Cutting Edge: Sanglifehrin A, a Novel Cyclophilin-Binding Immunosuppressant Blocks Bioactive IL-12 Production by Human Dendritic Cells

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Sanglifehrin A (SFA) is a novel cyclophilin-binding immunosuppressant with an unknown mechanism of action. 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 dendritic cells, 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 1 h to inhibit (80–95%) IL-12p70 production by differentiated dendritic cells. Experiments with Toll-like receptor 3 and 4 ligands show a stimulus-independent suppression. Competitive experiments with a molar excess of cyclosporin A indicate a cyclophilin A-independent blockade of IL-12p70 production. We confirm potent inhibition of IL-12p70 production by SFA using purified human blood DC. Real-time RT-PCR reveals 84–94% suppression of IL-12p70 production. We confirm potent inhibition of IL-12p70 production by SFA using purified human blood DC. Real-time RT-PCR reveals 84–94% suppression of IL-12p70 production. We confirm potent inhibition of IL-12p70 production by SFA using purified human blood DC. Real-time RT-PCR reveals 84–94% suppression of IL-12p70 production. We confirm potent inhibition of IL-12p70 production by SFA using purified human blood DC. Real-time RT-PCR reveals 84–94% suppression of IL-12p70 production.
affecting DC differentiation, phenotypic maturation, or cell viability. Thus, in comparison to the calcineurin inhibitor CsA and the mTOR inhibitor Rapa, SFA targets DC uniquely by rapid suppression of bioactive IL-12 production.

Materials and Methods

Compounds

SFA, kindly provided by Novartis Pharmaceuticals (Basel, Switzerland), and Rapa and CsA, purchased from Sigma-Aldrich (Seelze, Germany), were used at the indicated concentrations and time points. Stock solutions were prepared in absolute ethanol (vehicle) and diluted on the day of the experiment with culture medium. Control DC were treated with drug vehicle.

Generation of DC

Human PBMC were isolated from buffy coats of healthy blood donors by Ficoll-Paque (Pharmacia, Freiburg, Germany) density gradient centrifugation. CD14+ monocytes were purified (>95%) using CD14 immunomagnetic microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) and 3 × 105 cells were cultured in six-well flat-bottom plates, in 3 ml of DC medium, comprising RPMI 1640, 1-glutamine, penicillin/streptomycin, sodium-pyruvate, nonessential amino acids, 10% heat-inactivated FCS Gold (PAA Laboratories, Linz, Austria), 1000 IU/ml recombinant human (rh) GM-CSF (Novartis Pharmaceuticals, Vienna, Austria), and 1000 IU/ml rhIL-4 (Promo Cell, Heidelberg, Germany). After 3 days, 50% supernatant was replaced with fresh cytokine-containing medium. On day 6, CD1a+ DC represented >90% of cultured cells. The studies of human blood samples were approved by the Institutional Review Board.

Isolation of peripheral blood DC

CD11c+ blood DC (purity >90%) were isolated by immunomagnetic bead sorting (AutoMACS) using the CD11c DC isolation kit (Miltenyi Biotec). Briefly, in a first step, PBMC from buffy coats of blood donors were depleted of B cells with CD19 microbeads and subsequently CD11c+ DC were positively selected after indirect labeling with biotinylated CD11c mAbs and antibiotin microbeads (17).

DC stimulation and detection of cytokine production by ELISA

DC maturation was stimulated with 100 ng/ml LPS (Sigma-Aldrich) in DC medium with cytokines. For stimulation of cytokine production, DC were incubated at 2 × 105/ml in 96-well plates in DC medium with cytokines and stimulated for 24 h with 1 µg/ml LPS (Escherichia coli 0111:B4; Sigma-Aldrich) or 100 µg/ml polyinosinic-polycytidylic acid (poly I:C; Sigma-Aldrich) plus 20 ng/ml rhIFN-γ (BD PharMingen, San Diego, CA). Human IL-12p70 and TNF-α were measured using BD OptiEIA ELISA sets (BD PharMingen).

In vivo experiments

SFA stock solution was diluted freshly in 2.5% polysorbate 80, 51% polyethylene glycol (PEG) 300 (Sigma-Aldrich) and 46.5% sterile water. Eight- to 12-wk-old male C57BL10 mice (Charles River Breeding Laboratories, Sulzfeld, Germany) were injected i.p. with SFA (10 mg/kg/day, 3 days) or vehicle. On day 0, mice were injected i.p. with 0.5 µg of murine IL-4 and 10 µg of LPS, dissolved in PBS with 1% FCS. Four hours later, the animals were killed, blood was taken, and plasma IL-12p70 levels were measured by ELISA (R&D Systems, Wiesbaden, Germany).

