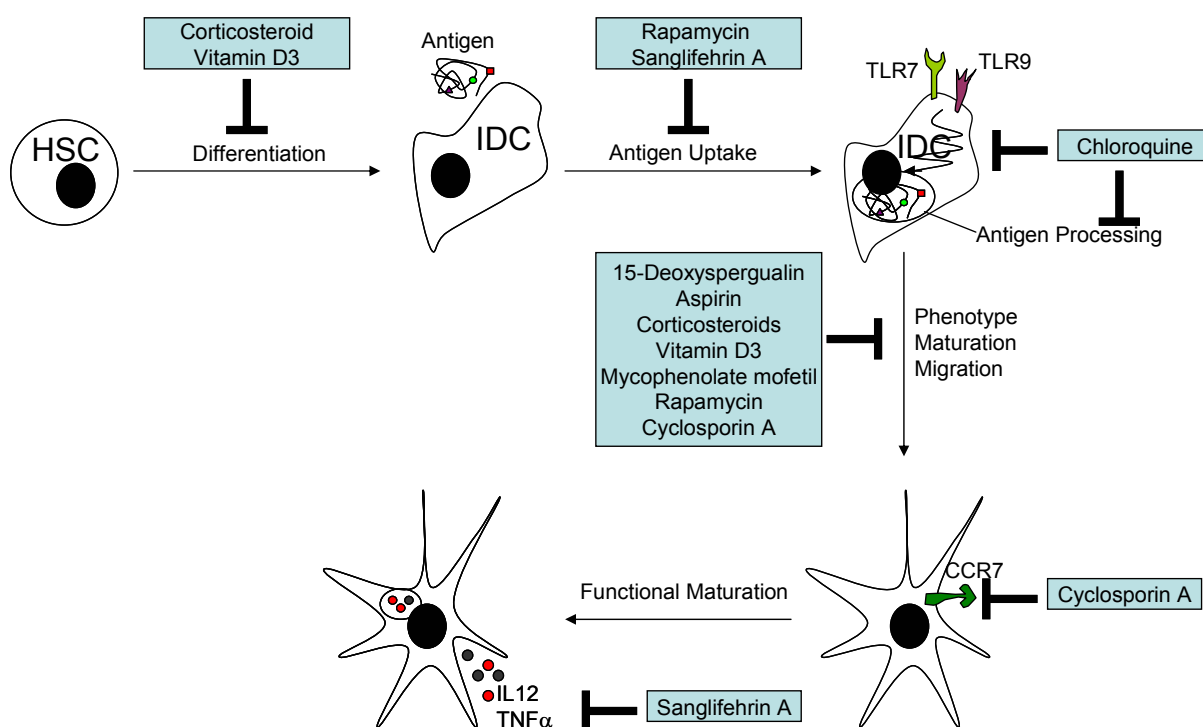
Corticosteroids and Vitamin D3 receptor ligands suppress DC differentiation and maturation, respectively. In contrast, RAPA suppresses Ag uptake and maturation, whereas the new cyclophilin-binding immunosupressant SFA mainly blocks the production of bioactive IL-12. SFA inhibits in parallel, but to a lesser extent, the production of TNF by DC as well as the Ag uptake. Chloroquine blocks acidification of the lysosomes and therefore impairs the endosomal pathway of MHC class-II-restricted Ag processing as well as Toll-like receptor-7 (TLR-7) and TLR-9 signalling. The immunosuppressant mycophenolate mofetil, 15-deoxyspergualin and the anti-inflammatory drug substance acetylsalicylic acid (aspirin) target phenotypic and functional DC maturation, whereas cyclosporine A impairs DC migration.

For instance, RAPA suppresses in vitro the generation of GM-CSF-expanded human monocyte derived DC [136, 137]. In vivo studies revealed that the drug substance inhibits the generation of fms-like tyrosine 3 kinase ligand (Flt3L)-expanded DC [138]. In parallel, it was identified that RAPA is the first clinically relevant substance that inhibits DC Ag uptake in a maturation independent matter [138]. At low concentrations, RAPA impairs macropinocytosis and mannose receptor-mediated endocytosis of murine bone marrow derived DC [139]. Furthermore, inhibition of DC Ag uptake by RAPA was confirmed with human monocyte derived DC [137] and after in vivo administration of the drug [138] .

MMF represents a potent inhibitor of iosine monophosphate dehydrogenase [140], an essential enzyme in the de novo synthesis of guanine and is thought to represent a selective agent for lymphocytes. However, in addition to its effect on lymphocytes, MMF has been shown to suppress phenotypical DC maturation and the capacity of Ag-bearing murine DC to induce a delayed hypersensitivity response in vivo [141].

Recently CsA has been reported to inhibit the migration of DC in vitro and in vivo [142]. CsA was published to suppress expression of COX-2, a critical enzyme involved in the synthesis of Prostaglandin E_2 (PGE_2) as well as to inhibit CCR7 expression [142]. PGE_2 is a major inducer of DC migration and has been reported to mediate its promigratory effects by facilitating CCR7 signal transduction [143]. CCR7 switches DC responsiveness to its ligands, the chemokines CCl 19 and 21, that lead to migration to secondary lymphoid tissues [144]

1.4   Pharmacological manipulation of Dendritic Cells

Pharmacological programming is a safe and predictable approach to the manipulation of DC [97]. In general, two different concepts have been pursued to generate tolerogenic DC:

(i)   the generation of immature DC and

(ii)   the generation of phenotypically mature DC that are insufficient producers of pro-inflammatory cytokines (partially mature DC).

The generation of immature DC that express low levels of MHC class II and T-cell costimulatory molecules (CD40, CD80, CD86) can be achieved via maturation inhibitors, i.e. Vitamin D3, corticosteroids, IL-10 and the NF-κB inhibitor LF15-0195 [135]. "Semi mature" DC can be generated by the combination of agents that promote phenotypical maturation (TNF-α, LPS) with agents suppressing the production of proinflammatory cytokines (e.g. IL-10, TGF-β) [102]. The application of immature DC has been shown to promote T-cell anergy and to prolong organ allograft survival in different rodent models [145, 146]. Recent findings indicate that semi-mature DC, expressing

- high levels of MHC class II

- different amounts of costimulatory molecules

- low levels of proinflammatory cytokines, in particular IL-12

have excellent tolerogenic properties and can induce Ag-specific regulatory T-cells in vivo [99, 103, 135, 147-151]. Regulatory T-cells play a central role maintaining self tolerance and controlling immune responsiveness to allo Antigens [13, 146]. Menges et al. have demonstrated that repeated injections of DC matured with TNF-α induce IL-10 producing peptide-specific regulatory T-cells in vivo as well as Ag specific protection of experimental autoimmune encephalomyelitis [152]. The tolerogenic DC were characterized as MHC II[high] and costimulatory[high], being weak producers of inflammatory IL-12p70. A similar approach was successfully employed by Sato et al. expanding DC in the presence of IL-10 and TGF-β and "maturation" of these Ag-presenting cells with either LPS or TNF-α [153, 154]. By using murine model graft versus host disease (GVHD) and leukaemia relapse, it was shown that host matched semi mature DC protected mice from GVHD lethality in an Ag-specific manner and induced the expansion of IL-10-producing CD4+ CD25+ suppressor T-Cells [153].

1.5   Aims of this work

DC are well-equipped Ag-presenting cells that initiate and regulate immune responses. They initiate adaptive immunity by the activation of naïve lymphocytes and represent

powerful stimulators of NK-cells. DC induce central and peripheral tolerance by mechanisms that include deletion, anergy and induction of regulatory lymphocytes. They possess capacity to produce bioactive IL-12, a major proinflammatory cytokine that promotes the expansion of T-helper 1 and NK-cells, bridging innate and adaptive immunity. In addition, evidence by different investigators suggests that IL-12p70 is the most important decisive factor switching tolerogenic into immunogenic DC. With respect to the central role of DC in immunity and tolerance, they are interesting therapeutic targets for pharmacological manipulation of immune responses.

The aim of this work is to investigate the pharmacological effects of the novel immunophilin-binding agent SFA on DC in vitro and in vivo.
For it, the effect of SFA on the typical DC differentiation and maturation markers as well as the impact of SFA on cytokine production after stimulation, the endocytotic and phagocytotic capacity of DC under the influence of SFA is to be investigated.
Another major aim is to reproduce in vitro findings in an in vivo mouse model in order to clarify the clinical relevance of these findings.

With respect to the importance of proinflammatory IL-12p70 one major goal of this study is to evaluate the effectiveness and selectivity of the novel substance SFA to block IL-12p70 production by DC in vivo.

The impact of SFA as a novel means for pharmacological programming of tolerogenic DC in models of alloimmunity and solid organ transplantation is to be evaluated.

SFA may be a unique immunosuppressive agent with relative selectivity for professional Ag presenting cells. Investigation of the immunosuppressive potential of SFA with emphasis on DC may provide a novel pharmacological approach for the therapy of allograft rejection.

Detailed knowledge about pharmacological modulation of DC function by SFA may improve the clinical therapy of transplant patients for two reasons: **(i)** it may provide a rational basis for selection and combination of immunosuppressive drugs in different clinical settings, and **(ii)** it represents a clinically approach for the generation of tolerogenic DC in the laboratory.

2.   MATERIALS AND METHODS

2.1   Materials

2.1.1   DC Medium and wash buffer

In order to produce DC Medium RPMI 1640 medium (PAA Laboratories, Linz, Austria; Cat. No. E15-840 Lot R5NN07195) was supplemented with 10% heat inactivated Fetal Calf Serum (FCS) Gold (PAA Laboratories, Linz, Austria, Cat. No. A15-649), 200mM L-Glutamine (PAA Laboratories, Linz, Austria, Cat. No. M11-004, Lot.No. M00406-0289) 10.000 u/ml Penicillin, 10 mg/ml Streptomycin (PAN Biotech GmbH, Aidenbach, Germany; Cat. No. P06-07100 Lot. No. 44606), 100mM sodium-pyruvate (Gibco/Invitrogen, Cat. No. 11360-039; Lot. 3095446), 1M HEPES (Gibco/Invitrogen, Cat. No. 15630-056, Lot. No. 44606) and 100x MEM nonessential amino acid solution (Sigma Aldrich Chemie GmbH, Steinheim, Germany, Cat. No. M7145, Lot. No. 105K2440). All supplements are 100x concentrations and were diluted to the appropriate concentration in the RPMI media.

As wash buffer Dulbeccos Phosphate Buffered Saline (PBS) was taken (PAA Laboratories, Linz, Austria, Cat. No. H 15-011, Lot. No. H01106-0289, DPBS). In order to stabilize the vitality of cells for certain experiments 1% of BSA (bovine serum albumine for cell culture, Serva Electrophoresis GmbH, Heidelberg, Germany, Cat. No. 47330, Lot. No. 17724) was added to the wash buffer.

2.1.2   Cytokines

Recombinant human (rehu) GM-CSF was purchased from Novartis (Leukomax, Basel, Switzerland) and from Berlex (Leukine, Seattle, Washington; Cat. No.: 6051601, Lot. No. c 031103) and rehu IL-4 was purchased from Promocell (Heidelberg, Germany, Cat. No. rh IL-4 500 µg, Lot. No 19040401-A). Both were diluted in DC Media to an end concentration of 1000 international units (IU)/ml. Recombinant human FMS-like tyrosine kinase 3 (Flt3L) was kindly provided by Amgen (Thousand Oaks, CA, USA) and used in vitro at a concentration of 25ng/ml and in vivo at a concentration of 10µg/animal/day.

2.1.3   Stimulants, Drugs and Chemicals

Rapamycin (Wyeth, Taplow, Maidenhead UK) Cyclosporin A (Sigma Aldrich, St.Louis, USA), LPS (E. coli strain 026:B6, Cat. No. L-2654, Lot. 064K4077), Polysorbate 80

(P-1379, Lot 032K0182), PEG 300 (Cat. No. P3140-500G, Lot. No. 063K0131) and Polyinosinic-Polycytidylic acid (Poly[I]-Poly[C], Cat. No. P-0913, Lot 014K4025) were purchased from Sigma Aldrich (Seelze, Germany). Sanglifehrin A was kindly provided by Novartis Pharma (Basel, Switzerland), IFN-γ from BD Pharmingen (Cat. No. 552138; Lot. No. 522d314, San Diego, California, U.S.A). Sterile water was purchased from Braun and Baxter (Aqua ad injectabilia, Cat. No. 2351544, Lot. No. 5094C12, Melsungen, Germany; Cat. No. 001428, Lot. No. 05L28S13) and Ficoll Paque Plus from Amersham Biosciences AB (Cat. No. 17-1440-3, Lot. No. 305516, Uppsala, Sweden).

2.1.4   Sanglifehrin A and Flt3L for in vivo use

SFA and Flt3L were used at the indicated concentrations and timepoints. SFA was predissolved in 96% Ethanol in a concentration of 35mg/ml. Shortly before injection the stock solution was further diluted in 2.5% Polysorbate 80 (Merck KgaA, Darmstadt, Germany, Cat. No. 8.17061.1000, Lot. No. S4112361443), 51% PEG 300 (2.1.3) and 46.5% sterile water for injection (2.1.3). Alcohol concentration in this dilution was about 4%. Stock solutions were prepared in absolute ethanol (vehicle) and diluted on the day of the experiment with drug vehicle. Flt3L was dissolved in PBS to a concentration of 1 mg/ml. For in vivo use it was further dissolved in PBS to a concentration of 20 µg/ml. Control animals were injected with drug vehicle.

2.1.5   Flow Cytometry Antibodies

2.1.5.1   Human Antibodies

HLA-DR-PE (clone L343) and rat IgG$_{2a}$ (clone R35-95), CD40-PE (clone 5C3) and isotype mouse IgG$_{1\kappa}$ (clone MOPC-21), CD83-PE (clone HB15e) and isotype mouse IgG$_{1\kappa}$ (clone MOPC-21), CD86PE (clone GL1) and isotype rat (Louvain,IgG$_{2a}$), CD14-FITC (clone M5E2) and isotype control IgG$_{2a}$ (cloneG155-178), CD1a-CY (clone HI149) and isotype mouse IgG$_{1\kappa}$ (clone MOPC-21) were purchased from BD Pharmingen (San Diego, California, USA).

2.1.5.2   Mouse Antibodies

FITC-, PE, CyChrome-conjugated or biotinylated mAb were used to detect expression of *I-A^b*(A$_\beta$b) (clone AF6-120.1), *CD 4* (clone L3T4;GK1.5), *CD 54* (ICAM-1/clone 3E2),

H-2K^{b} (clone AF6-88.5), *CD 80* (clone B7-1;16-10A1), *CD 86* (clone B7-2;GL1), *CD 45R/B220* (clone RA3-6B2), *CD 11b* (Mac-1 α chain; clone M1/70) *CD11c* (clone HL3), CD 40 (clone HM40-3;3/23), as well as isotype-matched control mAb. All antibodies were purchased from BD Pharmingen (San Diego, California, USA).

2.1.6   Elisas and Cell proliferation kits

2.1.6.1   Human Elisas

IL-2 (Cat. No. 555190, Lot. No. 82391), IL-5 (Cat. No. 555202; Lot. No. 78034), IL-6 (Cat. No. 555220; 70030); IL-8 (Cat. No. 555244; Lot. No. 56953), IL-10 (Cat. No. 555157; Lot. No. 52548), IL-12p70 (Cat. No. 555183; Lot. No. 82375), IL-15 (Cat. No. 559268; Lot. No. 54871), TNF-α (Cat. No. 555212 : Lot. No. MF 31447), IFN-γ (Cat. No. 555142; 60200). All human cytokine Elisa BD OptEIA^{TM} sets were purchased from BD Biosciences Pharmingen (San Diego, California, USA).

2.1.6.2   Murine Elisas

IL-2 (Cat. No. 555148; Lot. No. 75890), IL-4 (Cat. No. 555232; Cat. No.: 71222), IL-5 (Cat. No. 555236; Lot. No. 63843), IL-6 (Cat. No. 555240; Lot. No. 73078), IL-10 (Cat. No. 552635; Lot. No.442571), IL-12p70 (Cat. No. 555156, Lot. No.81817), TNF-α (Cat. No. 555671; Lot. No. 44328/05) and IFN-γ (Cat. No. 555138, Lot. No. 57286). All murine cytokine Elisa BD OptEIA^{TM} sets were purchased from BD Biosciences Pharmingen (San Diego, California, USA).

2.1.6.3   Cell Proliferation Biotrak ELISA

The Cell Proliferation Biotrak ELISA from Amersham Biosciences (UK, England; Cat. No. RPN 250, 15/rx5602.) was used according to the manufacturers instructions. The technique is based upon the incorporation of the thymidine analog 5'-bromo-2'-deoxyuridine (BrdU) into the DNA of proliferating cells cultured in a 96-well microtiterplate.

