Immunosuppressant Effect of IDS 30, a Stinging Nettle Leaf Extract, on Myeloid Dendritic Cells *in Vitro*

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ABSTRACT. ***Objective***. Dendritic cells are important antigen presenting cells that play a role in the initiation of rheumatoid arthritis (RA). The stinging nettle leaf extract IDS 30 (Hox alpha®) has been recommended for adjuvant therapy of rheumatic diseases. We investigated the immunomodulating effect of IDS 30 extract on the maturation of hematopoietic dendritic cells.

Methods. Human dendritic cells were generated from peripheral blood mononuclear cells cultured in granulocyte macrophage-colony stimulating factor and interleukin 4 (IL-4). Dendritic cell maturation was induced by keyhole limped hemocyanin (KLH). Dendritic cell phenotype was characterized by flow cytometric analysis; dendritic cell cytokine production was measured by ELISA. The ability of dendritic cells to activate naive autologous T cells was evaluated by mixed leukocyte reaction.

Results. IDS 30 prevented the maturation of dendritic cells, but did not affect their viability. IDS 30 reduced the expression of CD83 and CD86. It increased the expression of chemokine receptor 5 and CD36 in a dose dependent manner. The secretion of tumor necrosis factor-α was reduced. Application of IDS 30 to dendritic cells in culture caused a high endocytosis of dextran and a low capacity to stimulate T cell proliferation.

Conclusion. Our *in vitro* results showed the suppressive effect of IDS 30 on the maturation of human myeloid dendritic cells, leading to reduced induction of primary T cell responses. This may contribute to the therapeutic effect of IDS 30 on T cell mediated inflammatory diseases like RA. (J Rheumatol 2002;29:659–66)

Key Indexing Terms

RHEUMATOID ARTHRITIS	THERAPY	DENDRITIC CELLS
DIFFERENTIATION	IMMUNE RESPONSE	TUMOR NECROSIS FACTOR-α

Rheumatoid arthritis (RA) is a chronic inflammatory disease in which the synovial tissue (ST) and cartilage of joints are most strongly affected. Dendritic cells seem to play an important role during the development of RA. It is postulated that dendritic cells present T cells with autoantigens, leading to autoreactivity of T cells in the synovium[1-4]. A number of possible antigens have been described, but their putative roles have not been clarified[5-7].

In an inflamed joint endothelium is activated, for example, by fibroblast or monocyte derived tumor necrosis factor-α (TNF-α) or interleukin 1 (IL-1)[8]. Endothelial cells guide dendritic cells to the inflamed region, where T cells are stimulated. These T cells initiate or enhance the local inflammation, promoting the recruitment of granulocytes and/or macrophages, leading to degradation of the joint.

Between the process of antigen uptake and the presentation of generated antigen fragments to T cells, dendritic cells undergo differentiation from immature to mature status. Immature dendritic cells express a high amount of the receptor CD36. CD36 is important for the uptake of antigens[9]. In addition they express receptors for inflammatory chemokines such as chemokine receptor 1 (CCR 1), CCR 2, and CCR 5. These molecules guide them to the region of inflammation.

The uptake of antigen by dendritic cells in a context of cytokine exposure, particularly granulocyte macrophage-colony stimulating factor (GM-CSF) and IL-4, leads to maturation and presentation of the processed peptides as a complex with MHC molecules on the dendritic cell surface. The maturation is triggered either by inflammatory cytokines or bacterial or viral products. It is known that the nuclear transcription factor NF-κB is important during this development[10]. Mature dendritic cells express low amounts of cell surface molecules like CD36 and CCR 5[11].

In contrast to this, other surface molecules are upregulated, like the maturation associated protein CD83 as well as adhesion and costimulatory molecules including CD80 and CD86. In this developmental stage dendritic cells secrete cytokines like IL-12 and TNF-α[12] and chemokines like MIP-1α[10], causing further inflammation[13-15].

In rheumatoid synovial tissue and synovial fluid an increased number of blood dendritic cells with a potent antigen presenting function is reported, and their role in

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initiating rheumatic diseases has been discussed[6,16,17]. Thus it might be beneficial to reduce this proinflammatory influence of dendritic cells during RA.

