Aus der Universitäts-Hautklinik Tübingen
Abteilung Dermatologie (Allgemeine Dermatologie mit Poliklinik)
Ärztlicher Direktor: Professor Dr. M. Röcken

IL-4 mediated conditioning of human dendritic cell precursors to become IL-12p70 producing DC

Inaugural-Dissertation
zur Erlangung des Doktorgrades
der Medizin

der Medizinischen Fakultät
der Eberhard-Karls-Universität
zu Tübingen

vorgelegt von
Emmanuella Svetoslavova Guenova
aus Sofia

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Abbreviations

Ab	antibody
APC	allophycocyanin
CD	cluster of differentiation
DC	dendritic cell(s)
ELISA	enzyme-linked immunosorbent assay
(IC-) FACS	(intracytoplasmatic) fluorescence activated cell scanning
FITC	fluorescein isothiocyanate
GM-CSF	granulocyte-macrophage colony stimulating factor
IFN	interferon
Ig	immunoglobulin
IL	interleukin
LPS	lipopolysaccharide
mAb	monoclonal antibody
MHC	major histocompatibility complex
MoDC	monocyte-derived dendritic cell(s)
PAMP	pathogen associated molecular pattern
PBMC	peripheral blood mononuclear cell(s)
PE	phycoerythrin
PerCP	peridinin-chlorophyll-protein
PMA	phorbol 12-myristate 13-acetate
SD	standard deviation
SEB	staphylococcal enterotoxin B
Th	T helper
TNF	tumor necrosis factor

Introduction

Dendritic cells (DC) are antigen-presenting cells, characterised by their potency and ability to initiate primary MHC-restricted T cell responses [1, 2]. Several distinct subtypes of human dendritic cells, different in location, migratory route and immunological functions, were characterized and the developmental pathways of these most potent antigen presenting cells are beginning to be understood [3-5]. DC are cells of haematopoietic origin and precursor cells migrate from the bone marrow to peripheral tissues via blood stream [5]. In peripheral tissues, immature DC act as sentinels by continuously sampling antigens from the environment [6, 7]. Upon encounter of diverse danger signals such as microbial products or tissue injury, DC mature to migrate into the draining lymph nodes, where they efficiently activate antigen specific naïve or memory T cells [8]. Particularly at sites of chronic inflammation, early DC precursors are constantly recruited and differentiate into tissue resident immature DC under the influence of the local tissue milieu. Among other cell types, monocytes are well known to develop into DC *in vitro* [9, 10] or *in vivo* under certain inflammatory conditions [11-14]. This process may be of utmost importance for the regulation of immune responses both of the innate and adaptive immune system.

Interleukin (IL)-4 is a pleiotropic cytokine present in certain types of inflammation and is produced by $CD4^+$ T helper (Th), $CD8^+$ T cells, eosinophils, basophils, and activated mast cells. Initially described as B-cell stimulatory factor, at present, IL-4 is largely known for its capacity to initiate Th2 cell differentiation and to suppress Th1 responses and protective immunity against intracellular pathogens [15-17]. Recent murine studies, however, challenged the concept of IL-4 as classical Th2 driving cytokine and demonstrate opposing effects of IL-4 on the polarization of DC and T cells [18, 19]. Thereby, IL-4, when present during the initial activation of DC, led to a protective Th1 immune response in *Leishmania* infected mice [20]. In contrast, when also present during the period of T cell priming, IL-4 induced Th2 differentiation and susceptibility to *Leishmania major* [20, 21]. In humans, high IL-4 levels are also

associated with parasitic infections, but with increasing prevalence even more so with atopic diseases. Dominant expression of IL-4 is well documented for the initial phase of cutaneous inflammation in atopic dermatitis, which is followed by predominant IFN-γ expression in the chronic lesions. However the underlying mechanisms for this Th2 to Th1 switch are still not completely understood [22-25]. One possible explanation may be a dynamic process, orchestrated by the inflammatory milieu, determining the transition from DC precursors to functionally polarized DC subtypes [20, 26, 27].