Flow cytometry

Surface phenotype of cultured cells was analyzed on a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA) with the following mAbs: HLA-DR (clone L343), CD40-PE (clone 5C3), CD83-PE (clone HB15e), CD86PE (clone GL1), CD14-FTTC (clone M5E2), and CD1a-CY (clone HI149). All mAbs, including isotype-matched controls, were purchased from BD PharMingen. Apoptosis and necrosis were analyzed by staining of phosphatidylserine translocation with Annexin VPE in combination with the vital dye 7-AAD (BD PharMingen).

Real-time RT-PCR

The RNA of 5 × 106 cells was isolated by using the Qiagen Rneasy Mini kit (Hilden, Germany) and transcribed into cDNA using the Ready-To-Go You-Prime First-Strand Beads according to the manufacturer’s instructions (Amerham, Freiburg, Germany). Real-time RT-PCR was performed on an ABI Prism 7000 PCR cycler (Applied Biosystems, Foster City, CA). The following validated PCR primers and TaqMan MGB probes (6FAM-labeled) were used: IL-12p35 (sense ID: Hs00189034_m1), IL-12p40 (Hs00232688_m1), IL-23p19 (Hs00372322_m1). As endogenous controls, two independent primer sets with TaqMan probes were used: eukaryotic 18s ribosomal RNA (Hs99999901_s1) and GAPDH (Hs99999905_m1). PCR mix was prepared according to the manufacturer’s instructions (Assay on demand; Applied Biosystems) and thermal cycler conditions were as follows: 1 × 10 min 95°C, 40–50 cycles denaturation (15 s, 95°C) and combined annealing/extension (1 min, 60°C). Relative quantification was performed by comparison of threshold cycle values of samples with serially diluted standards.

Results and Discussion

SFA does not affect DC differentiation and phenotypic maturation

Analysis of GM-CSF/IL-4-expanded human monocyte-derived DC 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 (Fig. 1A). To induce

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**FIGURE 1.** SFA does not affect DC differentiation (A) or phenotypic maturation (B), but blocks bioactive IL-12 production (C) and inhibits TNF-α production (D). DC were generated in the presence of GM-CSF and IL-4, and SFA (500 nM) was added on day 2 (A–C). After 6 days, DC were harvested and analyzed for surface CD14 and CD1a expression (A) or stimulated with LPS and subsequently analyzed for CD40, CD80, CD83, and HL-DR expression by flow cytometry (B). Specific staining is indicated by gray shaded histograms, and isotype control staining by open histograms. C and D. Immature DC were stimulated on day 6 (LPS, poly I:C/IFN-γ) and IL-12p70 production (C) and TNF-α (D) production were analyzed by ELISA. Control values were 1340 pg/ml IL-12p70 (C, LPS), 2456 pg/ml IL-12p70 (C, poly I:C/IFN-γ), and 845 pg/ml TNF-α (D). The results are representative of seven (A and B), five (C), and three (D) separate experiments.
DC maturation, immature cells were stimulated with LPS. SFA did not affect the up-regulation of costimulatory molecules (CD40, CD80, CD86) or HLA-DR (Fig. 1B), nor did it affect de novo expression of CD83 (Fig. 1B). Similar results were obtained after TNF-α (1000 U/ml) stimulation of DC.

**SA blocks bioactive IL-12 production by human DC independent of the nature of the eliciting stimulus**

Addition of SFA to DC cultures on day 2 suppressed 80–90% of inducible IL-12p70 production after LPS stimulation (Toll-like receptor (TLR) 4 ligation) when compared with control DC (Fig. 1C). Because IL-12p70 production can be induced by many different cytokines and microbial agents, we confirmed these findings by a second mode of stimulation through TLR3 with poly I:C and IFN-γ (Fig. 1C). TNF-α production was also suppressed, but to a lesser extent (Fig. 1D). 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 (18), we consistently found a low incidence of DC death in SFA-treated cultures. 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).

**SA acts rapidly on already differentiated DC**

To investigate whether SFA needed to be present during DC differentiation to block IL-12p70 production, we added SFA at different time points during DC generation (days 2–6). SFA inhibited IL-12p70 production after LPS stimulation at all time points with similar potency (Fig. 2A). 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 (IC50 108 ± 45 nm; Fig. 2B). SFA blocked IL-12p70 production potently when added as late as day 6, 60 min before stimulation (Fig. 2C). The results are representative of three separate experiments.