Clinical efficacy data of *Urtica dioica foliorum* have been reported[18,19] and reviewed[20-23]. Data from *in vitro* trials of an ethanolic extract of *Urtica dioica foliorum* indicate a reduction of inflammation during RA. There have been several studies of administration in humans, which showed a significant reduction of cytokine secretion in whole blood from healthy donors. The *ex vivo, in vitro* data show reduced TNF-α/IL-1ß secretion of activated monocytes from human volunteers[24-26]. *In vitro* investigations described dose dependent T_H1-specific IL-2 and interferon-γ (IFN-γ) inhibition of phytohemagglutinin stimulated peripheral blood mononuclear cells (PBMC) and Jurkat T cells[27].

With the knowledge of the inhibitory activity of this extract a new extract from *Urtica dioica foliorum*, IDS 30 (Hox alpha®), has been developed[28]. It is registered in Germany for adjuvant therapy of rheumatic diseases.

Dendritic cells might be the most important cells in the initiation of RA[1-3]; we therefore investigated their differentiation stage as well as their ability to present antigens in the presence of the stinging nettle leaf extract IDS 30.

MATERIALS AND METHODS

Cell culture. Dendritic cells were generated as described by Thurner, *et al*[13] from PBMC by ficoll (Histopaque-1077 Hybri-Max, Sigma, Deisenhofen, Germany) density gradient centrifugation from buffy coats of healthy blood donors (Blood bank, University Hospital, Hamburg Eppendorf) and washed 3 times in phosphate buffered saline (PBS). PBMC were cultured in RPMI (Biochrom, Berlin, Germany) with 10% fetal calf serum (FCS) (Biowhittaker, Verviers, Belgium) and seeded at maximal 2×10^6/ml in T75 culture flasks (Greiner, Frickenhausen, Germany) at 37˚C and 5% CO_2. After 4 h, loosely adherent and nonadherent cells were removed. These cells were frozen in RPMI with 10% FCS (v/v) and 10% DMSO (v/v) for the isolation of autologous T cells as described below. Adherent monocytes were incubated overnight in RPMI with 10% FCS. Meanwhile, the majority of the cells detached. Monocytes were harvested, washed, and replated at 1×10^7 cells/ml. The cells reattached within 45 min. Again nonadherent cells were removed and the complete medium supplemented with 800 U/ml recombinant human GM-CSF, and 500 U/ml recombinant human IL-4 (Strathmann Biotech, Hannover, Germany) was added. On Day 2 the medium was changed. On Day 4, cells were harvested and seeded at 1×10^6/ml in 24 well plates (Greiner) with GM-CSF and IL-4, then either left unpulsed (negative control) or pulsed (positive control) with 500 ng keyhole limped hemocyanin (KLH; Sigma) and several concentrations of IDS 30 for 48 h (Day 6). Alternatively to KLH, 500 pg/ml lipopolysaccharides (LPS) from *Salmonella typhimurium* or *Salmonella enteritidis* (Sigma) were used to generate mature dendritic cells.

IDS 30 is an extract of *Urtica dioica foliorum* generated by using 95% (v/v) isopropanol (Hox alpha®; Strathmann AG, Hamburg, Germany). For *in vitro* studies IDS 30 was dissolved in ethanol (50 mg/ml), then transferred into RPMI with 10% FCS and sonicated for 10 min. Hydrocortisone (Sigma) was dissolved in DMSO (10 mg/ml) and transferred into RPMI with 10% FCS.

Flow cytometric analysis. Analyses were performed on a Coulter Epics XL (Coulter Beckmann, Krefeld, Germany). On Day 6 the cells were harvested, washed once in PBS, and incubated 20 min at room temperature (RT) with murine monoclonal antibodies (Mab) against the following human antigens: CD36-fluorescein isothiocyanate (FITC), CD80-phycoerythrin (PE) conjugated, CD83-PE, CD86-PE, CCR 5-PE. A quantity of 5×10^3 dendritic cells were gated for quantification. The percentage of positive cells was determined by gating cells stained with appropriate istoypic control IgG1-FITC, IgG1-PE, IgG2b-PE (Coulter), and IgG2a-PE (Pharmingen, San Diego, CA, USA).

Propidium iodide. Cells were resuspended in 50 µg/ml propidium iodide (Boehringer Ingelheim Bioproducts) in PBS and incubated 10 min in the dark at RT. The percentage of viable cells was directly determined by flow cytometry.

Quantification of endocytosis. The internalization of dextran-FITC by dendritic cells was tested as described[29]. On Day 6 the cells were resuspended in 100 µl RPMI with 10% FCS. The cells and dextran-FITC were preincubated for 10 min at 37˚C separately (or at 4˚C for controls). Dextran-FITC was added to dendritic cells at a final concentration of 0.5 mg/ml. After 30 min endocytosis was stopped by adding 2 ml cold PBS, 10% FCS, and the cells were washed 3 times at 4˚C with the same buffer. Dendritic cells were analyzed on a Coulter Epics XL.