Our challenge was to study the role of various IL-4 levels at sites of inflammation on differentiation and function of the newly arriving DC precursors using human monocytes as potential *in vivo* precursors.

In order to elucidate the role of IL-4 on the development and maturation of monocyte-derived DC, we differentiated human DC from peripheral blood. High but physiological concentrations of IL-4 [28] during DC precursor development resulted in a DC-phenotype with significantly enhanced IL-12p70 production that primed autologous naïve human $CD4^+CD45RA^+$ T cells towards a Th1 phenotype. In contrast, lower levels of IL-4 concentrations promoted IL-10 production and matured DC developed a phenotype producing low amounts IL-12p70 allowing the development of IL-4 producing Th2 cells. Equivalent results were obtained with DC from multiple donors. Since effective adaptive immune responses to microbes, tumours or self- and environmental antigens all depend on IL-4 [29, 30], this predicted role of IL-4 in orchestrating immune responses by fine tuning IL-12p70 and IL-10 produced by DC may be a general mechanism that determines the outcome of adaptive immunity at the time of initiation.

Materials and Methods

Apparatuses and instruments

- Cell Quest Pro® software (Becton Dickinson, Mountain View, CA, USA)
- Ascent Software® v2.6 (Thermo Fisher Scientific, Inc. Waltham, USA)
- CO_2 incubator (Heraeus, Vienna, Austria)
- Confocal laser scanning microscope (CLSM 510, Zeiss, Jena, Germany)
- Cooling centrifuges (Heraeus)
- FACSAria Cellsorter (BD Biosciences, San Jose, USA)
- Fluorescence-activated cell sorter (BD Biosciences)
- Freezers (-20°C, -80°C) (Heraeus)
- Fridges (Heraeus)
- Laminar flow (Holten, Allerod, Denmark)
- Liquid scintillation beta counter Wallac 1409 (PerkinElmer Waltham, Massachusetts, USA)
- Microcentrifuges (Eppendorf, Hamburg, Germany)
- Microplate photometer (Multiskan Ascent 51118307, Thermo Fisher Scientific, Inc. Waltham, USA)
- Optical microscope (Laborlux S, Leitz GmbH & Co. KG, Oberkochen, Germany)
- Pipetaid (Costar, Cambridge, MA, USA)
- Pipetboy (1-1000 µl; Costar; Eppendorf)
- Scale (Sartorius, Vienna, Austria)
- Scissors (Ethicon GmbH, Deutschland)
- Water bath (Telna Instruments, St-Orens de Gameville, France)

Plastic material

- Culture flasks (25, 75, 125, 220 cm^2; Costar; Nunc, Roskilde, Denmark)
- Culture plates (6-, 24-, 48-, and 96-well; Costar)
- Gloves (Smith & Nephew plc, London, England)
- Microtubes for flow cytometry (Micronic, Lelystad, The Netherlands)
- Petri dishes for tissue culture (100x20 mm; Corning, NY, USA)
- Pipettes (1, 2, 5, 10, 25, 50 ml; Costar)
- Polypropylene tubes (14 and 50 ml; Falcon, Lincoln Park, New Jersey, USA)
- Sterile filters Millex-GS (0.20 and 0.40 mm; Millipore S.A., Molsheim, France)
- Sterile tips (1-100 and 200-1000 ml; Costar)