2.1.7   Animals

Male C57 BL/10J CRL mice and female Balb/cAnN Crl mice, 8-12 weeks old, were purchased from Charles River Laboratories and maintained in the animal facility of the

Justus Liebig University, Department of Surgery, Giessen, Germany. Animals were treated in a humane manner in compliance with the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 86-23, revised 1985).

2.1.8   Buffy Coats

Buffy coat is the fraction of a centrifugated blood sample that contains most of the white blood cells. After centrifugation, one can distinguish a layer of clear fluid (the blood plasma), a layer of red fluid containing most of the red blood cells, and a thin layer in between, the buffy coat (so-called because it is usually buff in hue), with most of the white blood cells and platelets. Buffy coats were kindly provided from healthy blood donors after giving informed consent.

2.1.9   Materials for Realtime Polymerase Chain Reaction (PCR)

DC isolated RNA was transformed to cDNA according to the manufacturers instructions of Amersham Biosciences "Ready-To-Go You-Prime First-Strand Beads" and $pd(N)_6$ random hexamer 5`phospate as primer (Freiburg, Germany). Real Time PCR was performed with an Assay on Demand on an ABI-Prism 7000 Taqman (Applied Biosystems, Foster City, California, USA).

2.2   Methods

2.2.1   Generation of Monocyte derived DC

Human peripheral blood monocytes (PBMC) were isolated from buffy coats of healthy donors by Ficoll-Paque Plus^{TM} density grade centrifugation. Before centrifugation, the buffy coat was first diluted with PBS in a ratio 20 ml/30 ml. Subsequently 15 ml Ficoll-Paque Plus^{TM} was carefully overlayed. After centrifugation the white ring including the lymphocytes is carefully removed with a pipette. For DC generation, CD 14$^+$ monocytes were purified (Purity > 95%, evaluated with CD14 FITC) using the MACS CD14 isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany) and $3x10^6$ cells were cultured in 3 ml DC Medium (2.1.1), rehu GM-CSF (1,000 IU/ml), and rehu IL-4 (1,000 IU/ml) in 6 well flat bottomed plates. After three days, 50% supernatant was replaced with fresh cytokine-containing medium. On day 6, CD1a$^+$ cells represented > 90 % of cultured cells.

Figure 4   Purity of CD14$^+$ sorted cells
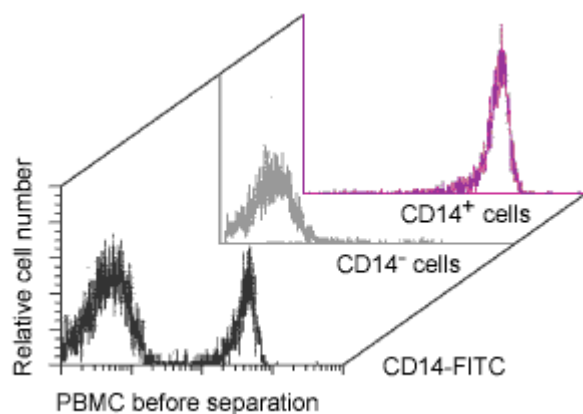
In order to control the purity of the affinity purification the cells were magnetically and fluorescence labelled. The samples were measured before and after purification to assure the quality of the experiments. In front the labelled cells in a histogramm blot before purification are shown (black histogram). Behind that there are two histogram blots: the CD 14$^-$ fraction (grey histogram) and the CD14$^+$ fraction (magenta histogram) (Histogramm from Miltenyi Biotec, Bergisch Gladbach, Germany).

2.2.2   In vitro application of SFA and stimulation of Monocyte derived Dendritic Cells (MoDC)

SFA was dissolved in absolute ethanol (Riedel de Haen Cat.No I/62513, Seelze, Germany) at a concentration of 10 µg/µl for stock solution. This stock solution was further diluted with DC media to prepare the working solution (50 nM/µl or 100 nM/µl). A control solution was produced at the same dilutions containing solely alcohol. Depending on the test SFA (100-2000 nM/ml) or control were given to the cells on day 1 to day 6 or even one hour before stimulation. The cells were harvested, washed, counted with Trypan Blue Solution (Cat. No. T8154, Lot. No. 73K2420, Sigma Aldrich, Steinheim, Germany) and put into fresh DC Medium with Cytokines and SFA or control solution. After one hour the cells were stimulated with either 1 µg/ml LPS or with 100 µg/ml Polyinosinic-Polycytidylic acid plus 20 ng/ml rehu IFN-γ. The supernatant was removed for cytokine analysis. Apoptosis was analysed by staining of phosphatidylserine translocation with Annexin-V-PE in combination with the vital dye 7-AAD.

2.2.3   Isolation of human CD 1c$^+$ (BDCA 1) Blood CD

To control the results with monocyte derived Dendritic Cells, Cd1$^+$ Blood Dendritic Cells were isolated. For CD1c$^+$ separation, PBMC were purified with the MACS CD1c$^+$

(BDCA 1) Blood Dendritic Cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). Briefly: PBMC from buffy coats were depleted of B-cells with CD19 microbeads. After indirect labelling with biotinylated CD1c mAb and antibiotin microbeads and subsequently CD1$^+$ DC were positively selected after indirect labelling with biotinylated CD1c mAb and antibiotin microbeads [43]. The cells showed a purity of at least 90%. 4×10^5 cells were transferred into a 96 well plate with 200 µl DC medium including IL-4 and GM-CSF (each at a concentration of 1000 IU/ml) per well. Immediately 100 to 500 nM SFA was added and after 12 hours the cells were stimulated as indicated.

2.2.4   In vivo DC expansion with Flt3L

Balb/c or C57 BL/10 mice were injected with Flt3L to increase the population of DC from 1 to over 30% in the spleen with regard to the lymphocytes. In murine experiments, animals were injected with either SFA (10 mg/kg/day, i.p.) or with drug vehicle for 10 days. In some murine experiments DC were expanded in vivo over 10 days using rhuFlt3L (CHO cell-derived; 10 µg/day, i.p.) kindly provided by AMGEN (Thousand Oaks, CA, USA).

A more detailed description concerning the in vivo DC expansion is given below (2.2.4.1 to 2.2.4.3).

2.2.4.1   Isolation of spleen DC for in vivo experiments

Spleen cells and bone marrow (BM) cells were prepared with fine scissors and passed through a nylon cell strainer (70 µm Nylon; 1 per package; Cat. No. 661233, Becton Dickinson, NJ, USA) to obtain a single cell suspension.

After 3 minutes in red blood cells (RBC) lysis solution (Cat. No. D-50K1; Lot. No. 0804315, Gentrasystems, Hess. Oldesdorf, Germany), the CD11c$^+$ spleen DC (purity >90%) were isolated by immunomagnetic-bead sorting via Auto-MACS (Miltenyi Biotec, Bergisch Gladbach, Germany).

2.2.4.2   In vivo administration of Sanglifehrin A and expansion of DC via Flt3L

Animals were injected with SFA (10mg/kg/d, i.p.) or vehicle, respectively for three or ten days. For a dynamic DC development the animals were treated in addition with 10 µg of

human recombinant Flt3L for 10 days. This constant application of Flt3L leads to an increase of DC from one to over thirty percent [155, 156].

2.2.4.3   Cytokines after in vivo stimulation

After in vivo administration of SFA or vehicle (± Flt3L) for ten days, the animals were injected i.p. with 10 µg LPS (0111:B4 strain, Sigma Aldrich, Seelze, Germany) and 0,5 µg murine IL-4 (Peprotech, Rocky Hill NJ) dissolved in PBS with 1%. In additional experiments, in vivo cytokine production was stimulated after injection of CpG ODN 1826 (10 mg/500µl PBS; Invivogen, Toulouse, France). 4 hours later the animals blood was taken and centrifuged 10 minutes at 10.000 rpm, RT. Plasma supernatant was taken avoiding erythrocytes for measurement of murine cytokines. Erythrocytes lead to high background absorption rates and to false positive results. Murine bioactive IL-12p70, TNF-α and IL-10 were analyzed by ELISA according to the manufacturer's instructions (BD Pharmingen and R&D Systems, Wiesbaden, Germany).

2.2.5   Apoptotic Cell Death

The apoptotic program is characterized by certain morphologic features, including loss of plasma membrane asymmetry and attachment, condensation of the cytoplasm and nucleus and internucleosomal cleavage of DNA. Loss of plasma membrane is one of the earliest features. In apoptotic cells, the membrane phospholipids phosphatidylserine (PS) is translocated from the inner to the outer leaflet of the plasma membrane, thereby exposing PS to the external cellular environment. In order to investigate apoptosis Annexin V staining was applied. Annexin V is a 35-36 kDa Ca^{2+}-dependent phospholipid binding protein that has a high affinity for PS and binds to cells exposing PS. In order to differentiate between apoptosis and cell death, the cells were also stained with 7-AAD, a vital dye binding to DNA fragments. The Annexin V Apoptosis Detection Kit was purchased from BD Pharmingen (Cat. No. 662901; San Diego, California, USA) and was applied according to the manufacturers instructions.

2.2.6   Flow Cytometry

Wallace Coulter laid 1949 the cornerstone for Flow Cytometry. He patented the counting of particles and simplified therefore the counting of leucocytes. Nowadays the FACS analysis is used to analyse certain attributes of cells, i.e. markers on the surface of cells for differentiation of similar cell subtypes or of their maturation status. Since recently even intracellular pathways and cytokines can be measured via FACS. The principal way of a FACS is based on an antibody antigen reaction. The monoclonal antibody is linked to a fluorescence molecule and binds in the best case only to one specific antigen on or in the cell. This specific binding made it possible to detect a few unique cells in millions of other cells. That's why these surface markers have the abbreviation "CD" for Cluster of differentiation. The stained cells get fixed in formaldehyde, while without fixation some clusters might alter at the surface of the cell and lead to false positive or negative results. These fixed cells get resuspended and are passed by a liquid flow through the light of a laser ray. Via different fluorescence colours, many surface markers, the size and granularity of the cells can be analysed simultaneously (Figure 5). The concentration of cells should not exceed 5×10^6 cells/ml in order to have a sharp differentiation between the cells. All flow cytometric analyses were performed with a FACSCalibur^{TM} (Becton Dickinson, San Jose, California, USA). The system possesses two lasers: The first laser is an air-cooled argon-ion laser (488 nm) that allows the measurement of three colours: FITC (Flouresceinisothiocyanate), PE (Phycoerythrin) and CY (Cychrome). The second laser is a red diode laser (635 nm), that allows the measurement of a fourth colour: Allophycocyanin (APC). Normally the measuring of 10000 cells per sample is sufficient, for certain seldom cell subtypes the measurement is increased up to 100.000 cells per sample. For simple analysis the *Cellquest Research Software* (Becton Dickinson, California, USA) and the freeware *WinMDI Version 2.8 (*Scripps Research Institute, Florida, USA*)* were used. For complex analyses the *FloJo* software (Treestar Inc., California) was applied. The results were shown either in histograms or Dot-Plots. Via *Dot-Plot* analysis even cell subtypes could be securely detected. Within the experiments 5×10^5 cells were blocked with 10% v/v normal goat serum (10 min; 4°C)

then stained with mAb (30 min; 4°C). Appropriate isotype-matched antibodies were used as negative controls. The cells were washed with PBS containing 1% sodium-azide and then fixed in 10% PBS formaldehyde solution. These cells were analyzed using a FACS Calibur flow cytometer as described above.

Figure 5   Principals of flow cytometry in a double fluorescence system
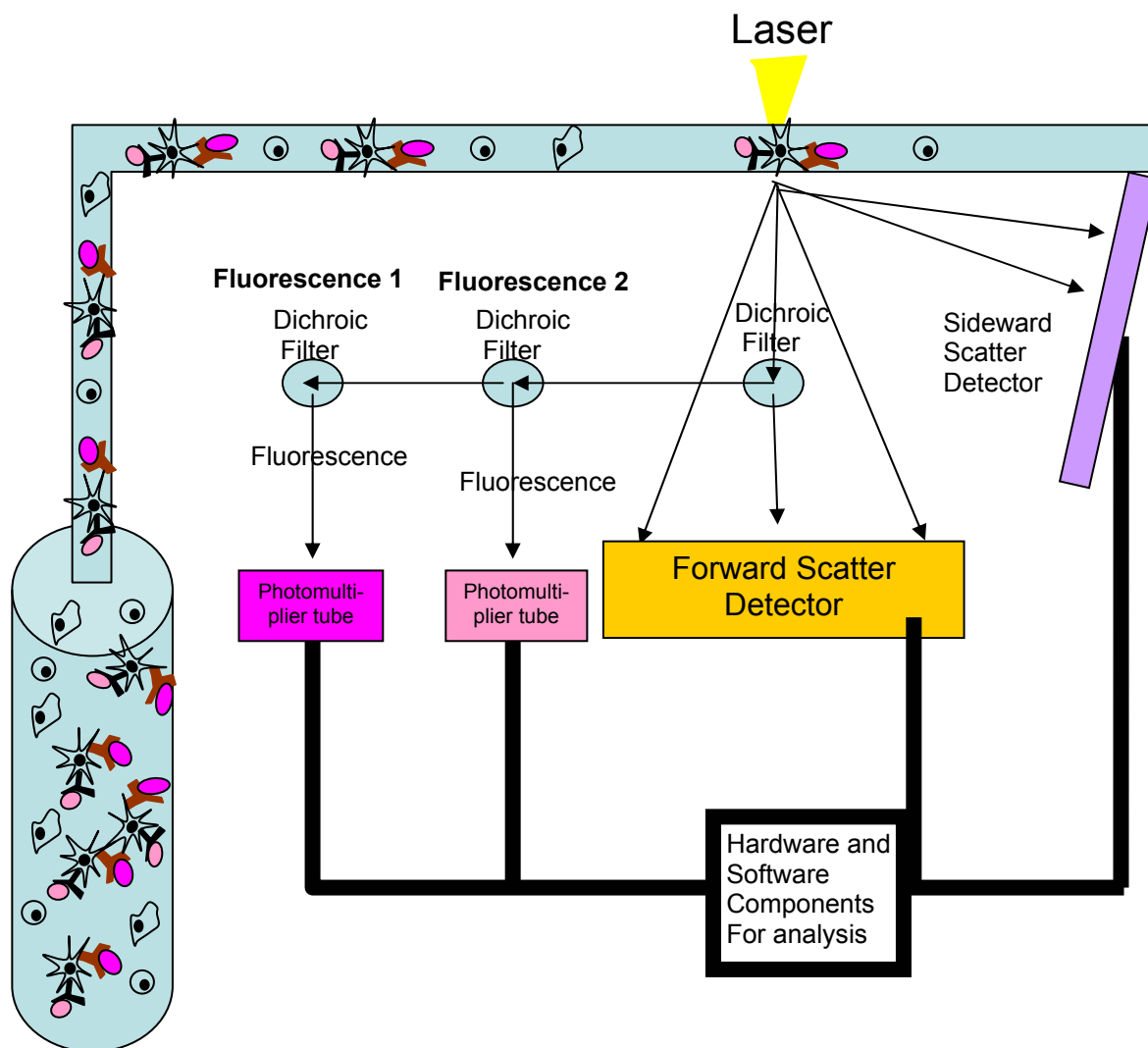
The sideward scatter intensity is measured in a 90° angle to the incident light and defines the granularity of the cells.The forward scatter intensity is measured via the axis of the incident light and defines the size/surface of the cells. The fluorescence intensities of each cell is proportional to the amount of bound fluorochrome molecules and is measured in a 90° angle to the in falling light.

2.2.7   ELISA (Enzyme Linked Immuno Sorbent Assay)

The principle of an Immunoassay is based on having a pure antigen whose quantity can be measured by an indicator molecule. The indicator molecule (an antibody) is covalently coupled to an enzyme (e.g. a horseragish peroxidase) and is quantified via spectrophotometer. The amount of antibody coupled enzyme bound to existing antigen is measured by the rate the enzyme converts a clear substrate to a coloured product. Several types of ELISA exist, but the most commonly used version is the sandwich assay (Figure 6).