Mixed lymphocyte reaction. On Day 6 the dendritic cells were harvested, washed 3 times with RPMI, irradiated with 20 Gy (6 min with 3.35 Gy/min), and seeded in triplicates in 96 well flat bottom flasks (Greiner) in serially diluted concentrations. Frozen autologous cells (see cell culture) were thawed. T cells were isolated by CD4 coated magnet beads according to manufacturer's instructions (Dynal) and added to the dendritic cells at 2×10^5 cells/well. As positive control for T cell proliferation, triplicates of T cells were incubated with 4 µg/well Phytohaemagglutinin-L (Sigma) and 40 U rhIL-2/well (Strathmann Biotec). On Day 5 cells were pulsed with 0.2 µCi/well tritiated thymidine (^{3}H-thymidine; Amersham Buchler, Braunschweig, Germany) for 18 h, then harvested and washed 3 times on Beckmann ready filters (Micro Cell Harvester; Skatron Instruments, USA); ^{3}H-thymidine incorporation was measured on a liquid scintillation counter (1205 Betaplate, Wallac, Finland).

Determination of TNF-α. Supernatants of dendritic cell cultures were harvested on Day 6, cultivated with medium, KLH with or without IDS 30, and stored at –20˚C until the assay procedure. ELISA was applied for quantitative determinations of TNF-α release and measured according to the manufacturer's instructions (Medgenix, Ratingen, Germany). IDS 30 showed no crossreactivity in the assay.

Statistical analysis. An unpaired single tailed Student t test was used to analyze experimental results. A p value < 0.05 was considered to indicate a significant deviation from the control measurements. The value of correlation (r^2) was determined.

RESULTS

The extract IDS 30 was applied on dendritic cells in a concentration range of 1.56 up to 25 µg/ml. It showed no toxic effect on any cell type when measured with propidium iodide (data not shown). The expression rates of specific proteins in immature dendritic cells like the differentiation marker CD83 or costimulatory molecules like CD40, CD80, CD86, HLA-ABC, and HLA-DR were similar to described levels[13]. There was no effect of IDS 30 on the expression of these proteins in dendritic cells cultured in RPMI containing IL-4 and GM-CSF lacking KLH (data not shown).

Influence of IDS 30 on expression of maturation associated surface molecules. The influence of IDS 30 on the expression of surface molecules on maturing dendritic cells was measured. Maturation was determined by adding KLH, described to be used to generate mature dendritic cells[30,31]. In all cases the inhibitory effect was significant from 1.56 µg/ml compared to the positive control ($p < 0.05$). Figure 1

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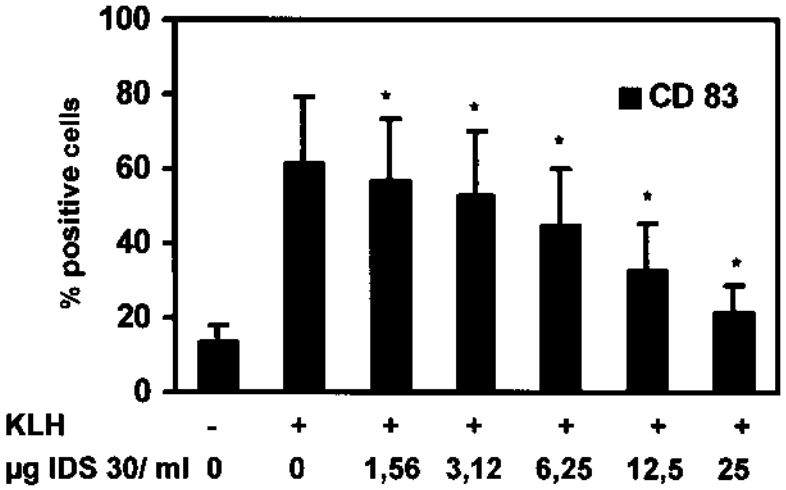

Figure 1. Dose dependent downregulation of CD83 of dendritic cells in response to IDS 30. On Day 4, dendritic cells (1×10^6/ml) were incubated without (negative control, –) or with (positive control, +) KLH with the noted concentrations of IDS 30. On Day 6, the percentages of cells expressing CD83 were determined by flow cytometry (n = 6; *p < 0.05).