**Suppression of human DC IL-12 production by SFA is unique compared with 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 (19, 20). The mTOR inhibitor Rapa has been shown to inhibit DC IL-12 production if present for several days during DC differentiation, either in vitro or in vivo (9, 11). We next questioned whether the rapid and potent suppressive effect of SFA on bioactive IL-12 production by differentiated DC was unique in direct comparison to the immunophilin-binding immunosuppressants CsA and Rapa. Neither Rapa nor CsA was able to inhibit bioactive IL-12 production by DC significantly when added shortly before the stimulation (Fig. 2C). In direct contrast to CsA and Rapa, SFA was the only immunophilin-binding immunosuppressant that strongly inhibited IL-12p70 production of differentiated DC (Fig. 2C).

**Blockade of IL-12 production by SFA is unlikely to be dependent of cyclophilin binding**

Both SFA and CsA bind to cyclophilin in cells with high affinity (5), 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 (Fig. 2C). To assess whether suppression of IL-12 production was dependent on binding of SFA to cyclophilin, we performed competitive experiments with SFA and a molar excess of CsA. We found that SFA still blocked 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 or at least not inhibited by CsA (Fig. 2D).

**Confirmation of the suppressive effect of SFA 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 (21), 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 (17, 22). We purified CD1c+ peripheral blood DC (purity >90%) from blood donors after depletion of B cells by immunomagnetic cell sorting (17). DC were treated with SFA and stimulated with either LPS or poly I:C/IFN-γ. SFA-treated blood DC exhibited a striking decrease in bioactive IL-12 production compared with control DC (Fig. 2D).

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**FIGURE 2.** SFA acts rapidly on differentiated DC to inhibit bioactive IL-12 production (A and B). Its suppressive effect is unique when compared directly to the immunophilin-binding immunosuppressants CsA and Rapa (C). Blockade of IL-12 production by SFA is independent of cyclophilin binding (D). DC were generated in the presence of GM-CSF and IL-4. Immature DC were stimulated on day 6 with LPS (A and B) or poly I:C/IFN-γ (C and D) and IL-12p70 production was analyzed 24 h later by ELISA. SFA (500 nM) 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). SFA, CsA, and Rapa were added on day 6, 1 h before stimulation (C). In competitive experiments, SFA alone, CsA alone, and SFA after a 60-min preincubation with a molar excess of CsA were added 60 min before stimulation (D). Control values were 1478 pg/ml IL-12p70 (A) and 1298 pg/ml IL-12p70 (B). The results are representative of three (C), four (A and D), and five (B) separate experiments.
decrease in bioactive IL-12 production, suggesting that SFA was effective on freshly isolated peripheral blood DC (Fig. 3 A).

**SFA effectively suppresses IL-12 production in vivo**

To investigate whether SFA suppresses IL-12 production in vivo, we injected mice with SFA (10 mg/kg/day). 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 demonstrate that a short course of SFA blocks 70% of bioactive IL-12 production in vivo in comparison to vehicle-injected animals (Fig. 3 B).

**SFA blocks IL-12 expression by human DC at 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 (23), whereas mTOR inhibition by Rapa results in inhibition of translation initiation and cell cycle arrest (4). To investigate whether SFA inhibited IL-12 production by DC 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 (Fig. 4, A-D).

**FIGURE 3.** Confirmation of the suppressive effect of SFA on bioactive IL-12 production using human purified peripheral blood DC (A) and after in vivo administration in mice (B). A, CD1c⁺ blood DC were purified freshly from healthy donors by immunomagnetic sorting, incubated with SFA (500 nM), and stimulated after 12 h with LPS or poly I:C/IFN-γ. IL-12p70 production was analyzed by ELISA. The results are representative of three separate experiments. B, Mice were injected with SFA (10 mg/kg/day, 3 days) or vehicle and IL-12 production was stimulated in vivo by LPS/IL-4 injection. Four hours later, animals were killed and plasma IL-12p70 was determined by ELISA. Results are representative of three animals per treatment group.

Relative quantification of IL-12p35 and p40 transcripts in relation to diluted standards indicated ≥90% inhibition in the presence of 500 nM SFA (Fig. 4 F). Recently, IL-23 has been discovered as a member of the IL-12 cytokine family, has been described. IL-23 is a heterodimer, comprising IL-12p40 and the IL-23-specific p19 subunit (24). 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, we decided to analyze p19 mRNA in SFA-treated DC (24, 25).

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 is unique among immunophilin-binding immunosuppressants. Furthermore, our in vivo experiments confirm the marked suppressive effect of SFA on IL-12p70 production. Given the clinical importance of CsA and Rapa in therapy of transplant rejection and autoimmune diseases (26, 27), these findings are likely to impact on the introduction of SFA into clinical therapy.

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**References**