Figure 6   Enzyme linked immuno sorbent assay
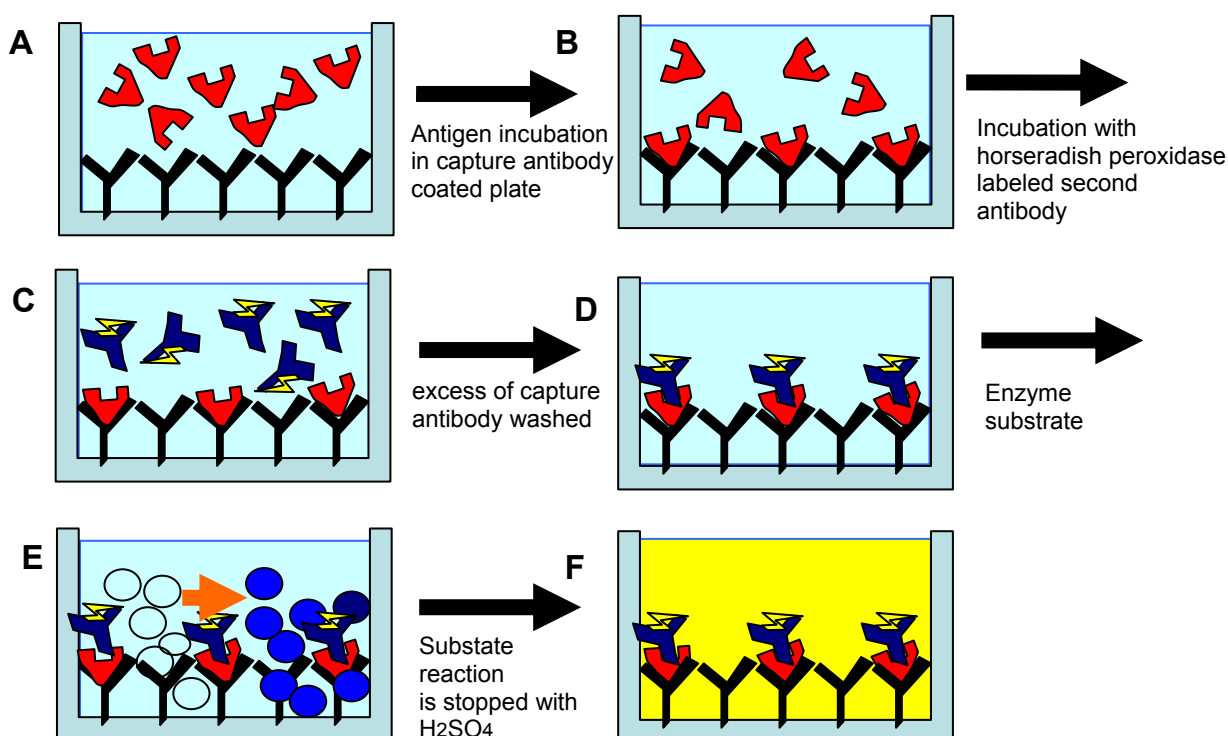
An antibody coated 96 well plate is incubated for a certain amount of time (usually over night) with the antigen to allow the binding to the 96 well plate (A and B). The plate is washed and a second antibody coupled to horseradish peroxidase is incubated to bind to an allosteric binding site of the antigen (C and D). The plate is washed again and the enzyme substrate is added to the 96 well plate (E). The substrate reaction is stopped with H_2SO_4. The pH-sensitive colour system changes the colour and the absorption is measured in a photometer (F).

The sandwich assay uses two different antibodies reacting with different epitopes on the antigen whose concentration needs to be determined. A fixed quantity of one antibody is attached to a 96 microtiter well plate. Test solutions containing antigen at an unknown concentration as well as a series of standard solutions with known concentrations are

added to the wells and are allowed to bind. Unbound antigen is removed by washing, and the second enzyme linked antibody is allowed to bind. The antigen serves as a bridge, so the more antigen is in the test or standard solutions, the more enzyme-linked antibody will bind. The results from the standard solutions are used to construct a binding calibration curve for second antibody as a function of antigen concentration, from which the quantities of antigen in the test solutions are quantified.

Nowadays a 96 well plate is coated with an antibody over night. The following day not bound antibody is washed away. The wells are filled with the sample fluids (sera, cell culture supernatants etc.) and incubated for a certain amount of time according to the manufacturers advice. After washing the sample fluids away, a second antibody conjugated to a horseradish peroxidase is transferred into the plates and incubated. Not bound antibody is washed away after incubation time. The detection solution is given to the cells and stopped after 30 minutes.

The amount of cytokine is optically quantified. All reagents are provided in the Elisa sets. The 96 well plates (Nunc F96 MAXISORP No 442404 Nunc-Immunoplate; Nunc A/S Kumpstrupvey 90 P.O. Box 280 DK-400 Roskilde, Denmark) have to be covered with the capture antibody over night before use.

2.2.8   Polymerase Chain Reaction and real time RT-PCR

The PCR is a rapid and simple method for copying and amplifying specific DNA sequences. Short regions of DNA on each end of the larger sequence that is to be copied have to be known. These short sequences are used to specify oligonucleotide primers. The method consists of repetitive cycles of DNA melting, DNA annealing and DNA synthesis. Double stranded DNA containing the sequence to be copied and amplified is mixed with a large molar excess of two single stranded DNA oligonucleotides as primers. The melting steps are used to divide the double stranded DNA, the DNA annealing step in order to bind primers and within the polymerase step the DNA synthesis takes place. Every time the amplification growth exponentially, because in every step the originals and the copies get re-amplified.

For amplification of RNA, the RNA transcripts of the IL-12 genes first had to be reconverted to a complementary DNA copy using the reverse transcriptase (RT). For relative quantification an adaption of this method, the so called RT-PCR is used.

Within the RT-PCR fluorescent probes are used to follow the accumulation of amplified product during each PCR cycle in contrast to the end point detection by conventional PCR methods.

In order to gain the IL-12 RNA isolated and purified monocytes are cultured for 6 days in DC-medium and cytokines as described in section 2.1.1 and 2.1.2. 500 nM Sanglifehrin (SFA) was added to the DC on day 6 of cultivation, 1 hour before stimulation with 1μg/ml LPS. In parallel, the control solution including DC medium and ethanol as drug vehicle was given. 6 hours after stimulation the cells were washed with PBS, centrifuged (1200 rpm, 6 minutes) and the supernatant was removed. The RNA of 5x10^6 cells of each group was isolated according to the manufacturers instructions (Qiagen Rneasy Mini Kit, Qia Shredder columns and RNAse free DNAse, Hilden, Germany). The isolated RNA was then transformed to cDNA according to the manufacturers instructions of Amersham Biosciences Ready-To-Go You-Prime First-Strand Beads and pd(N)$_6$ random hexamer 5`phosphate as primer. Real Time PCR was performed with Assay on Demand on an ABI-Prism 7000 Taqman according to the manufacturers instructions.

2.2.9   Endocytosis

Efficient Antigen internalization, either through receptor mediated endocytosis or fluid phase endocytosis (i.e. macropinocytosis) is a specific attribute of immature DC. Macropinocytosis represents an antigen uptake pathway allowing DC to rapidly and non-specifically sample large amounts of surrounding fluid. Receptor mediated endocytosis, in contrast, is initiated by the engagement of specific receptors, triggering a cascade of signal transduction that is required for actin polymerization and effective engulfment.

2.2.9.1   In vitro endocytosis

For the in vitro investigations the DC on day 6 of culture were washed, transferred into 37°C tempered DC Medium and incubated for 30, 60 or 90 minutes with 1 mg/ml FITC-Albumin or 10mg/ml FITC-Dextran. To stop endocytosis immediately after the indicated times all isolation and washing steps were performed with ice-cold 0.1% sodium azide/ 1% FCS/PBS. The cells were washed and centrifuged (1200 rpm, 6

minutes, 4°C) three times. Human cells were stained with CD1a mAb as described in section above and analysed immediately by flow cytometry.

2.2.9.2   In vivo endocytosis

After in vivo administration of SFA or vehicle ± Flt3L for ten days (for details see section above, the animals were either injected with 12.5 µg FITC Albumin or with 25 µg FITC Dextran i.p. (Sigma Aldrich Chemie GmbH, Steinheim, Germany). Four hours later the spleen cells were isolated as described above. Washing steps and quantification were carried out as described in the in vitro section above. Murine cells were stained for CD 11c (HL3) as described and analyzed immediately by flow cytometry.

2.2.10   Mixed Lymphocyte Reaction and detection of T-Cell Cytokines by ELISA

The response of alloreactive T-cells to foreign MHC molecules can be analysed via in vitro reaction called mixed lymphocyte reaction (MLR). The MLR is a model of direct T-cell recognition of allogeneic MHC molecules and is used as a predictive test of T-cell mediated allograft rejection. Studies of the MLR were among the first to establish the role of class I and class II MHC molecules in activating distinct populations of T-cells (CD8$^+$ and CD4$^+$, respectively) [157].

The MLR is induced by culturing mononuclear leucocytes (including T-cells, B-cells, NK cells) from different species. Mononuclear leukocytes (purified DC from mouse lymph nodes) from one individual with mononuclear leukocytes derived from another individual are mixed in order to induce proliferation. If the two individuals have differences in the alleles of the MHC genes, a large proportion of the mononuclear cells will proliferate during a period of 4 to 7 days, called allogeneic MLR. To be sure that the stimulator cells are incapable of proliferation, they were pretreated with γ-irradiation. In this one-way MLR, the treated cells serve exclusively as stimulators while the untreated cells still capable of proliferation serve as responders.

For stimulation of cytokine production, irradiated (IPF IRRADIATOR, Model OB29 STS-Buchler, Germany) lymphocytes (2x10^6/ml) or purified DC (2x10^5/ml) were added in 96-well plates in DC medium at a volume of 100 µl/well.

$2x10^6$/ml allogenetic T-cells (100ul/well) were added as responder cells and incubated for 36 h to take culture supernatants or for three days for MLR. Mouse IL-2, IL-4, IL-6 and IFN-γ were measured employing BD OptEIA™ ELISA Sets (BD Pharmingen, see section 2.1.6). MLR was measured with the Amersham Cell Proliferation Kit after a 16 h preincubation with 10 μM BrdU. The test was performed as described by the manufacturer`s instructions. The readout was done on the Elisa Reader (Plattenphotometer SLT Spectra Lab Instruments Deutschland GmbH) similar to cytokine Elisa's via optical density at a wavelength of 488 nm.

2.2.11   Adoptive transfer experiments

Adoptive DC transfer was performed in three different animal models:

i)   Transfer of purified, unpulsed DC into fully allogeneic recipients in order to investigate the direct immunostimulatory capacity (Figure 7 A);

ii)   Transfer of allo AG-pulsed DC into syngeneic animals to analyse indirect antigen presentation capacity (Figure 7 B),

iii)   Repeated injection of allo AG-pulsed DC into syngeneic animals to analyse boosting of T-cell activation (Figure 7 C).

To obtain highly-purified DC populations for adoptive transfer experiments, DC were labelled with magnetic bead-conjugated anti-CD1c$^+$ mAb (Miltenyi Biotec, Auburn, CA) followed by positive selection through paramagnetic columns (LS columns, Miltenyi Biotec) prior to injection in recipient animals. A DC purity of 90-95% was consistently achieved.

Figure 7   Adoptive transfer experiments
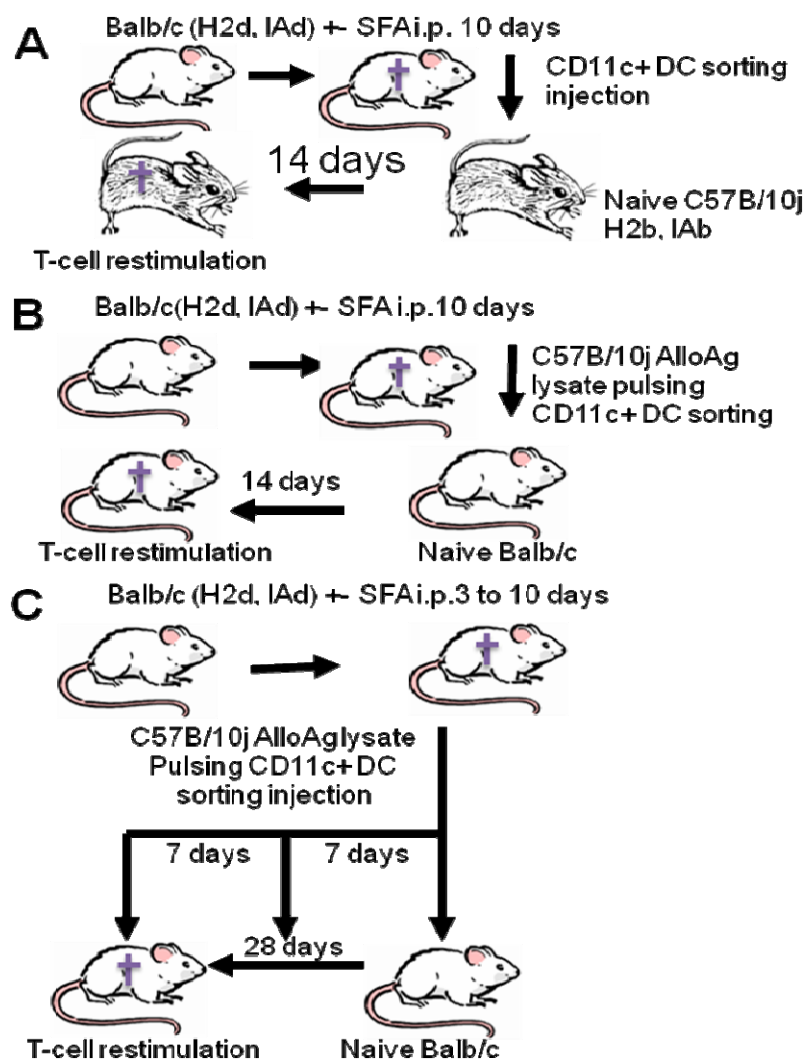
Adoptive transfer of freshly isolated, purified BALB/c (H2^d/IA^d) CD11c$^+$ DC from SFA (10 mg/kg/d; 10 d or drug vehicle-injected donor animals (+Flt3L) into naïve and non-immunosuppressed allogeneic C57BL/10 (H2^b/IA^b; A) or syngeneic recipients, BALB/c DC pulsed with C57BL/10 spleen lysate. In all experiments CD11c$^+$ DC were purified before injection into recipient animals by bead-sorting to >95% purity. Recipient T-cell proliferation (A, B, C) and cytokine production were analyzed after restimulation with donor splenocytes. In C, recipient animals were boosted with two additional injections of allo Ag-pulsed, bead purified CD11c$^+$ DC.

3.   RESULTS

3.1   SFA does not affect DC differentiation and phenotypical maturation

Analysis of GM-CSF/ IL-4 expanded human monocyte-derived DC that were cultured from day 2 in the presence of SFA (100 – 1000 nM) and harvested at day 6 showed no significant effects of SFA on DC differentiation as demonstrated by similar numbers of CD14$^-$ CD1a$^+$ immature DC (Figure 8 A). To induce DC maturation, immature cells were stimulated with LPS. SFA did not affect the upregulation of costimulatory molecules CD40, CD80, CD86 and HLA-DR (Figure 8 B), nor did it affect de novo expression of CD83 (Figure 8 B). Similar results were obtained after TNFα (1000 U/ml) stimulation of DC.

Figure 8   Effect of SFA on CD Markers of Dendritic Cells
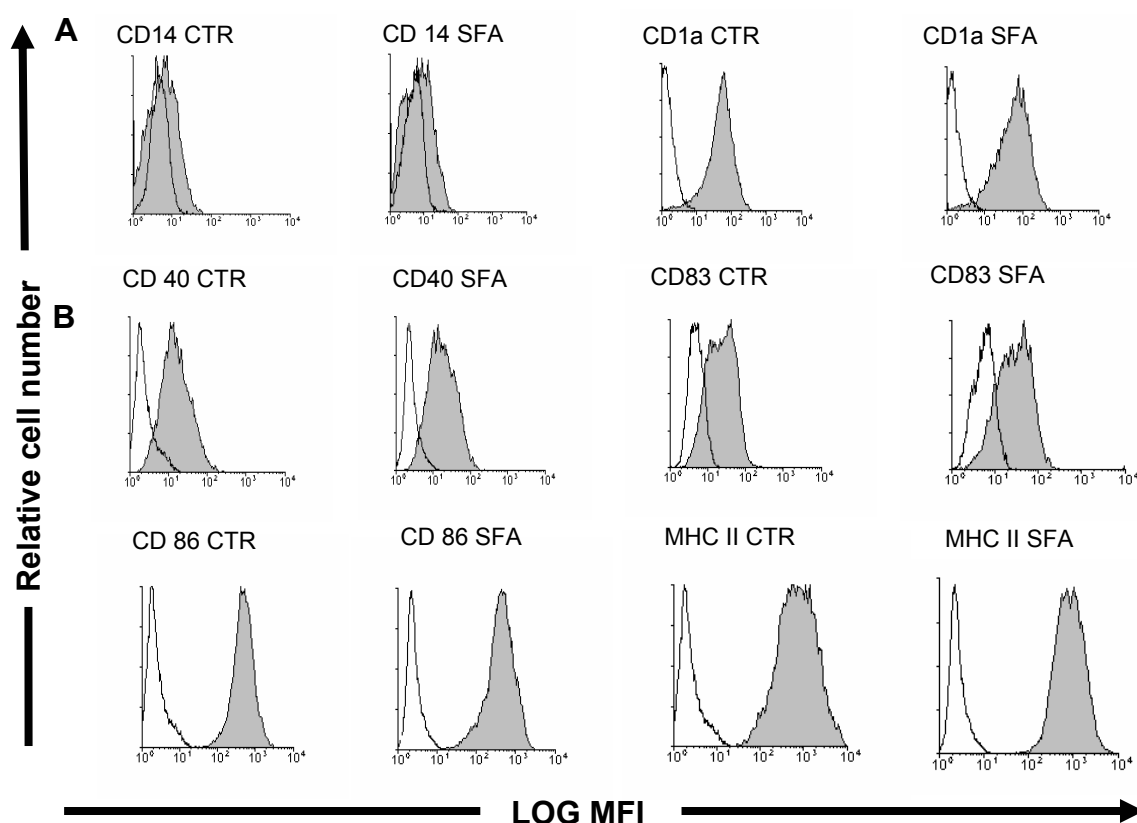
DC were generated in the presence of GM-CSF, IL-4 and SFA (500nM) was added on day 2. After 6 days, DC were harvested and analyzed for their CD14 and CD1a expression (A) or stimulated with LPS and subsequently analyzed for expression of CD40, CD80, CD83, and HLA-DR by flow cytometry (B). SFA does not affect DC differentiation (A) and phenotypical maturation (B) [158]. Specific staining is indicated by gray shaded histograms, and isotype control staining by open histograms. The results are representative of five separate experiments.