shows that IDS 30 reduced the percentage of positive cells expressing the maturation associated protein CD83 in a dose dependent manner up to 61.5 ± 11.6% at highest IDS 30 concentration. These data show a strong correlation between the concentration of IDS 30 and the inhibitory effect on the expression of CD83 ($r^2 = 0.94$). Figure 2 shows the expression of the costimulatory molecules CD80 and CD86. The mean fluorescence intensity (MnX) of both was reduced. CD86 was lowered to 70.3 ± 37.5% ($r^2 = 0.79$). IDS 30 had a mild effect on the MnX of the costimulatory molecule CD80 ($r^2 = 0.91$). It was 28 ± 25% lower at the highest IDS 30 concentration compared to the control. The expression of the costimulatory molecules CD40, HLA-ABC, and HLA-DR IDS 30 were weak or not affected by IDS 30 (data not shown).

In contrast to CD83 and CD86, the CCR 5 and CD36 (collagen type I receptor, thrombospondin receptor) were highly expressed by immature dendritic cells and expressed in very low measure by mature dendritic cells. After incubation with KLH and the stinging nettle leaf extract a high percentage of dendritic cells were stained positively (Figure 3). The expression of CCR 5 was as high as the expression rate of the immature form of dendritic cells. The expression of CD36 was raised to 81.1 ± 11.7% at highest IDS 30 concentration compared to 26.6 ± 7.1% of mature dendritic cells incubated without IDS 30 in the presence of KLH. These data show good correlation between the concentration of IDS 30 and the stimulatory effect on CD36 ($r^2 = 0.84$) and CCR 5 ($r^2 = 0.96$).

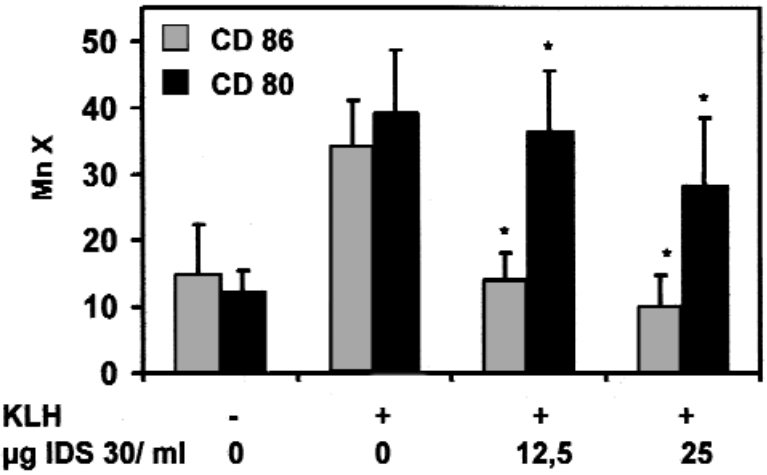

Figure 2. Dose dependent downregulation of CD80 and CD86 of dendritic cells in response to IDS 30. On Day 4, dendritic cells (1×10^6/ml) were incubated without (negative control, –) or with (positive control, +) KLH with noted concentrations of IDS 30. On Day 6, the mean fluorescence intensity of cells expressing CD80 and CD86 were determined by flow cytometry (n = 6; *p < 0.05).

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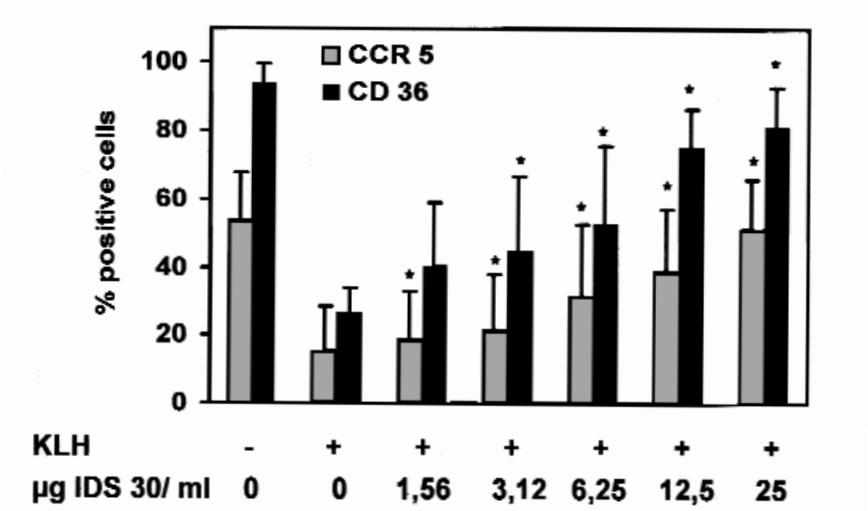