Reagents

- ^{3}H-thymidine [(^{3}H) TdR; Amersham, Arlington Heights, IL, USA]
- Anti-IL-10 neutralizing antibody (MAB217; RnD Systems, Inc., Minneapolis, MN USA)
- Bovine serum albumin (BSA; Sigma-Aldrich, SL Louis, USA)
- Brefeldin A (Sigma-Aldrich)
- Dimethyl sulphoxide (DMSO; Sigma-Aldrich)
- Ethanol (70%; Merck KGaA, Frankfurter Str. 250, 64293 Darmstadt, Germany)
- Fetal calf serum (FCS; PAA Laboratories, Linz, Austria)
- Ficoll/Paque (Biochrom, Berlin, Germany)
- GM-CSF (Novartis Institutes for Biomedical Research, Vienna, Austria)
- IL-2 (Chiron GmbH, Munich, Germany)
- IL-4 (RnD Systems, Inc.)
- Ionomycine (Sigma-Aldrich)
- Paraformaldehyde (PFA; 37%; Merck)
- Phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich)
- Phosphate-buffered saline (PBS 1x; Gibco Life Technologies GmbH, Eggenstein, Germany)
- Propidium iodide (PI; Sigma-Aldrich)
- Saponine (Sigma-Aldrich)
- Sodium azide (NaN_3; Merck)
- Sodium citrate (Merck)
- staphylococcal enterotoxin B (SEB; Sigma-Aldrich)
- Trypan blue (0.4%; Sigma-Aldrich)
- X-VIVO 15 cell culture medium (BioWhittaker; Walkersville, Maryland, USA)

Source of primary cells

Peripheral blood mononuclear cells (PBMC) were obtained from leukapheresis products of healthy volunteers from the blood bank of the University of Tübingen. After Ficoll/Paque density gradiert separation (Biochrom, Berlin, Germany) monocytes were directly isolated and used for dendritic cell culture, as described below. PBMC for DC/T-cell co-cultures were preserved in X-VIVO 15 culture medium (BioWhittaker; Walkersville, Maryland, USA) under minimal serum-deprived conditions (1% heat-inactivated autologous human plasma), supplemented with 10U/ml rhIL-2 and stored at 37°C in a humidified atmosphere containing 5% CO_2 until use. $CD4^+$ or $CD4^+CD45RA^+$ T cells were sorted from PBMC and used for subsequent experiments.

Generation and activation of human monocyte derived DC

DCs were generated from adherent PBMCs as previously described [31]. Briefly, to obtain immature DC, PBMCs were allowed to adhere at $5x10^6$ cells/ml onto 6-well plates (Corning Inc., Costar, Cambridge, USA) in X-VIVO 15 culture medium under minimal serum-deprived conditions. After 1 hour of incubation at 37°C, non-adherent cells were removed by stringent rinsing and adherent cells were cultured in dendritic cell culture medium (X-VIVO 15, supplemented with 1% autologous human plasma, various concentrations IL-4 [from 5 to 50ng/ml] (RnD Systems, Inc., Minneapolis, MN USA) and 50ng/ml GM-CSF (Novartis International AG, Basel, Switzerland). At day 6, the culture consisted of uniformly $CD11c^+$ $CD14^-$ HLA-$DR^+CD86^+CD83^-$ immature DC. Cell differentiation was monitored by light microscopy and the expression of cell surface molecules was flowcytometrically analyzed after 6 days of culture. To induce DC maturation, day 6 immature DC were cultured for additional 24 hours in the presence of LPS (1µg/ml; Alexis Biochemicals, San Diego, USA). Control DCs were left untreated. Anti-IL-10 neutralizing antibody (10µg/ml; RnD Systems, Inc.) was added when indicated. After 24 hours, IL-12p70, IL- 12p40 and IL-10 were measured by ELISA using matched Abs pairs from BD Pharmingen according to the manufacturer's recommendations. The limits of

detection of these ELISAs were as follows: 3,9 pg/ml (IL-12p70); 31,2 pg/ml (IL-12p40); 7,8 pg/ml (IL-10).

Flow cytometry

Cell surface staining of DC and T cells was performed using FITC-, PE-, PerCP- or APC-conjugated mouse monoclonal antibodies (mAbs) against CD4, CD14, CD11c, MHC II (HLA-DR) and CD86 (Caltag/Invitrogen Corporation, California, USA), CD83 (Beckman Coulter Inc., Fullerton, USA) and CD3 (BD Biosciences, San Jose, USA). Appropriate mouse IgG mAbs served as isotype control. The results were evaluated by FACSCalibur equipped with CellQuestPro® Software, both from BD Biosciences.