3.2   SFA blocks bioactive IL-12 production by human DC and its suppression of IL-12 production is stimulation independent

Addition of SFA to DC cultures on day 2 suppressed 80-90% of inducible IL-12p70 production after LPS stimulation compared to control DC (Figure 9 A). Since IL-12p70 production can be induced by many different cytokines and microbial agents, these findings were confirmed by a second mode of stimulation through TLR3 with Poly I:C and IFN-γ (Figure 9 A). TNF-α production was also suppressed, but to a lesser extent (Figure 9 B). To analyze whether these effects were related to apoptotic or necrotic cell death, we performed Annexin V / 7-AAD staining. In accordance with a recent publication, suggesting that SFA can even act as an inhibitor of cell death [159], a consistently low incidence of DC death in SFA-treated cultures was found in comparison to control DC: In SFA treated cultures, the mean incidence of apoptotic DC was 4.1± 1.1% (control DC 7.5± 1.6% n=3) and the mean incidence of necrotic DC was 0.9 ± 0.7% (control DC 1.6 ± 1%; n=3)(data not shown).

Figure 9   Effect of SFA on proinflammatory cytokines
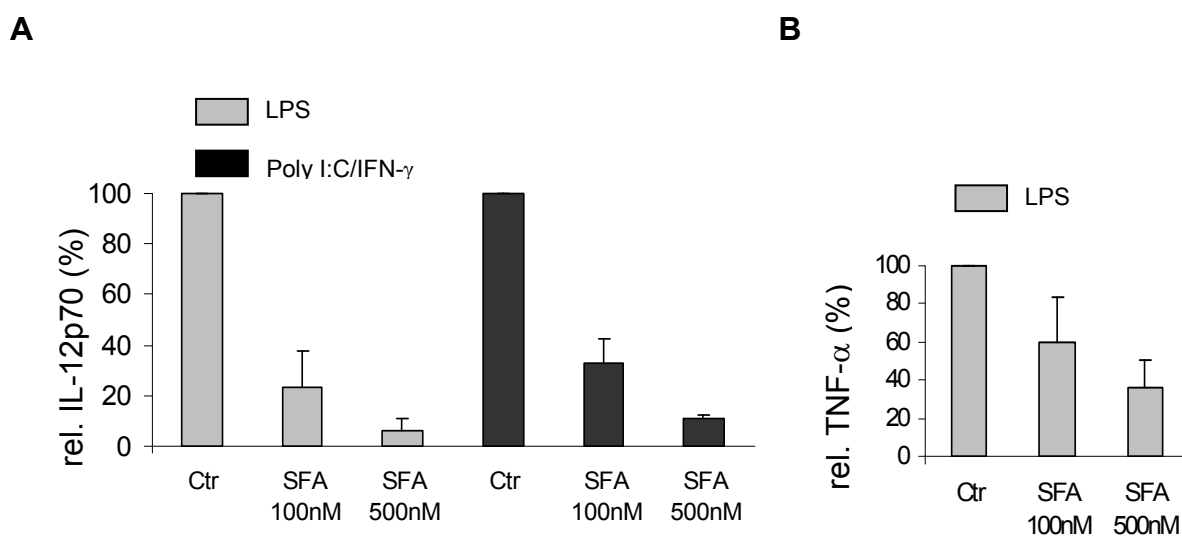
A   **B**

DC were generated in the presence of GM-CSF, IL-4 and SFA (500nM) was added on day 2. After 6 days, DC were harvested and stimulated with LPS to investgate cytokine production.
SFA blocks bioactive IL-12 production (A) and inhibits TNF-α production (B) [158]. Immature DC were stimulated on day 6 (LPS, Poly I:C/IFN-γ) and IL-12p70 production (A) and TNF-α (B) production were analyzed by ELISA 24 h later. The results are representative of seven (A) and three (B) separate experiments.

3.3   SFA acts rapidly on differentiated DC

In order to investigate whether SFA needs to be present during DC differentiation to block IL-12p70 production, SFA was added at different timepoints during DC generation (day 2-6). SFA blocked IL-12p70 production after LPS stimulation at all timepoints with similar potency (Figure 10 A) [158]. SFA blocked IL-12p70 production when added on day 6, 60 min before LPS stimulation (IC_{50} 108nm $\pm$ 45; Figure 10 B) [158]. Similar results were obtained after Poly I:C/ IFN-γ stimulation of DC (data not shown).

Figure 10   SFA acts rapidly on bioactive IL-12p70 even short before stimulation
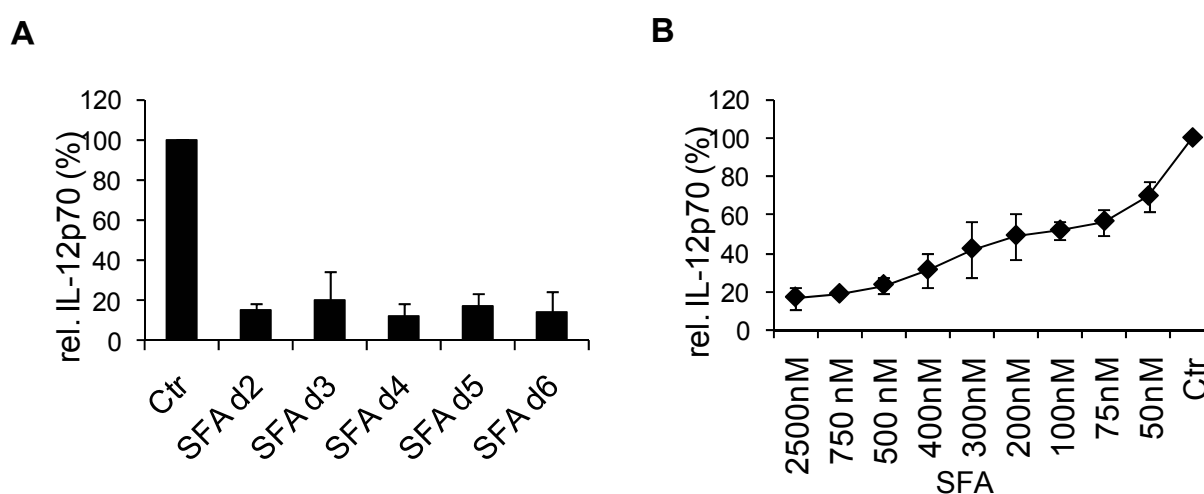
SFA acts rapidly on differentiated DC (A, B) and its suppressive effect on bioactive IL-12 production is present even if given to the culture shortly before stimulation.
DC were generated in the presence of GM-CSF and IL-4. Immature DC were stimulated on day 6 with LPS (A,B) and IL-12p70 production was analyzed 24 h later by ELISA. SFA (500nM) was added at different days during DC generation (A) or different SFA concentrations (50-2500 nM) were added late on day 6, 1 h before stimulation (B). The results are representative of three separate experiments[158].

3.4   SFA suppression of IL-12 production by human DC is unique in comparison to other immunophilin-binding immunosuppressants

The calcineurin-inhibitor CsA has been reported by different investigators to have a moderate or no suppressive effect on IL-12 production by DC [160, 161]. The mTOR inhibitor RAPA has been reported to inhibit DC IL-12 production if present for several days during DC differentiation either in vitro or in vivo [18, 137]. The effect of SFA on bioactive IL-12 production of differentiated DC should be investigated in comparison to the immunophilin-binding immunosuppressants CsA and RAPA under the same conditions. Neither RAPA nor CsA were able to inhibit significantly bioactive IL-12 production of DC when added shortly before the stimulation (A). In direct contrast to CsA and RAPA, SFA was the only immunophilin-binding immunosuppressant that strongly inhibited IL-12p70 production of differentiated DC (Figure 11 A) [158].

Both SFA and CsA bind to cyclophilin in whole cells with high affinity [129], but affected bioactive IL-12 production by DC in a different manner: SFA blocked IL-12 production whereas CsA did not inhibit IL-12 production [158] (Figure 11 A). To assess whether suppression of IL-12 production was dependent on binding of SFA to cyclophilin competitive experiments with SFA and a molar excess of CsA were performed. SFA blocked IL-12 production of DC in the presence of a 20-fold molar excess of CsA indicating that the activity of SFA was independent of cyclophilin-binding (Figure 11 B).

Figure 11   SFA acts uniquely on bioactive IL-12p70 compared to other immunophilin binding substances

SFA`s suppressive effect on bioactive IL-12 production is unique when compared directly to the immunophilin-binding immunosupressants CsA and Rapa (A). Blockade of IL-12 production by SFA is independe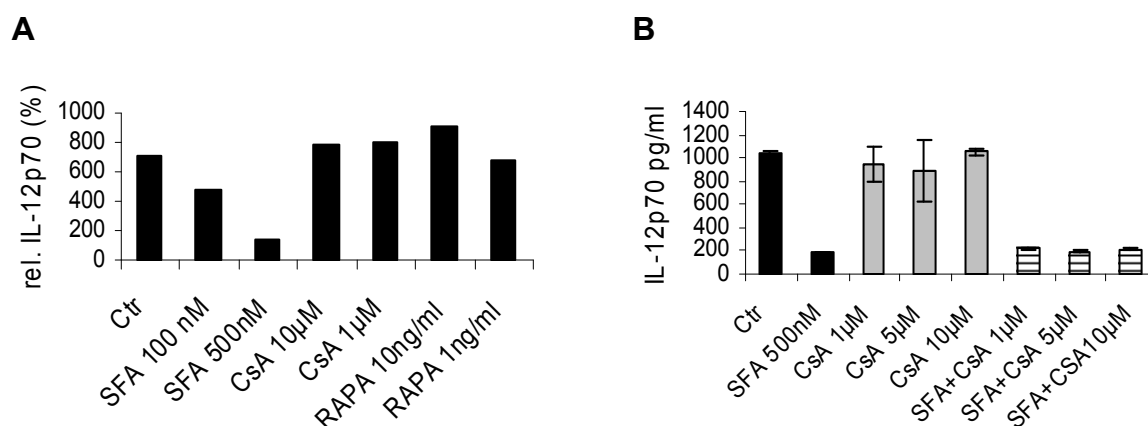nt of cyclophilin-binding (B). DC were generated in the presence of GM-CSF and IL-4. Immature DC were stimulated on day 6 with PolyI:C/ IFN-γ (A,B) and IL-12p70 production was analyzed 24 h later by ELISA. SFA, CsA and RAPA were added on day 6, 1 h before stimulation (C). In competitive experiments SFA alone, CsA alone, and SFA together with a molar excess of CsA were added 60 min before stimulation (D). The results are representative of three separate experiments[158].

3.5 Confirmation of SFA suppressive effect on bioactive IL-12 with sorted peripheral blood DC from healthy donors

Given the fact that monocyte-derived DC may display functional differences in comparison to preformed in-vivo generated DC [162] we questioned whether SFA was able to block IL-12 production of freshly isolated peripheral blood DC, purified from healthy blood donors. CD1c$^+$ (BDCA 1) is expressed on CD11c$^+$ CD123$^-$ dendritic cells and represents the major subset of myeloid DC in human peripheral blood [118, 159]. We purified CD1c$^+$ peripheral blood DC (purity >90%) from blood donors after depletion of B-cells by immunomagnetic cell sorting [159]. DC were treated with SFA and stimulated with either LPS or Poly I:C/ IFN-γ. SFA-treated blood DC exhibited a strong decrease of bioactive IL-12 production suggesting that SFA was effective on freshly isolated peripheral blood DC (Figure 12).

Figure 12   Confirmation of SFA suppressive effect on bioactive IL-12 with purified peripheral blood DC
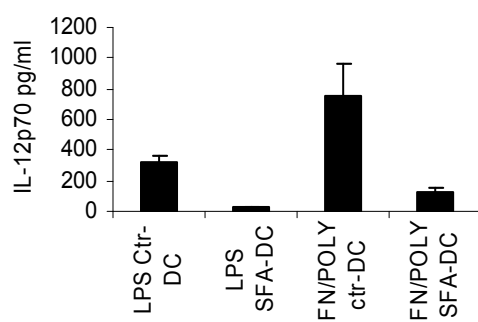
CD1c$^+$ blood DC were purified freshly from healthy blood donors by immunomagnetic sorting, incubated with SFA (500 nM) and stimulated with LPS or Poly I:C/ IFN-γ. IL-12p70 production was analyzed 24 h later by ELISA. The results are representative of three separate experiments [158].

3.6   SFA blocks IL-12 expression by human DC on the transcriptional level

The calcineurin-inhibitors CsA and FK506 exert potent immunosuppressive effects on the transcriptional level via inhibition of NFAT-induced cytokine gene expression [163] whereas mTOR inhibition by RAPA results in inhibition of translation initiation and cell cycle arrest [128]. To investigate whether SFA inhibited IL-12 production by DC on the transcriptional or translational level, we performed real-time RT-PCR and quantified IL-12p35 and IL-12p40 mRNA expression. The results revealed a strong suppression of IL-12p35 and p40 mRNA expression, whereas expression of two independent housekeeping genes 18sRNA and GAPDH was not affected (Figure 13 A-D).

Relative quantification of IL-12p35 and p40 transcripts in relation to diluted standards indicated $\geq$ 90% inhibition in the presence of 500nM SFA (Figure 13 F). Recently, IL-23 a novel member of the IL-12 cytokine family has been discovered. IL-23 is a heterodimer, comprising IL-12p40 and the IL-23-specific p19 subunit [164]. Short-term pre-treatment of DC with SFA (1h) was found to block 84% of p19 transcription (Figure 13 E, F).

Figure 13   SFA blocks IL-12 expression by human DC on the transcriptional level
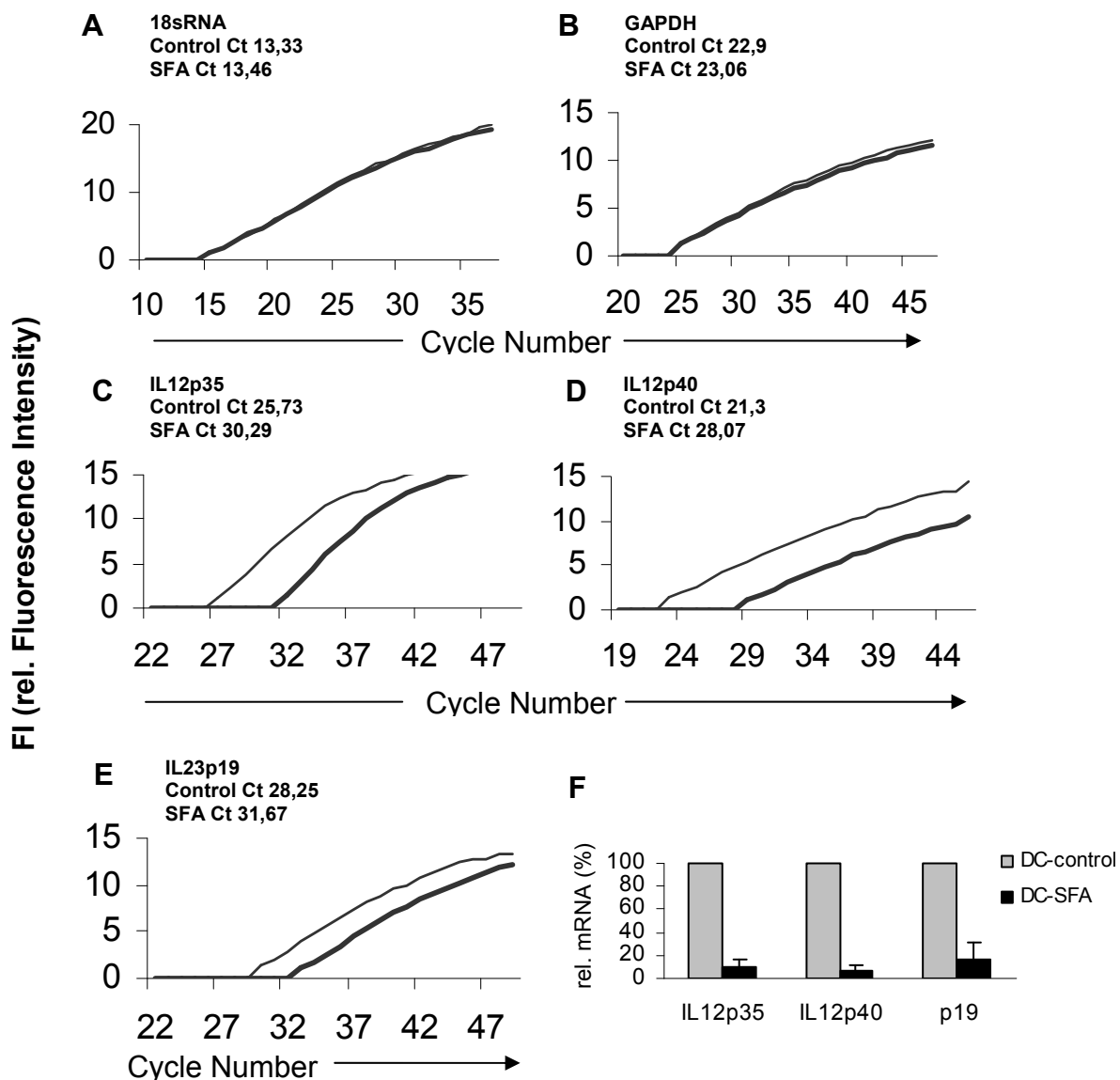
DC were generated in the presence of GM-CSF, IL-4 and SFA (500nM) was added after 6 days, 1 h before stimulation with LPS. 6 h later RNA was isolated and quantitative RT-PCR was performed as described in 2.2.8. Threshold cycle (Ct) values for the endogenous controls 18s RNA (A), GAPDH (B) and the specific IL-12p35 (C), IL-12p40 (D) and the IL-23-specific p19 (E) transcripts are shown. Relative mRNA quantification for IL-12p35, IL-12p40 and IL-23 specific p19 after normalization to the endogenous control GAPDH (F). The results are representative of five separate experiments [158].