Figure 3. Dose dependent upregulation of CD36 and CCR 5 of dendritic cells in response to IDS 30. On Day 4 dendritic cells (1×10^6/ml) were incubated without (negative control, –) or with (positive control, +) KLH with noted concentrations of IDS 30. On Day 6, the percentages of cells expressing CD36/CCR 5 were determined by flow cytometry (n = 6; *p < 0.05).

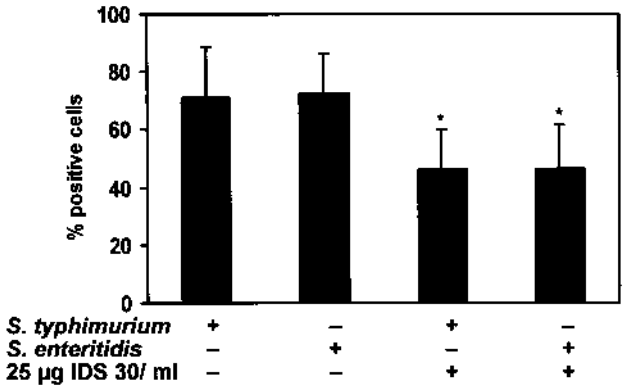

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Figure 4. Modulation of CD83 expression of dendritic cells matured using LPS in response to IDS 30. On Day 4 dendritic cells (1×10^6/ml) were incubated without (negative control, –) or with (positive control, +) 500 pg LPS from *S. typhimurium* or *S. enteritidis* in the presence or absence of 25 μg/ml of IDS 30. On Day 6, the percentages of cells expressing CD83/CD36 were determined by flow cytometry (n = 6; *p < 0.05).

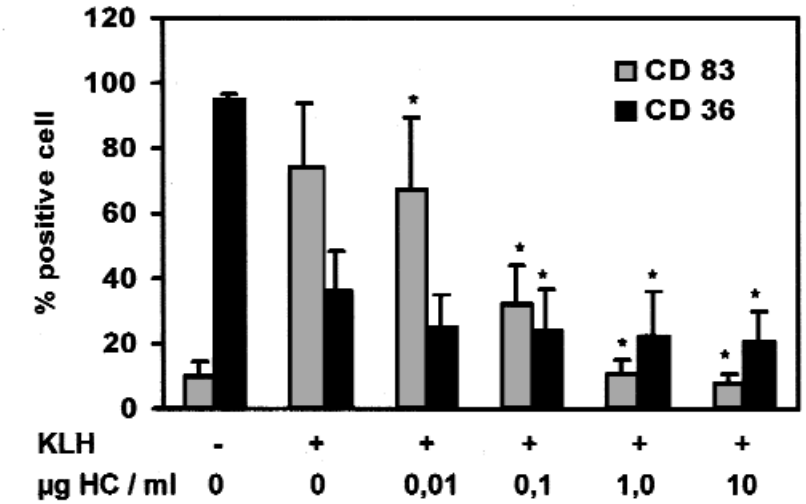

Figure 5. Dose dependent reduction of CD83 but no upregulation of CD36 of dendritic cells in response to hydrocortisone (HC). On Day 4 dendritic cells (1×10^6/ml) were incubated without (negative control, –) or with (positive control, +) KLH with noted concentrations of HC. On Day 6, the percentage of cells expressing CD83/CD36 was determined by flow cytometry (n = 6; *p < 0.05).

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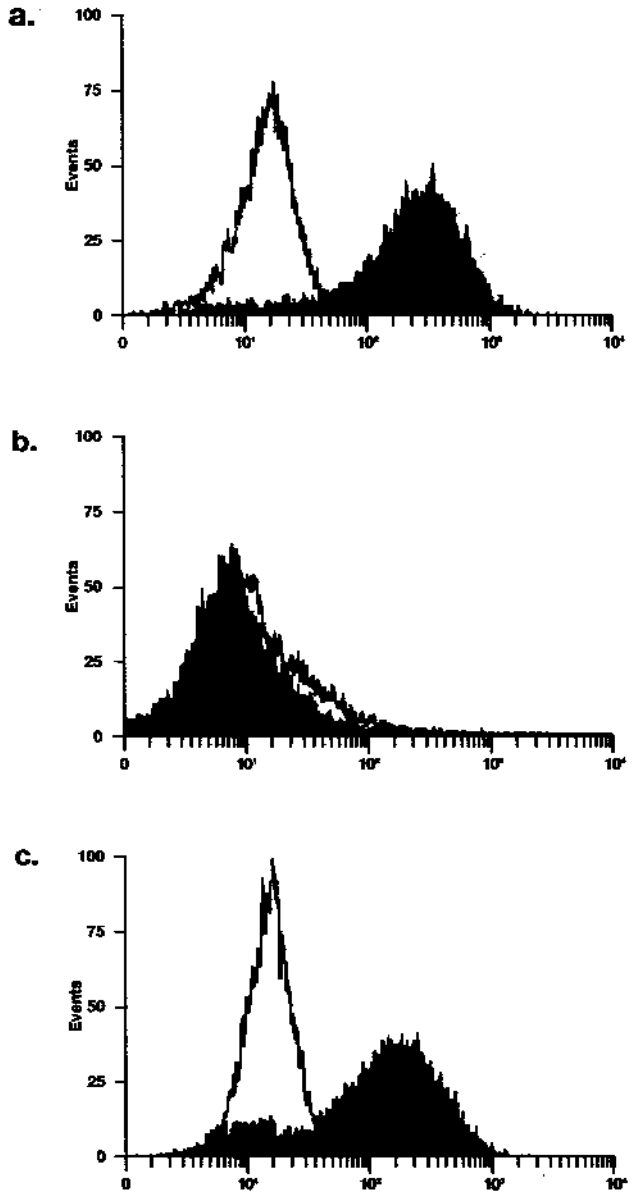

Figure 6. IDS 30 enhances the endocytic activity of dendritic cells. On Day 4 dendritic cells (1×10^6/ml) were incubated 48 h in medium (a), with KLH (b), or with KLH and 25 μg IDS 30/ml (c). On Day 6 the dendritic cells were incubated at 4°C as control (white histograms) and at 37°C (black histograms). Data represent one of 6 independent experiments ($p < 0.05$).

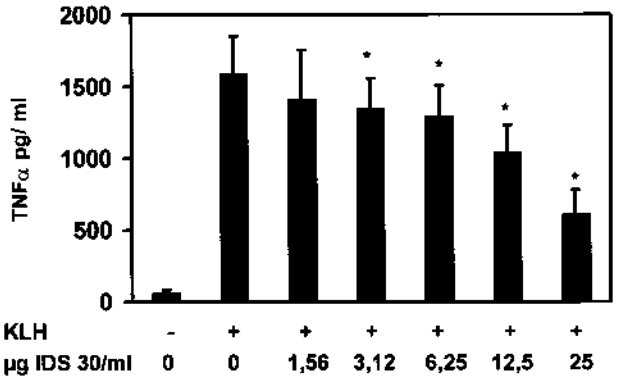

Figure 7. IDS 30 decreases the expression of TNF-α by dendritic cells. On Day 4 dendritic cells were incubated without (negative control, –) or with (positive control, +) KLH with noted concentrations of IDS 30. On Day 6 dendritic cell culture supernatants were harvested and analyzed for TNF-α by ELISA. Mean values ± SD of 6 independent experiments are shown ($n = 6$; *$p < 0.05$).

There were also effects of IDS 30 using other maturation methods. Mature dendritic cells generated using lipopolysaccharides of *Salmonella typhimurium* and *S. enteritidis* were influenced in their expression of CD83. Its expression was 34 ± 19.3% *(S. typhimurium)* and 35 ± 21% *(S. enteritidis)* lower at 25 μg Hox 30/ml, a somewhat milder effect than on dendritic cells matured using KLH (Figure 4).

Glucocorticoids are known to suppress the maturation of dendritic cells. To compare the effect of IDS 30 and a glucocorticoid we examined the influence of hydrocortisone on the expression of CD83 and CD36. Figure 5 shows the divergent results of the tested substance compared to IDS 30. Like IDS 30, hydrocortisone also inhibited the KLH induced expression of CD83 ($r^2 = 0.38$). The KLH induced expression was reduced from 74.12 ± 19.8% in medium to 7.55 ± 3.09% with 10 μg/ml hydrocortisone. But the effect of hydrocortisone on the expression of CD36 differed from the effect of IDS 30. In contrast to IDS 30, hydrocortisone did not enhance the expression of CD36 ($r^2 = 0.23$). Instead, the expression of the CD36 receptor was inhibited. The CD36 receptor is normally highly expressed by immature dendritic cells. Similar effects in the same concentration range were seen using methylprednisolone (data not shown).