3.7   SFA abrogates bioactive IL-12 production in vivo

IL-12 is a heterodimeric pro-inflammatory cytokine that activates Th1, cytotoxic T-cells and NK cells, bridging innate and adaptive immunity. DC represent the major producers of this cytokine in the immune system and several reports demonstrate that bioactive IL-12 production is a critical signal transforming putative immunoregulatory DC into immunogenic DC [74]. To investigate the maximal capacity of SFA inhibiting bioactive IL-12 production in vivo, we expanded DC with Flt3L, and injected the animals with SFA 10 mg/kg/d or drug vehicle for 10 days. In the first model, using a protocol described by Hochrein et al. [165], massive in vivo IL-12p70 production (> 1000 pg/ml plasma) was stimulated by combined LPS (10 μg) and IL-4 (0.5 μg) injection on day 10 (Figure 14 C). SFA-treatment resulted in 93.6% inhibition of in vivo IL-12 production compared to vehicle-injected control animals (Fig. 14 C $p<0.0002$). In contrast, TNF-α and IL-10 production were not affected significantly by SFA administration (Figure 14 A,B) excluding the possibility that the profound IL-12 inhibition was related solely to a non-specific or general toxic effect. To confirm these findings we used a different IL-12 inductor (TLR was applied), we injected animals with the Toll-like receptor (TLR) 9 ligand CpG ODN and measured in vivo IL-12 production. Again, CpG ODN injection resulted in massive in vivo IL-12 production (>600 pg/ml plasma) while SFA treatment suppressed IL-12p70 production by 92.8% compared to vehicle-injected controls (Figure 14 D; $p{\leq}0.05$); TNF-α and IL-10 levels were not affected (Figure 14 E and F).

Figure 14   In vivo administration of SFA abrogates systemic IL-12p70 production
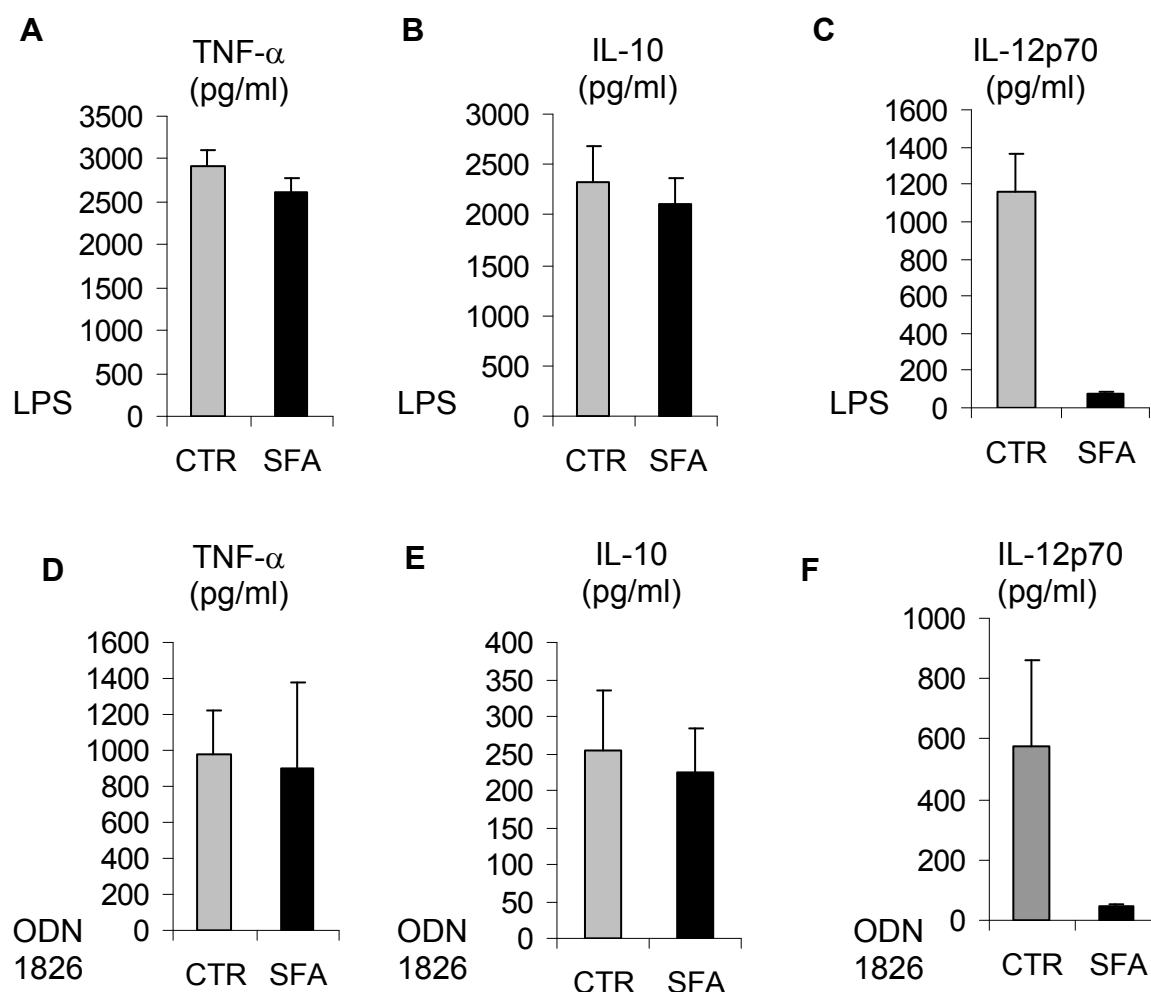
Mice were injected with SFA (10 mg/kg/d) or vehicle (plus Flt3L, 10 days). On day 10, systemic cytokine production was induced after injection of LPS (10µg) and IL-4 (0.5 µg). LPS/IL-4 injection resulted in massive systemic IL-12p70 (>1000 pg/ml), TNF-α (>2000 pg/ml) and IL-10 (>2000 pg/ml) production in drug-vehicle injected control animals (A-F). In contrast, in SFA-injected animals, >92% of systemic IL-12p70 production (A) was abrogated, whereas TNF-α. (B) and IL-10 (C) were not affected. In control experiments, similar results were obtained after injection of the TLR-9 ligand CpG ODN 1826. Again, SFA exposure of animals resulted in >90% inhibition of systemic IL-12p70 production (D) whereas TNF-α (E) and IL-10 (F) were not affected. Results are representative of 3-6 animals/treatment group, means ± SD; **p=0.0002; *p<0.05, 2-tailed Student's t test) [166].

3.8   SFA does not influence the in vivo expansion and phenotypic Maturation of DC subsets

With respect to the massive inhibition of bioactive IL-12 production, we questioned whether this effect was related to interference by SFA with DC expansion in vivo. RAPA, a related immunophilin-binding immunosuppressant, has been reported recently to suppress the expansion of DC in vivo [138]. Analysis of total leukocyte numbers and CD11c^{+} DC in spleen and bone marrow of normal and Flt3L-treated animals did not indicate a significant suppressive effect of SFA on total DC expansion in vivo (Figure 15 A-E).

Figure 15   SFA exerts no major inhibitory effects on total DC populations in vivo
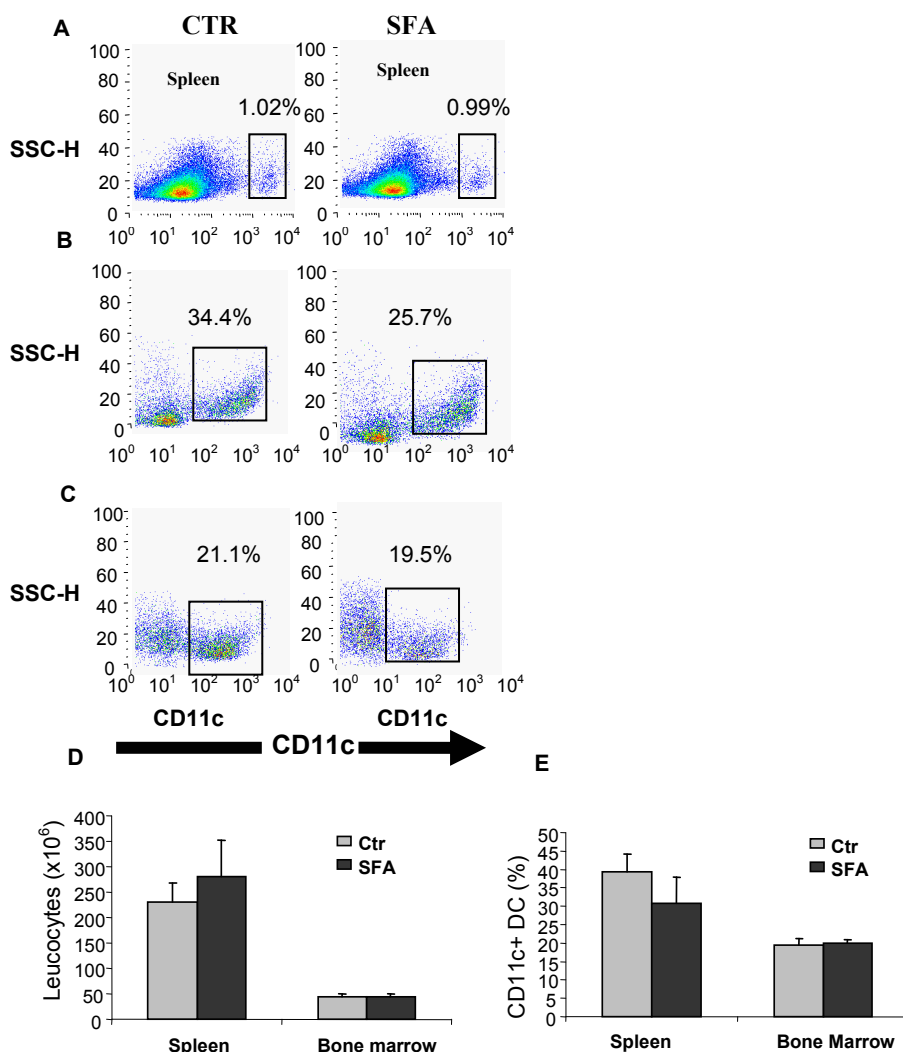
Bulk DC and the three major DC subsets (myeloid DC, plasmacytoid DC, lymphoid related DC) were enumerated in different tissue compartments of SFA-injected (10 mg/kg i.p; 10 d) and drug vehicle-injected control (CTR) mice under steady-state (without Flt3L) and dynamic (+10 μg/d Flt3L i.p., 10 d) conditions by flow cytometry. Under steady state (A) and dynamic conditions (B, C, E) CD 11 c$^+$ DC numbers did not differ significantly between SFA- and vehicle-injected controls. SFA also did not significantly influence absolute leukocyte numbers in either the bone marrow or spleen of Flt3L-injected animals (D) [166].

Furthermore, detailed analysis of plasmacytoid, myeloid and lymphoid-related DC subsets in bone marrow and spleen also did not indicate a significant suppressive effect of SFA on DC subset development in vivo (Figure 16 A-D). Additionally, analysis of surface expression of CD40, CD80, CD86, and MHC class II of in vivo-expanded splenic and bone marrow CD11c$^+$ DC after LPS stimulation did not indicate a significant effect of SFA on phenotypic DC maturation (data not shown). These findings were confirmed with CD11c$^+$ DC stimulated with allogeneic cell lysates (data not shown). The results indicate that SFA does not suppress the differentiation, expansion or phenotypic maturation of DC in vivo.

Figure 16   SFA exerts no major inhibitory effects on DC subpopulations

A

B

CD11b

C

D
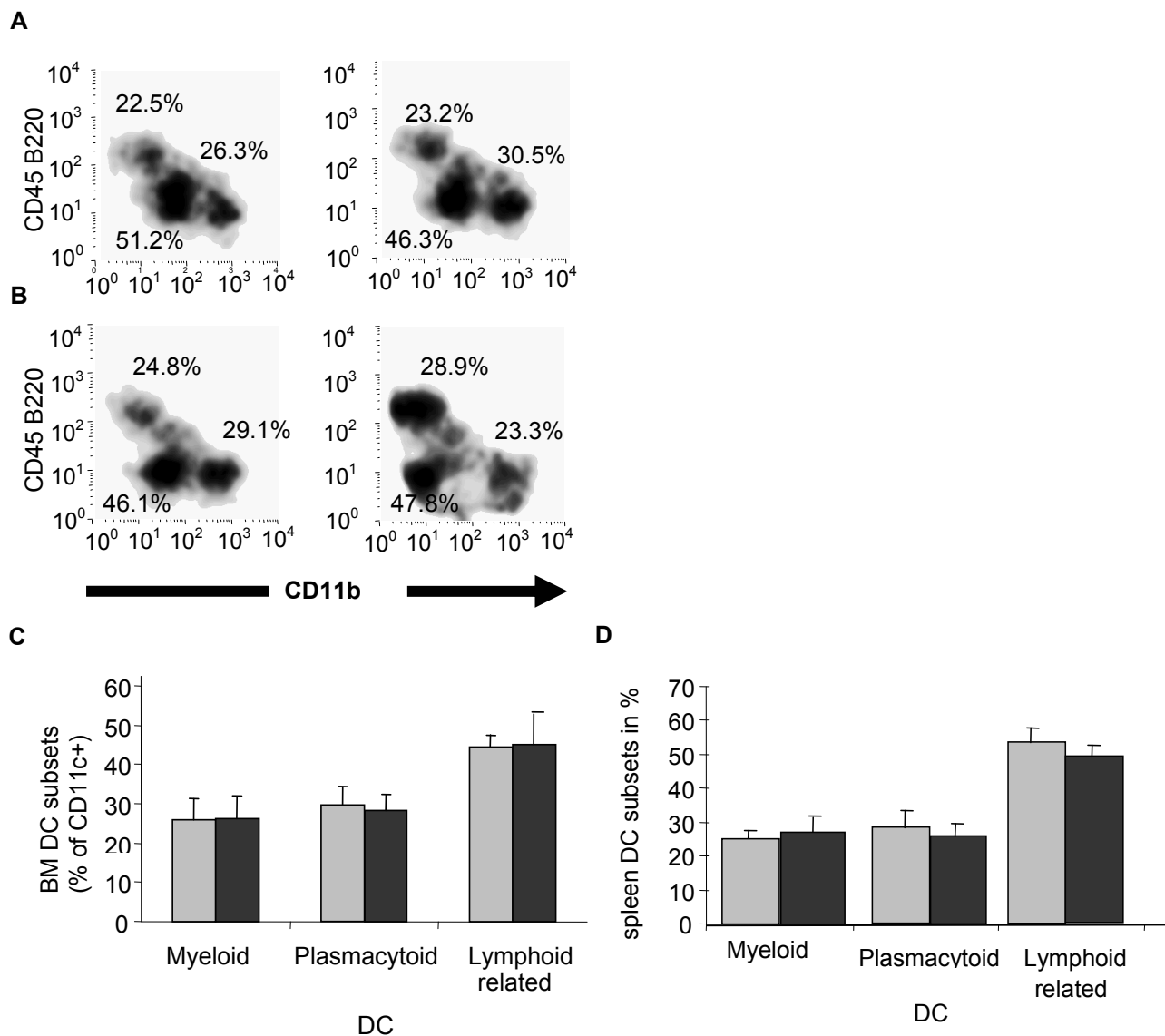
For details of generating in vivo DC see 2.2.4. The three major DC subsets were differentiated by analysis of surface expression of CD45b220 and CD11b after gating on $CD11c^+$ cells in Flt3L-injected animals. Plasmacytoid DC are CD11c+, $CD45b220^{bright}$, CD11b; lymphoid-related DC are $CD11c^+$, $CD45b220^{dim}$, $CD11b^{dim}$ and myeloid DC are $CD11c^+$, $CD45b220^{dim}$, $CD11b^{bright}$. SFA did not significantly influence the in vivo expansion of plasmacytoid, lymphoid-related or myeloid DC in spleen (A, D) and bone marrow (B, C) in comparison to age- and gender-matched control mice (vehicle-injected). Results are representative of 6 to 10 animals/treatment group [166].

3.9   SFA suppresses DC receptor-mediated endocytosis and DC macropinocytosis in vivo

A critical prerequisite for DC to function as sentinels of the immune system is efficient endocytosis. Two major mechanisms for efficient antigen capture by DC are fluid phase uptake through macropinocytosis and receptor-mediated endocytosis [46]. Recently, Woltmann et al. [167] reported that SFA inhibits expression of endocytosis receptors and endocytotic activity of human monocyte-derived DC in vitro. To assess the potential relevance of SFA as a novel DC endocytosis inhibitor we investigated DC receptor-mediated antigen uptake in vivo using FITC-Dextran, and DC fluid phase uptake by employing FITC Albumin in SFA-injected animals. After 10 day exposure to SFA, animals were injected with either FITC-Albumin or FITC-Dextran and FITC-uptake was quantitated in $CD11c^{+}$ DC by flow cytometry (Figure 17). The results revealed that SFA significantly impaired in vivo macropinocytosis of FITC-Albumin ($p=0.006$; Figure 17 C, D) and receptor-mediated endocytosis of FITC-Dextran ($p=0.0001$; Figure 17 A, B). To date, the related immunophilin-binding immunosuppressant RAPA has been reported to significantly impair endocytosis by murine and human DC [137, 139]. RAPA has been reported to inhibit 30% macropinocytosis (in terms of mean fluorescence intensity reduction) of in vivo expanded DC [168]. In contrast, our present data indicate that SFA inhibits >80% of DC macropinocytosis in vivo.

**Figure 17   SFA profoundly inhibits DC receptor mediated endocytosis and DC
                   macropinocytosis in vivo**

A

B

C

D
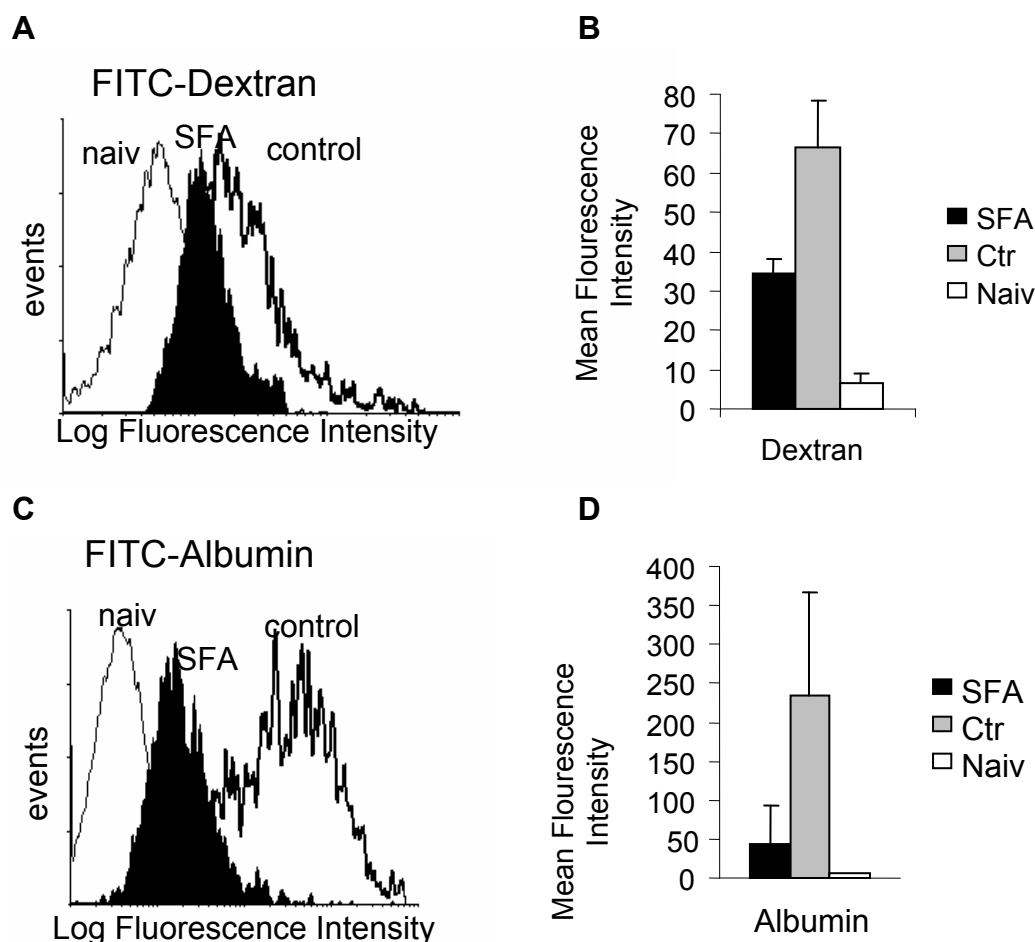
Mice were treated with SFA (10 mg/kg/d) or vehicle (CTR) plus Flt3L. On day 10, they were injected i.p. with FITC-Dextran (A, B), to analyze in vivo receptor-mediated endocytosis or with FITC-Albumin (C, D) to analyze macropinocytosis in vivo. Four hours later, spleen cells were prepared as described in Materials and Methods, DC stained with CD11c mAb were analyzed by flow cytometry. Negative controls included animals not injected with FITC to determine background fluorescence. In contrast to vehicle-injected control animals, SFA suppressed both receptor-mediated endocytosis (A, B; p=0.0001, mean ± SD; 2-tailed Student's t test) and macropinocytosis (C, D; mean ± SD; p=0.006, 2-tailed student´s t test) of CD 11c[+] DC in vivo. In quantitative terms, SFA suppressed 52% of DC receptor mediated endocytosis and 81% of DC macropinocytosis in vivo. The results are representative of 6 animals/ treatment group [166].

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3.10   SFA inhibits indirect antigen presentation of DC in vivo

To address the impact of SFA specifically on DC T-cell stimulatory capacity in vivo, we performed adoptive transfer experiments of sorted CD11c$^+$ DC from SFA-exposed donor animals into naive, non-immunosuppressed recipients (Figure 18). These experiments allowed us to precisely quantitate the impact of SFA on DC antigen presenting capacity without interfering with SFAs effects on other cells. Adoptive DC transfer was performed in three different animal models: i) transfer of purified, unpulsed DC into fully allogeneic recipients to investigate direct immunostimulatory capacity; ii) transfer of allo-AG-pulsed DC into syngeneic animals to analyse indirect antigen presentation capacity, and iii) repeated injection of allo-AG-pulsed DC into syngeneic animals to analyse boostering of T-cell activation. In all experiments, CD11c$^+$ DC were purified before injection by bead-sorting to >95% purity and T-cell activation was analysed after restimulation ex vivo with donor splenocytes.

When SFA-exposed, sorted BALB/c CD11c$^+$ DC were injected into fully allogeneic C57BL/10 animals, only a moderate decrease in T-cell proliferation was detected after restimulation in comparison to controls (Figure 18 A). In contrast, naive non-immunosuppressed recipients of syngeneic SFA-exposed DC pulsed with allogeneic C57BL/10 cell lysate showed markedly reduced T-cell proliferation after restimulation with C57BL/10 splenocytes (Figure 18 B; p<0.0001 at all stimulator/responder ratios). Moreover, when we boosted T-cell activation in vivo with two additional injections of syngeneic DC pulsed with allogeneic C57BL/10 cell lysate, recipients of SFA-exposed DC showed a stronger decrease in T-cell proliferation in comparison to recipients of control DC (Figure 18 C; p<0.0001 at all stimulator/responder ratios). The analysis of cytokine production by T-cells from latter animals revealed significantly reduced production of IL-2, IL-4 and IFN-y, p<0.0001; Data not shown) [166] . Taken together, these DC adoptive transfer experiments indicate that SFA profoundly impairs indirect antigen presentation and T-cell priming capacity of DC in vivo.

Figure 18   SFA inhibits indirect antigen presentation of CD11c⁺ DC in vivo
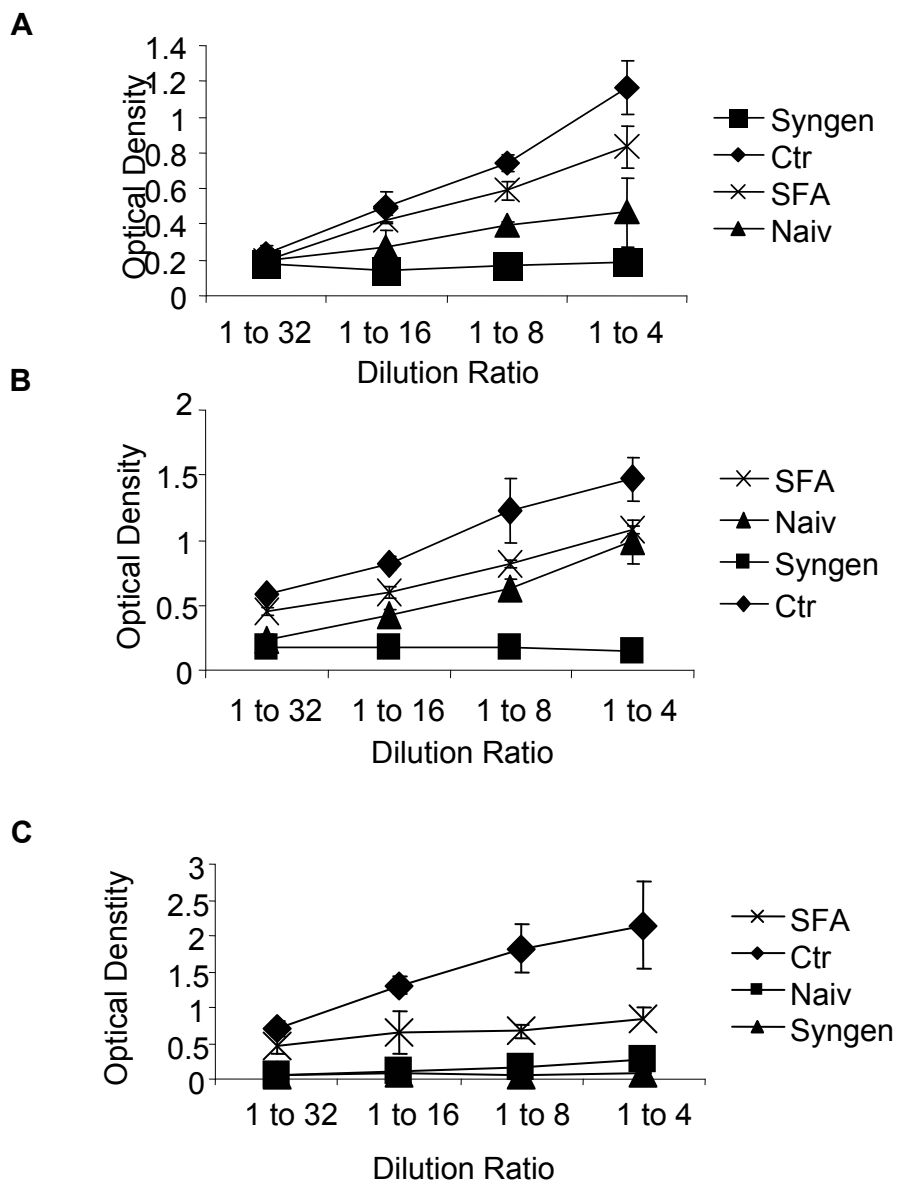
Adoptive transfer of freshly-isolated, purified BALB/c ($H2^d/IA^d$) CD 11 c+ DC from SFA (10 mg/kg/d; 10d) or drug vehicle-injected donor animals (+ Flt3L) into naive and non immunosuppressed allogeneic C57BL/10 ($H2^b/IA^b$; A; p=0.006) or syngeneic BALB/c (B, p<0.0001; C: p<0.0001) recipients. For adoptive transfer into syngeneic recipients, BALB/c DC were pulsed with C57BL/10 spleen lysate, as described in 2.2.11. In all experiments, CD11c⁺ DC were purified before injection into recipient animals by bead-sorting to >95% purity. Recipient T-cell proliferation (A, B, C) were analyzed after restimulation with donor splenocytes, as described in Materials and Methods. In C recipient animals were boosted with two additional injections of allo-AG-pulsed, bead-purified CD11c⁺ DC. Additional controls included primary T-cell proliferation and cytokine production by naive non-immunized animals and syngeneic controls (A-C). Results are representative of 6 (A, B) to 9 animals (C) per treatment group (mean ± SD; 2-tailed Student's t test) [166].

4.   DISCUSSION

Sangelifehrins, first described by Sanglier et. al [125, 126] produced by the acinomycetes strain Streptomyces A92-308110 belong to the group of immunophilin binding immunosuppressants with a yet unknown mechanism of action.

The first immunophilin binding substance introduced into therapy was Cyclosporin A (CsA) [114]. CsA binds to cyclophilin and inhibits activity of the calcineurin phosphatase, whereas both FK506 and RAPA bind to the FK506 binding protein but inhibit two different effector proteins: calcineurin phosphatase and mammalian target of Rapamycin (mTOR), respectively [127, 128].

Although SFA, like CsA, binds with high affinity to cyclophilin but unlike the latter, SFA does not inhibit calcineurin phosphatase activity [129]. SFA has been reported to exhibit lower immunosuppressive activity in MLR when compared with CsA [126, 129].

Studies of the immunosuppressive effects of SFA have been focused on T and B-lymphocytes [129, 130]. Thus, SFA inhibits IL-2 induced T-cell proliferation and mitogen-induced B-cell proliferation [129, 130]. Similar to RAPA, SFA has been shown to inhibit cell cycle progression. SFA was suggested to be related to NF-κB-dependent activation of p53 [130]. Unlike the calcineurin inhibitors CsA and FK506, SFA does not suppress IL-2 transcription [129, 130]. Taken together these data suggest that SFA exerts immunosuppressive effects on T and B-cells via a novel mode of action that partially resembles the effects of RAPA. There is increasing evidence, that immunophilin-binding agents such as RAPA exert immunosuppressive effects at the level of the APC. Recently it was reported that RAPA impairs antigen uptake of murine dendritic cells [169], a finding that was subsequently confirmed in human DC [170]. Moreover, it was suggested that RAPA promotes in vitro apoptosis of human DC by interfering with GM-CSF signalling [136] and suppresses in vivo DC development and function [171].

As SFA apparently has no major impact on T-Lymphocytes [129, 130], the aim of this work was to investigate if SFA, like other immunophilins i.e. RAPA has an impact on DC. Many immunosuppressive drugs like Rapamycin have a major impact on DC differentiation and maturation, by inhibiting the upregulation of expression of DC co-stimulatory molecules (CD40, CD80, CD86 and MHC class II) [170-178].

It is important that Rapamycin has to be added latest by 5 of a 7 day culture to show a major effect. Our first experiments were performed to evaluate the effective doses of SFA. We used SFA concentrations beginning at 50 nanoMol exceeding to 2 µMol that were given 24h after the start to the culture. No significant changes in either differentiation (CD14 or CD1a) or maturation (CD40, CD83, CD 86 or HLR-DR) could be found [158]. For RAPA at therapeutic ranges [179] MHC class II was 57% decreased, CD 86 was decreased 50% compared to the control group. The inhibition of differentiation and maturation was increased the earlier the substance was given to the culture. As no impact on differentiation as described for RAPA could be determined the biological effect/mechanism of SFA seems to be different. In contrast to RAPA other immunophilins like for CsA and Tacrolimus moderate [180] or no effects [155, 174, 181, 182] on DC differentiation or maturation have been described.