Endocytic capacity of dendritic cells. Mannose receptor mediated endocytosis is a method used to detect antigen uptake by dendritic cells[7]. Dextran-FITC internalization was examined. As shown in Figure 6, the percentage of internalization of dextran-FITC in dendritic cells was raised to 73.4 ± 34.7% in the presence of 25 μg/ml IDS 30.

Expression of TNF-α from mature IDS 30 treated human dendritic cells. Stimulation of dendritic cells by KLH resulted in increased release of TNF-α. Figure 7 shows that after addition of IDS 30 to KLH stimulated cells for 48 h, the expression was lowered about 61 ± 28.9% at the highest IDS 30 concentration. These data show a strong correlation between the concentration of IDS 30 and the inhibitory effect on TNF-α ($r^2 = 0.980$).

Effect of IDS 30 on dendritic cell stimulation of T cell proliferation. Dendritic cells are important for the stimulation of T cell proliferation since they act as antigen presenting cells. The influence of IDS 30 on the stimulating capacity of

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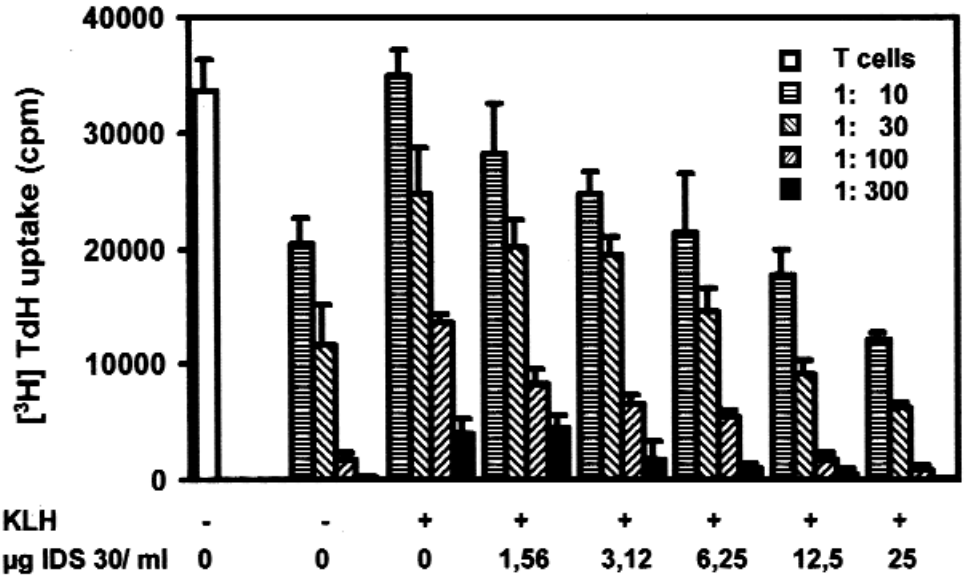

Figure 8. IDS 30 treated mature dendritic cells were studied for their potential to stimulate the proliferation of autologous T cells by mixed lymphocyte reaction. Triplicates of 2×10^5 T cells were cultured with serially diluted autologous irradiated dendritic cells. Dendritic cells were incubated without (negative control, –) or with (positive control, +) KLH with noted concentrations of IDS 30. The T cell control (white bar) was incubated with 4 μg PHA-L and 40 U IL-2/well. After 5 days of incubation T cells were pulsed with ^{3}H-thymidine and its incorporation was measured 18–20 h later. Data represent mean counts per minute (cpm) of triplicate wells ± SD. Results are typical of 4 independent experiments with cells from healthy volunteers.

mature dendritic cells on T cells was measured using the mixed lymphocyte reaction: 2×10^5 autologous T cells were incubated with serial dilutions of a number of dendritic cells, which were irradiated as described[32]. After 5 days the proliferation rate of the T cells was determined by ^{3}H-thymidine incorporation. Figure 8 shows the lower capacity of IDS 30 treated dendritic cells to trigger the proliferation of autologous T lymphocytes in a dose dependent manner.