DC represent the major producers of bioactive IL-12p70 in the immune system. It should be noted that Interleukin-12 (IL-12) is a heterodimeric cytokine, because only the heterodimer is bioactive. IL-12p70 induces the production of IFN-γ, favours the differentiation of T-helper 1 (Th1) cells and forms a link between innate resistance and adaptive immunity. Dendritic cells and phagocytes produce IL-12 in response to pathogens during infection [183]. Giving the importance of bioactive IL-12 in inflammation and response to alloantigens or cancer antigens [184] we started to perform experiments to evaluate the impact of SFA on IL-12 production of DC. We used for all our in vivo and in vitro experiments monoclonal antibodies to detect bioactive IL-12p70, which consists of the subunits IL-12p40 and IL-12p35. Not every stimulus or every inhibitory drug leads to a production of both subunits, and only existence of both together leads to bioactive proinflammatory IL-12. While DC mature, they start to secrete cytokines like TNF-α and IL-12p70. When the maturation is inhibited, it is very likely that the amount of produced cytokines is reduced. Sanglifehrin A in contrast to Rapamycin does not inhibit phenotypical maturation as discussed above but massively inhibits the production of IL-12p70 but not TNF-α or IL-10 [158]. GM-CSF/IL-4 expanded human monocyte derived DC cultured from day 2 in the presence of SFA (100 nM to 1000 nM) suppressed 80-90% of inducible IL-12p70 production after LPS stimulation (or Toll-like receptor 4 ligation) when compared with control DC [158]. In these

experimental setups we saw a maximum effect with 500 nM and therefore decided to perform all following experiments with this concentration. Because IL-12p70 production can be induced or inhibited by many different cytokines and microbial agents [74, 81, 184-195], we confirmed these findings by a second mode of stimulation through TLR3 with poly I:C and IFN-γ. This path of stimulation leads to the same results like LPS stimulation. The produced amounts of IL-12p70 and TNF-α were much higher with the TLR3 stimulus, but the relative inhibition of IL-12p70 again was over 90% compared to the control [158]. This finding was interestingly enough to question if this effect was selectively for IL-12p70 or if other inflammatory cytokines like TNF-α or immunoregulatory cytokines like IL-10 were also affected. For Rapamycin, due to the inhibitory effect on differentiation and maturation, the massive decrease of proinflammatory cytokines was logical [138]. To our own surprise, SFA does not inhibit maturation or TNF-α or IL-10 production [158], it selectively inhibits IL-12p70.

To exclude the possibility that the effects were related to apoptotic or necrotic cell death, we performed annexin V/7-AAD staining, a vital dye that intercalates with the core DNA of dead cells. In accordance with a recent publication suggesting that SFA can even act as an inhibitor of cell death [159], we consistently found a low incidence of DC death in SFA treated cultures [158]. These findings exclude the possibility, that the effect of SFA is a toxic effect. In SFA treated cultures, the mean incidence of apoptotic DC was significantly lower than in the control group. It may be of special interest that part of the inhibitory functions of RAPA on DC might originate from an increased apoptosis [196] which is controversially discussed and might also be untrue [138].

These results showed us, that SFA acts different compared to cyclosporine, tacrolimus or RAPA. SFA on the one hand does not have an effect on differentiation or maturation like Rapamycin. But unlike Rapamycin, SFA does not inhibit maturation but has very selectively a major impact on the production of proinflammatory IL-12p70 production of DC, while other proinflammatory or immunoregulatory cytokines like TNF-α or IL-10 remain unaffected. To our knowledge no other immunosuppressive substance is known that has a selective effect like SFA only on the amount of produced proinflammatory IL-12p70.

Our next question to solve was, whether this effect is only taking place very early on cells before differentiation or if differentiated cells are equally affected. For it, we added SFA at different time points during DC generation (days 2-6). SFA blocked IL-12p70 production after LPS stimulation at all time points with similar potency. SFA even blocked IL-12p70 production potently when added as late as day 6, 60 min before stimulation, suggesting that it was acting rapidly on differentiated DC (IC$_{50}$ 108+- 45nm) [158]. This finding differs to the findings in cells that were treated with Rapamycin, where the cytokine inhibition correlates with the inhibition of maturation. The reason for this effect is the reduced differentiation, when given at an early time point to the culture [138]. To make sure that this early and strong inhibition takes place stimulus independent we performed the same experiments with poly I:C / IFN-γ stimulation of DC and had similar results [158]. This is especially important because the alloantigen of the transplanted organ as stimulus should equally lead to a reduced inflammatory answer under influence of SFA in order to prevent transplant rejection.

In a next step we compared the uniqueness of our inhibitory effect on IL-12p70 production to other immunophilin binding immunosuppressives, in our case Rapamycin and Cyclosporin. We wanted to exclude the possibility, that CsA and RAPA may lead to similar results, solely based on an artefact in our experimental setup. The calcineurin inhibitor CsA has been reported by different investigators to have a moderate or no suppressive effect on IL-12 production by DC [160, 161]. The mTOR inhibitor RAPA has been shown to inhibit DC IL-12 production if present for several days during DC differentiation, either in vivo or in vitro [136, 197]. In our experiments neither RAPA nor CsA were able to inhibit bioactive IL-12 production by DC significantly when added shortly before stimulation [158]. In direct contrast to CsA and RAPA, SFA was the only immunophilin binding immunosuppressant that strongly and immediately inhibits IL-12p70 production of differentiated DC [158].

Based on the already published findings that SFA and CsA bind to cyclophilins [125, 198] we wanted to perform an experiment that would solve the question, if the cyclophilin binding of SFA is necessary for blockade of IL-12 production. We assumed a different pathway or an allosteric mode of action must take place, because CsA has no effect on IL-12 production [160, 161] compared to SFA. To assess whether suppression

of IL-12 production was dependent on binding of SFA to cyclophilin at the same or nearby at an allosteric site of CsA, we performed competitive experiments with SFA and a molar excess of CsA. We even gave the molar excesses of CsA up to four hours before SFA to the culture to give CsA enough time to bind to the cyclophilins. With this experimental setup we assured, that SFA could not displace CsA from its cyclophilin binding site due to faster and more fierce affinity to the receptor. We found that SFA still blocks IL-12 production of DC even in the presence of a 20-fold molar excess of CsA indicating that the activity of SFA was independent of cyclophilin binding and is independent of the CsA cyclophilin binding site for IL-12p70 inhibition [158].

Given the fact that monocyte-derived DC may display functional differences in comparison to preformed in vivo generated DC [199], we questioned whether SFA was able to block IL-12 production by freshly isolated peripheral blood DC, purified from healthy blood donors. CD1c+ (BDCA 1) is expressed on CD11c+CD123- DC and represents the major subset of myeloid DC in human blood [43, 199]. We purified CD1c+ peripheral blood DC (purity> 90%) from blood donors after depletion of B cells by immunomagnetic cell sorting [43]. DC were treated with SFA and stimulated with either LPS or poly I:C/IFN-γ to show again a stimulus independent effect. SFA-treated blood DC exhibited a decrease of over 95% of bioactive IL-12, showing that SFA was effective on freshly isolated peripheral blood DC. An experiment with peripheral blood DC excludes the possibility of an artificial result based on in vitro generated DC [158]. Using sorted blood DC is so far technically difficult due to the small amount, which excludes the possibility to run large experimental setups (less than 1 % of lymphocytes) and to the expensive purification.

The calcineurin inhibitors CsA and FK506 exert potent immunosuppressive effects on the transcriptional level via inhibition of NFAT-induced cytokine gene expression [118], whereas mTOR inhibition by RAPA results in inhibition of translation initiation and cell cycle arrest [128]. To investigate whether SFA inhibits IL-12 production at the transcriptional or translational level, we performed real-time RT-PCR and quantified IL-12p35 and IL-12p40 mRNA expression. The results revealed a strong suppression of IL-12p35 and p40 mRNA expression, whereas expression of two independent housekeeping genes, 18s RNA and GAPDH was not affected [158]. Relative

quantification of IL-12p35 and p40 transcripts in relation to diluted standards indicated ≥ 90% inhibition in the presence of 500 nM SFA [158].

Recently, IL-23, a novel member of the IL-12 cytokine family, has been discovered. As IL-23 is a heterodimer, comprising IL-12p40 and the IL-23-specific p19 subunit [163]. Because IL-23 is produced by DC and is suggested to play a unique role in the activation of memory T-cells, as well as in autoimmune inflammation of the brain [164], we decided to analyze p19 mRNA in SFA-treated DC [163, 200] in order to see, if SFA has an effect on the gene transcription of this novel cytokine family. Again only a short term pretreatment of DC with SFA (1h) was enough to block 84% of p19 transcription [158].

In conclusion these data provide evidence, that the novel cyclophilin binding immunosuppressant SFA acts rapidly on human DC and blocks bioactive IL-12 production at the transcriptional level. Additionally, we provide evidence, that SFA suppresses expression of the IL-12 related, IL-23-specific p19 subunit. Direct comparison with the related drugs CsA and RAPA demonstrates that the rapid action of SFA on DC production of proinflammatory IL-12 is selective and even takes place very early in gene transcription. These results might show that the inhibition of SFA effects a complete group of inflammatory cytokine genes that are tightly linked together and may play an important role in inducing immunity or when inhibited lead to an enhanced and long-lasting tolerance.

Although we had consistent results with peripheral blood DC, the real proof of principle are in vivo experiments. For this purpose, we injected mice with SFA (10mg/kg/day). In a first experiment we used a short application of SFA and only a small amount of animals, measuring just the IL-12p70 levels. On day 3, IL-12 production was stimulated in vivo by injection of LPS/IL-4 and plasma IL-12p70 levels were measured 4 h later. Results show that a short course of SFA blocks 70% of bioactive IL-12 production in vivo in comparison to vehicle injected animals. Before starting a large amount of animal experiments this simple and small experiment confirmed our in vitro findings in vivo and encouraged us to make larger and more complex experiments to understand the in vivo effects of SFA.

To investigate the maximal capacity of SFA to inhibit bioactive IL-12 production in vivo, especially to have a stronger cytokine signal, we expanded DC with Flt3L and injected the animals with SFA 10mg/kg/d or drug vehicle for a longer period of time, for 10 days. In the first model, using a protocol described by Hochrein et. al. [165], massive in vivo stimulation (>1000 pg/ml plasma) was achieved by combined LPS (10 μg) and IL-4 (0.5 μg) injection on day 10. SFA treatment resulted in 93.6% inhibition of in vivo IL-12 production compared to vehicle-injected control animals. To ensure the selectivity we also performed ELISAs for TNF-α and IL-10 production. Both cytokines were not affected significantly by SFA administration, excluding the possibility that the profound IL-12 inhibition was related solely to a non-specific or general toxic effect. To confirm these in vivo findings using a different IL-12 inductor, we redid the experimental setup and injected animals with the TLR 9 ligand CpG ODN and measured in vivo IL-12 production. Again, CPG ODN injection resulted in massive in vivo IL-12 production (>900 pg/ml plasma) while SFA treatment suppressed IL-12p70 production by 92.8% compared to vehicle-injected controls; TNF-α and IL-10 levels were not affected. Based on a recent review, SFA is one of the most potent selective pharmacological inhibitors of bioactive IL-12 production in vivo [135]. In our research only clobetasol-17-propionate, [194] a synthetic glucocorticosteroid has been demonstrated to inhibit, among many other cytokines,>90% of bioactive IL-12 in vitro.

With respect to the massive inhibition of bioactive IL-12 production we questioned whether this effect was related to interference by SFA with DC expansion in vivo.

Our in vitro effects at the beginning could not show any significant alteration of differentiation or maturation markers. For Rapamycin a suppression of DC in vivo expansion has been reported recently [171]. Analysis of total leucocytes numbers and CD11c$^+$ DC in spleen and bone marrow of normal and Flt3L-treated animals did not indicate a significant suppressive effect on total DC expansion in vivo. Furthermore, detailed analysis of plasmacytoid, myeloid and lymphoid related DC subsets in bone marrow and spleen also did not indicate a significant suppressive effect of SFA on DC subset development in vivo [201]. This was especially interesting, because this excludes the possibility that our in vivo findings could be solely related to a selective inhibition of just a single DC type that is the major producer of bioactive IL-12p70.

Additionally, analysis of surface expression of CD40, CD80, CD86 and MHC class II of in vivo expanded splenic and bone marrow CD11^{+} DC after stimulation did not indicate a significant effect of SFA on phenotypic DC in vivo maturation. These findings were confirmed with CD11c^{+} DC stimulated with allogeneic cell lysates. These results indicate that SFA does not suppress differentiation, expansion or phenotypic maturation of DC in vivo. It correlates with our in vitro findings and confirms that inhibition of cytokine production is not related to its maturation or differentiation status.

A critical prerequisite for DC to function as sentinels of the immune system is efficient endocytosis. Two major mechanisms for efficient antigen capture by DC are fluid phase uptake through macropinocytosis and receptor mediated endocytosis [202].

The function of immature DC in the body is to recognize and take up a wide range of self and non self Ag. DC differentiate between allo and autoantigens using conserved pattern recognition receptors, which recognize molecular patterns at the cell surface of all micro organisms. Receptors of this type include Toll like receptors and C type lectins. Recently Woltman et.al. [167] reported that SFA inhibits expression of endocytosis receptors and endocytotic acticity of human monocyte-derived DC in vitro. SFA is the first immunosuppressive drug that shows inhibitory effects on the expression of DC-SIGN [167].

The substance strongly inhibits Ag uptake by DC by at least two different mechanisms: the fluid phase endocytosis (macropinocytosis) as shown by FITC-Albumin, as well as the receptor mediated endocytosis, as shown by FITC-Dextran. As it has already been published for in vitro data [167] the decreased uptake of FITC-Dextran can be explained by the inhibitory effect of SFA on the expression of C-type lectins, such as MR and DC-SIGN. All inhibitory effects are dose dependent and were achieved at nano Molar concentrations.

To assess the potential relevance of SFA as a novel in vivo DC endocytosis inhibtor we investigated DC receptor-mediated antigen uptake in vivo using FITC-Dextran, and fluid phase uptake by employing FITC-Albumin in SFA-injected animals. After 10 day exposure to SFA, animals were injected with either FITC-Albumin or FITC-Dextran and FITC-uptake was quantitated in CD11c^{+} DC selected by flow-cytometry. For the first

time it was shown that SFA suppresses DC receptor-mediated endocytosis and DC macropinocytosis in vivo.

The results revealed that SFA significantly impaired in vivo macropinocytosis of FITC Albumin ($p= 0.006$) and receptor-mediated endocytosis of FITC-Dextran ($p=0.0001$). To date, the related immunophilin-binding immunosuppressant Rapamycin has been reported to significantly impair endocytosis by murine and human DC [139, 203]. However, Rapamycin has been reported to inhibit 30% macropinocytosis (in terms of mean fluorescence intensity reduction) of in vivo expanded DC [139]. In contrast, our present data indicate that SFA inhibits >80% of DC macropinocytosis in vivo. Taken together, our in vivo experiments suggest that SFA displays a unique mode of action with respect to DC: strong inhibition of both antigen uptake and bioactive IL-12 production but no significant effects on DC differentiation, expansion and surface costimulatory expression. To our knowledge, no immunosuppressive agent has been reported to date that allows potent suppression of the two key DC functions antigen uptake and IL-12 production without interfering with DC differentiation or DC expansion [135]. Both, corticosteroids and Rapamycin have been reported to exert rather broad inhibitory effects including suppression of DC differentiation and expansion [135, 138, 160].

The purpose of DC and their unsurpassed antigen uptake and presentation is their interaction with other cells, especially T-cells. DC therefore are the early switch between differentiation of auto- and alloantigens. This differentiation takes place via cytokines and costimulatory molecules. As SFA inhibits endocytosis in vivo and in vitro, this might lead in conclusion to a decreased antigen presentation in vivo. Our next step was to investigate if SFA treated DC due to their decreased antigen uptake might lead to an inhibited antigen presentation and to a less pronounced T-cell proliferation.

To address the impact of SFA specifically on DC T-cell stimulatory capacity in vivo, we performed adoptive transfer experiments of sorted CD11c$^+$ DC from SFA-exposed donor animals into naïve, non-immunosuppressed recipients. These experiments allowed us to quantitate the impact of SFA on DC antigen presenting capacity without interfering with SFAs effects on other cells. Adoptive DC transfer was performed in three different animal models: **i)** we transferred purified, unpulsed Balb/c DC into fully

allogeneic nonimmunosuppressed C57BL/10 recipients. Compared to the control this setup showed us only a very moderate direct immunostimulatory capacity. This result seemed very likely, as a fully mismatched allograft would be rejected even under strongest immunsuppression; **ii)** Balb/c DC were pulsed with C57BL/10 cell lysate and after bead purification transferred into syngeneic animals. This commonly used model gives a good insight what would happen with a real allograft: Syngeneic DC take up allogeneic cell fragments and present them to T-cells. This experiment was made to analyze indirect antigen presentation capacity. Here we could show that SFA`s effects on DC endocytosis and IL-12 production had a significant effect on T-cell stimulation. The next logical step was to investigate; if a repeated injection of alloantigen pulsed DC multiplied the T-cell stimulatory capacity. Again the principle of an allograft in organ transplantation is that recipient DC take up allograft antigens and the reaction of the recipients immune system gets more fierce and after a certain time (i.e. for heart allograft 1-3 years) a significant amount of allografts is rejected **iii)** for this purpose we repeated injection of allo-Ag-pulsed DC into syngeneic animals to analyze boosting of T-cell activation. In all experiments, CD11c$^+$ DC were purified before injection by bead-sorting to >95% purity and T-cell activation was analysed after restimulation ex vivo with donor splenocytes. The purity of the DC was of special concern to minimize the possibility that solely the contamination with other lymphoctytes was the reason for our results in the adoptive transfer experiments.