DISCUSSION

Our data confirm that IDS 30 modulates the differentiation stage of dendritic cells *in vitro*. We measured a dose dependent inhibitory influence on the expression of the surface proteins, on the secretion of cytokines, on the phagocytic activity, and on their capacity to activate T cells.

The measured surface molecules are known to play an important role in the inflammatory process in the synovial tissue of RA[1,11]. The results of this study suggest a possible mechanism for the antiinflammatory effect of the IDS 30 extract in the inflamed tissue of rheumatic joints.

A comparison with the potent immunosuppressive agent hydrocortisone was made. Glucocorticoids like methylprednisolone or hydrocortisone are used as immunosuppressive and antiinflammatory therapy to treat autoimmune and allergic diseases[33-36]; hydrocortisone is often administered as a dermatological preparation. Glucocortiocoids are known to suppress the maturation of dendritic cells[37-39]. The comparison of IDS 30 with hydrocortisone showed a divergent effect on the expression of CD36 — expression of CD36 was raised only by the addition of IDS 30, and not by hydrocortisone. The expression of the CD36 cell surface epitope was raised upon addition of IDS 30 into the range found in immature dendritic cells. In the case of CD83, hydrocortisone was 100-fold more potent than the plant extract IDS 30, but this extreme effect of hydrocortisone may be a direct effect (related to undesired side effects of corticoid substances administered orally for the treatment of rheumatic diseases[40]), rather than the indirect result related to the dendritic cell maturational stage. There have not yet been any reports of negative side effects from the administration of stinging nettle leaf extract[41].

Immature dendritic cells have a high affinity to take up and process antigens. This ability is downregulated during the maturation process. In the presence of corticoid substances the endocytic capacity in presence of antigens is high. Our data show that in the presence of IDS 30, dendritic cell endocytosis is similarly increased. This high rate of endocytosis in the presence of antigen and the plant extract reflects inhibition of dendritic cell differentiation, and the consequently increased population fraction of immature dendritic cells.

One of the most important functions of dendritic cells is the stimulation of T cells by presenting the processed antigen. The subsequent clonal expansion of T cells leads to enhanced production of proinflammatory cytokines, causing the activation of macrophages in the vicinity. The inhibitory effect of corticoids on the differentiation of dendritic cells results in a reduction of the stimulating capacity with regard

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to T cells. The corticoids lower the expression of surface proteins, which are important for the interaction of dendritic cells with T cells in the initiation of inflammatory responses[37]. Reduced capacity to stimulate T cells was also observed with IDS 30 treatment of dendritic cells.

The immunosuppressive effect of IDS 30 on dendritic cells was milder in the presence of the immune stimulatory LPS from *S. typhimurium* and *S. enteritidis* than the effect of IDS 30 on dendritic cells in the presence of KLH. This could be traced back to the divergent maturation processes of dendritic cells when different immunostimulatory substances are used. KLH contains some endotoxin. It probably performs as an enhancer of the divergent maturation process of dendritic cells in the presence of the limped antigen.

The mechanism of action of IDS 30 is unknown. In comparison the mode of action of corticoids such as methylprednisolone is relatively clear. Methylprednisolone inhibits the transcription factor NF-κB, which controls the expression of genes of proinflammatory cytokines, for example. NF-κB is described as one of the most important transcription factors for the maturation of dendritic cells[10,37].

The inhibitory effect of an ethanolic stinging nettle leaf extract on the activity of transcription factor NF-κB has been described[42]. The extract inhibits the release of NF-κB from the cytosolic complex with IκB.

Several transcription factors other than NF-κB[9,43], e.g., Rel B (a member of the NF-κB/Rel family) and Oct-2[44], have been described to play a role in maturation of dendritic cells. The failed maturation of dendritic cells that leads to defective T cell stimulation may be involved in the development and maintenance of the autoimmunity characteristic of RA[1,11,45]. Further analysis is needed to determine which, if any, of these transcription factors is a target of the stinging nettle leaf extract IDS 30. The differential effects of IDS 30 and hydrocortisone on the expression of CD36 and CD83 indicate that there could be other putative targets than NF-κB, because CD36 is mainly regulated by the transcription factor Oct-2[10]. Further, the biologically active molecular components of the IDS 30 extract responsible for the immune suppression have yet to be isolated.

The results of this study strongly support the hypothesis that dendritic cells remain in their immature stage when under the influence of IDS 30. This *in vitro* investigation suggests a mechanism behind the antiinflammatory *in vivo* action of the stinging nettle leaf extract and its efficacy in the treatment of autoimmune diseases such as RA.

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