When SFA-exposed, sorted BALB/c CD11c DC were injected into fully allogeneic C57BL/10 animals, only a moderate decrease in T-cell proliferation was detected after restimulation in comparison to controls. In contrast, naïve non-immunosuppressed recipients of syngeneic SFA-exposed DC pulsed with allogeneic C57BL/10 cell lysate showed markedly reduced T-cell proliferation after restimuation with C57BL/10 with C57BL/10 splenocytes (p<0.0001 at all stimulator/responder ratios) [201]. It should be noted that secondary T-cell proliferation of recipients of SFA-exposed DC was so strongly decreased that it was similar to the primary T-cell proliferation of naïve BALB/c mice stimulated with C57BL/10 splenocytes. Moreover, when we boosted T-cell activation in vivo with two additional injections of syngeneic DC pulsed with allogeneic C57BL/10 lysate, recipients of SFA-exposed DC showed a further pronounced

significant decrease in T-cell proliferation in comparison to recipients of control DC. This boosting experiment shows a time and memory dependent effect of SFA in indirect antigen presentation: This helps to prevent a multiplying effect of repeatedly presented alloantigens by the recipient and may reduce the chronic allograft rejection. Naïve Balb/c mice get in contact with fully mismatched antigens from C57/BL 10 mice. They produce an immune reaction which is relatively mild. Memory cells are produced to help the organism to have a faster and fiercer reaction if there is a reoccurring contact to the antigen. This helps the organism in fight against infectious diseases, but is an unwanted reaction in organ transplantation. Therefore it is of major interest that alloantigen tissue leads to no or only a mild T-cell proliferation. Consequently it might be assumed that this reduced T-cell proliferation might lead to a reduced chronic allograft rejection. This assumption is also supported by the reduced cytokine levels of the T-cells [201]. Analysis of cytokine production by T-cells from latter animals revealed significantly reduced production of IL-2, IL-4 and IFN-γ. Taken together, these DC adoptive transfer experiments indicate that SFA profoundly impairs indirect antigen presentation and T-cell priming capacity of DC in vivo.

As the effects of SFA are very specific on certain effects of APCs it was interesting to see if SFA alone or in combination with CsA (that dramatically improved the outcome of transplant rejection and certain autoimmune diseases) would show effects especially on chronic allograft recection.

Reduced endocytotic capacity and low IL-12p70 production alone is not enough to avoid graft rejection. Graft vasculopathy is the leading cause of death between 1-3 years after heart transplantation and represents the major obstacle to long term survival [6].

The potential clinical relevance of SFA as a novel, cyclophilin-binding immuno-suppressant that does not inhibit the calcineurin phosphatase activity, was analyzed with SFA alone and in combination with CsA in fully-allogeneic rat vasculized heart transplantation [201]. These experiments showed that addition of SFA to low-dose CsA allowed long-term allograft survival (>100days) in 4 out of 6 recipients in comparison to absence of long term survival in the cyclosporine only group. These data undermines the different mechanisms of action and suggests a synergy between SFA and CsA to prevent allograft rejection. In the chronic rejection model, CsA doses were increased

(1.25 mg/kg/d) to allow long-term graft survival ≥ 100 days of all organ recipients. In this model, addition of SFA to CsA-treated organ recipients markedly suppressed development of cardiac allograft vasculopathy [201]. It should be noted that cardiac allograft vasculopathy in SFA+CsA-treated animals was similar to syngeneic control transplants+CsA. In contrast simple elevation of CsA dose to 2.5 mg/kg/d in animals receiving CsA monotherapy did not prevent cardiac allograft vasculopathy [201].

These data show that inhibition of DC endocytosis and bioactive IL-12 production by SFA is not sufficient to prevent acute allograft rejection [201]. Why is the use of SFA alone to prevent allograft rejection not sufficient? The result of insufficient protection against allograft rejection correlates with in vitro studies showing that SFA is > 15-fold less potent than CsA at inhibiting T-cell proliferation in the mixed leucocyte reaction [126, 198].

These results indicate a different mode of immunosuppressive action for SFA in comparison to immunophilin-binding agents CsA, tacrolimus and Rapamycin. Whereas calcineurin inhibitors CsA and Tacrolimus,as well as the mTOR inhibitor Rapamaycin, represent highly potent T-cell inhibitors and show less activity against DC, SFA exhibits potent inhibitory activity against key DC functions in vivo and low inhibitory activity with respect to effector lymphocytes. Accordingly, combination of SFA with CsA, representing a classical T-cell inhibitor [114, 204], resulted in additive effects with respect to inhibition of acute heart rejection supporting the observation that SFA and CsA target different cell types in the immune sytem. It should be noted that these novel data do not establish true synergy for SFA and CsA [201]. Future experiments might even show like for combinations of CsA and RAPA that the reason for synergy effects is related to pharmacokinetics and can even exacerbate toxicity [205, 206]. The next logical step is to investigate SFA`s pharmacological and toxic effects alone and in combination with CsA.

The inhibition of cardiac allograft vasculopathy by SFA in CsA-treated recipients [201] indicated that SFA qualitatively modulates the immunologogical effects of CsA. These results indicate that SFA may be used not only as a CsA-sparing but also as a CsA modulating agent in clinical therapy. These results indicate that the combination of SFA and CsA may be of clinical relevance for the suppression of chronic graft rejection.

Further studies directly comparing SFA+CsA with other combinations of immunosuppressants are necessary to evaluate the potency of SFA to inhibit chronic allograft rejection.

In conclusion, our data showed in vitro and in vivo immunosuppressive capacity of SFA to suppress key DC functions in vitro and in vivo and correlates with recent in vivo findings to potently inhibit acute and chronic allograft rejection in combination with CsA [201]. We provide novel insights into SFA`s activity to interfere with major cells and functions in vitro and in vivo forming a basis for further analyses at the molecular level. Additional molecular analyses are needed to identify major target genes of SFA with respect to DC and T-cell activation. These studies may provide additional mechanistic insight into SFA`s suppressive effects on transplant rejection. We propose that SFA represents a novel class of immunophilin binding immunosuppressants that exhibit potent DC inhibitory activity and additive effects with low dose CsA [201]. SFA`s inhibitory effect on the development of graft vasculopathy in CsA-treated recipients may represent a novel therapeutic strategy to overcome chronic rejection.

5.   SUMMARY

Dendritic Cells (DC) belong to the innate immunity and present professional Antigen (AG) presenting cells that initiate and regulate immune responses. For an efficient host defence both AG non-specific innate immunity and Ag specific adaptive immunity are needed. On the other hand it is desired that the host defence is selectively reduced preventing organ rejection after transplantation but leaves the desired immunity against bacteria and viruses or cancer unattached.

Acute organ rejection is very well controllable with the available immunosuppressive drugs, but new drugs still have to be developed for chronic allograft rejection.

A selective inhibition of dendritic cells desirably leads to less endocytotic capacity, Ag uptake and presentation and production of proinflammatory cytokines and is assumed delaying allograph rejection. With respect to the central role of DC in immunity, they are interesting therapeutic targets for pharmacological manipulation of immune responses. The impact of Sangliferin A (SFA) as a novel means for pharmacological programming of tolerogenic DC in models of alloimmunity and solid organ transplantation is to be evaluated. Therefore the pharmacological effects of the novel immunophilin-binding agent SFA on DC were investigated in vitro and in vivo.

This work shows that SFA does not affect DC differentiation and phenotypical maturation. IL-12p70 plays a critical role in the pathogenesis of inflammation and autoimmune diseases. We discovered that SFA abrogates bioactive IL-12p70 production by human DC, the major producers of this cytokine. In direct comparison to the related calcineurin inhibitor Cyclosporin A and the mammalian target of Rapamycin inhibitor Rapamycin, SFA acts uniquely within 1h to inhibit (80-95%) IL-12p70 production by differentiated DC. To show that the inhibition is stimulus independent further experiments with Toll-like receptor 3 and 4 ligands were performed. Competitive experiments with a molar excess of Cyclosporin A indicate a cyclophilin A independent blockade of IL-12p70 production. We confirm potent inhibition of IL12p70 production by SFA using purified human blood DC.Real-time RT-PCR reveals 84-94% suppression of IL-12p40, IL-12p35 and IL-23-specific p19 transcription.

Another major aim of this work was to reproduce the in vitro findings in an in vivo mouse model in order to clarify the clinical relevance of these findings. Within these in vivo

investigations it could be shown, that three day in vivo injections of SFA into mice and stimulation of the animals with LPS/IL-4 blocked 70% of bioactive IL-12 production compared to vehicle injected control animals. By using independent models, we provide evidence that SFA abrogates that SFA abrogates >90% IL-12p70 production in vivo while having no effect on IL-10 AND TNF-α. SFA treatment in vivo showed no influence on in vivo expansion or phenotypic maturation of DC subsets and had nor effects on DC subpopulations.

Furthermore, SFA profoundly decreases DC receptor-mediated endocytosis and macropinocytosis and inhibits indirect antigen presentation of CD11c$^+$ DC in vivo. Our results are the first to reveal the immunosuppressive activity of SFA in vivo. Taken together, our in vivo experiments suggest that SFA displays a unique mode of action with respect to DC: strong inhibition of both antigen uptake and bioactive IL-12 production but no significant effects on DC differentiation, expansion and surface costimulatory expression. To our knowledge, no immunosuppressive agent has been reported to date that allows potent suppression on the two key DC functions antigen uptake and IL-12 production without interfering with DC differentiation or DC expansion. We propose that SFA represents a novel class of immunophilin-binding immunosuppressants with high activity against DC and in addition has synergetic effects if combined with other immunopilins like CsA. For an effective prevention of chronic allograft rejection the combination of immunosuppressive drugs with SFA might be a powerful approach.

6.   ZUSAMMENFASSUNG

Dendritische Zellen gehören zum angeborenen Immunsystem und präsentieren professionelle Antigen-präsentierende Zellen, die immunologische Antworten initiieren und anregen. Für eine effiziente körpereigene Immunabwehr werden sowohl die Antigen-unspezifische angeborene Immunität als auch die humorale Immunität benötigt. In den bestimmten Fällen wie einer Transplantation ist es erwünscht, dass die körpereigene Abwehr selektiv reduziert wird, so dass der Körper das körperfremde Organ nicht abstößt, jedoch gleichzeitig eine ausreichende Immunität gegen Bakterien, Viren und Krebs erhalten bleibt.

Heutzutage kann mittels der derzeit erhältlichen immunosuppressiven Substanzen die akute Transplantatabstoßung im Gegensatz zur chronischen sehr gut kontrolliert werden. Um die chronische Transplantatabstoßung zufriedenstellend zu beherrschen, müssen neue Substanzen mit anderen Wirkmechanismen etabliert werden.

Ein therapeutischer Ansatz wäre die selektive Hemmung dendritischer Zellen. Sie spielen eine zentrale Rolle im Immmunsystem und präsentieren interessante Ziele für die pharmakologische Manipulation. Eine Hemmung dendritischer Zellen führt zu einer verringerten endozytotischen Antigenaufnahme, -präsentation und Produktion proinflammatorischer Zytokine und könnte folglich die chronische Organabstoßung verzögern oder verhindern.

In dieser Arbeit wurde untersucht, ob man Sanglifehrin A (SFA), eine neue immunophilin bindende Substanz, zur pharmakologischen Programmierung tolerogener Dendritischer Zellen nutzen kann, um einen Effekt in Alloimmunitätsmodellen und Organtransplantationsmodellen zu zeigen. Die pharmakologischen Effekte von SFA auf dendritische Zellen wurden in vitro und in vivo untersucht.

In ersten Versuchen zeigten wir, dass SFA keinen Effekt auf Differenzierung oder phänotypische Reifung von Dendriten hat.

Als nächstes untersuchten wir eine Reihe von Zytokinen, unter anderem IL-12p70, das eine wichtige Rolle bei der Pathogenese von Entzündungen und Autoimmunerkrankungen spielt. Wir fanden heraus, das SFA die Produktion von bioaktivem IL-12p70 in dendritschen Zellen reduziert, die Hauptquelle für bioaktives IL-12. Im direkten Vergleich zu dem verwandten Calcineurin-Inhibitor Cyclosporin A und

dem „mammalian Target of Rapamycin" (mToR) Hemmer Rapamycin, hemmt nur SFA schon innerhalb einer Stunde 80–95 % der IL-12p70 Produktion von differenzierten Dendriten. Um zu zeigen, das es sich um einen stimulusunabhängigen Mechanismus handelt, wurden die Versuche sowohl mit Toll-like Rezepor 3 als auch 4 Liganden durchgeführt. Es wurden Rezeptorverdrängungsexperimente durchgeführt, bei denen molare Überschüsse von Cyclosporin A vor der Zugabe von SFA zur Zellkultur gegeben wurden. Die Ergebnisse zeigen eine äqipotente Hemmung der IL-12p70 Produktion mit oder ohne Cyclopsorin A: Daraus lässt sich eine Cyclophilin- unabhängige Blockade der IL-12p70 Produktion durch SFA vermuten.

Die starke Hemmung der IL-12p70 Produktion wurde in einer Versuchsreihe mit gereinigten peripheren Blut-Dendriten bestätigt. Echtzeit-PCRs ergaben eine 84-95% Hemmung der IL-12p40-, IL-12p35- und der IL-23-spezifischen p19 Transkription.

In einem nächsten Schritt sollte untersucht werden, ob diese in vitro Ergebnisse im in vivo Mausmodell reproduzierbar sind, um die klinische Relevanz dieser Ergebnisse zu klären. Die in vivo Untersuchungen zeigten, das dreitägige Injektionen von SFA in Mäuse (Kontrolltiere erhielten eine wirkstofffreie Trägerlösungen injiziert) und die darauffolgende in vivo Stimulation mit LPS/IL-4 70% der Produktion von bioaktivem IL-12 hemmten. Mit weiteren unabhängigen Modellen zeigten wir, das SFA mehr als 90% der IL-12p70 Produktion hemmt, während es keinen Effekt auf IL-10 und TNF-α hat. Die in vivo Behandlung mit SFA zeigte keine Einfluß auf die in vivo Expansion oder die phänotypische Reifung von DC Subtypen und hatte keinen Effekt auf die DC Population.

Außerdem hemmte SFA die rezeptorvermittelte Endozytose und Makropinozytose und die indirekte Antigenpräsentation von CD11c$^+$ dendritschen Zellen in vivo. Unsere Ergebnisse zeigten zum ersten Mal den Effekt von Sanglifehrin A in vivo.

Die Ergebnisse lassen vermuten, insbesondere die Bestätigung durch die in vivo Experimente, das es sich bei Sanglifehrin A um eine Subanz mit einem einzigartigen Wirkprofil gegenüber dendritschen Zellen handelt: starke Hemmung sowohl der Antigen-Aufnahme als als auch der IL-12p70 Produktion, aber keinen signifikanten Einfluß auf Differenzierung, Expansion oder Expression von Oberflächenmarkern dendritischer Zellen. Unserem Wissen nach gab es bisher keine immunosuppressive

Substanz, die zwei Schlüsselfunktionen, IL-12 Produktion und Antigenaufnahme hemmt, aber keine Effekte auf Differenzierung oder Expansion hat.

Wir vermuten, das SFA eine neue Klasse immunophilinbindender Immunsuppressiva darstellt, die eine hohe Aktivität auf dendritische Zellen zeigt und in der Kombination mit anderen Immunsuppressiva synergistische Wirkungen entfaltet. Somit könnte die Kombination von immunsuppressiven Substanzen mit Sanglifehrin A ein wirkungsvoller Ansatz sein, um chronische Tranplantatabstoßung zu verhindern.

7.   LITERATURE

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8.   STATEMENT

"Ich erkläre: Ich habe die vorgelegte Dissertation selbständig, ohne unerlaubte fremde Hilfe und nur mit den Hilfen angefertigt, die ich in der Dissertation angegeben habe. Alle Textstellen, die wörtlich oder sinngemäß aus veröffentlichten oder nicht veröffentlichten Schriften entnommen sind, und alle Angaben, die auf mündlichen Auskünften beruhen, sind als solche kenntlich gemacht. Bei den von mir durchgeführten und in der Dissertation erwähnten Untersuchungen habe ich die Grundsätze guter wissenschaftlicher Praxis, wie sie in der "Satzung der Justus-Liebig-Universität Gießen zur Sicherung guter wissenschaftlicher Praxis" niedergelegt sind, eingehalten."

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