Isolation and activation of human $CD4^+$ T cells

Autologous T cells were sorted for CD3, CD4 and CD45RA expression as indicated using a FACSAria Cellsorter (BD Biosciences, San Jose, USA). Consecutively, LPS-stimulated dendritic cells were harvested, washed, and co-cultured with sorted autologous either $CD4^+$ or $CD4^+CD45RA^+$ T cells in the presence of 500 pg/ml staphylococcal enterotoxin B (Sigma Aldrich) in X-VIVO 15, supplemented with 5% autologous human heat-inactivated plasma and 10 U/ml hIL-2 (Chiron GmbH, Munich, Germany). After 24 hours, cell proliferation was assessed by determining the incorporation of ^{3}H-thymidine. Intracellular FACS-Analysis of T cell cytokine production was performed as described [32]. Briefly, on day 12 of the co-culture, after 6 hours stimulation with PMA (50ng/ml; Sigma-Aldrich) and ionomycine (1 µg/ml; Sigma-Aldrich) in the presence of Brefeldin A (10µg/ml; Sigma-Aldrich) during the last 4 hours, the cells were fixed with 2% paraformaldehyde, permeabilized with 0,1% saponine (Sigma-Aldrich) and labelled with PE-conjugated mAbs against IL-4, IL-2, IL-10, TNF-α and IFN-γ (BD Biosciences) for 30 minutes at room temperature. To perform cytokine analysis via ELISA, after 12 days of co-culture, T cells were restimulated with 2 µg/ml immobilized CD3 mAb and 1 µg/ml soluble CD28 mAb (both a kind gift from Prof. G. Jung, Tübingen, Germany) and cultured for another 48 hours.

Supernatants were analysed for IL-4 using an ELISA Set from RnD Systems Inc. (detection limit 31,2pg/ml) and IFN-γ (detection limit 4,7 pg/ml) and IL-10 (detection limit 7,8 pg/ml) using BD OptEIA ELISA Sets from BD Biosciences according to the manufacturer's instructions.

Statistical testing

Student's *t*-Test (two-tailed) was used for statistical analysis of differences between two groups. p-values ≤ 0,05 (*) were considered significant and p-values ≤ 0,01(**) - highly significant. Results represent means ± SD of at least five representative experiments, unless indicated otherwise.

Results

IL-4 dose-dependently regulates IL-12 producing capacity by human monocyte-derived DC

Activation of DC via TLR4 and other receptors for pathogen associated molecular patterns (PAMP) physiologically signals for the induction and secretion of IL-12.

In order to determine the optimal concentration of LPS for functional DC maturation in our experimental setting, we measured the level of secreted IL-12p40, IL-12p70 and IL-10 by human monocyte derived dendritic cells in response to LPS (Fig. 1). The optimal LPS concentration for all three cytokines was 1µg/ml. To determine the role of IL-4 for the development of DC and the potential crosstalk between IL-4 and LPS for IL-12 induction we differentiated human monocytes into monocyte-derived DC (MoDC) with varying doses of IL-4 and stimulated these cells with LPS for 24 hours (Fig. 2a).

$CD14^{+}CD11c^{-}$ monocytes were differentiated into DC using IL-4 concentrations ranging from 5 to 200 ng/ml, as these are concentrations suspected to be relevant for in vivo conditions [28]. Interestingly, adding raising IL-4 concentrations as late as for the last 3 days of the DC differentiation was sufficient to upregulate IL-12p40 levels. Differences in DC IL-12p40 production between low and high IL-4 concentrations were 6 fold and at IL-4 doses ≥ 200 ng/ml IL-12p40 production declined (Fig. 2b).

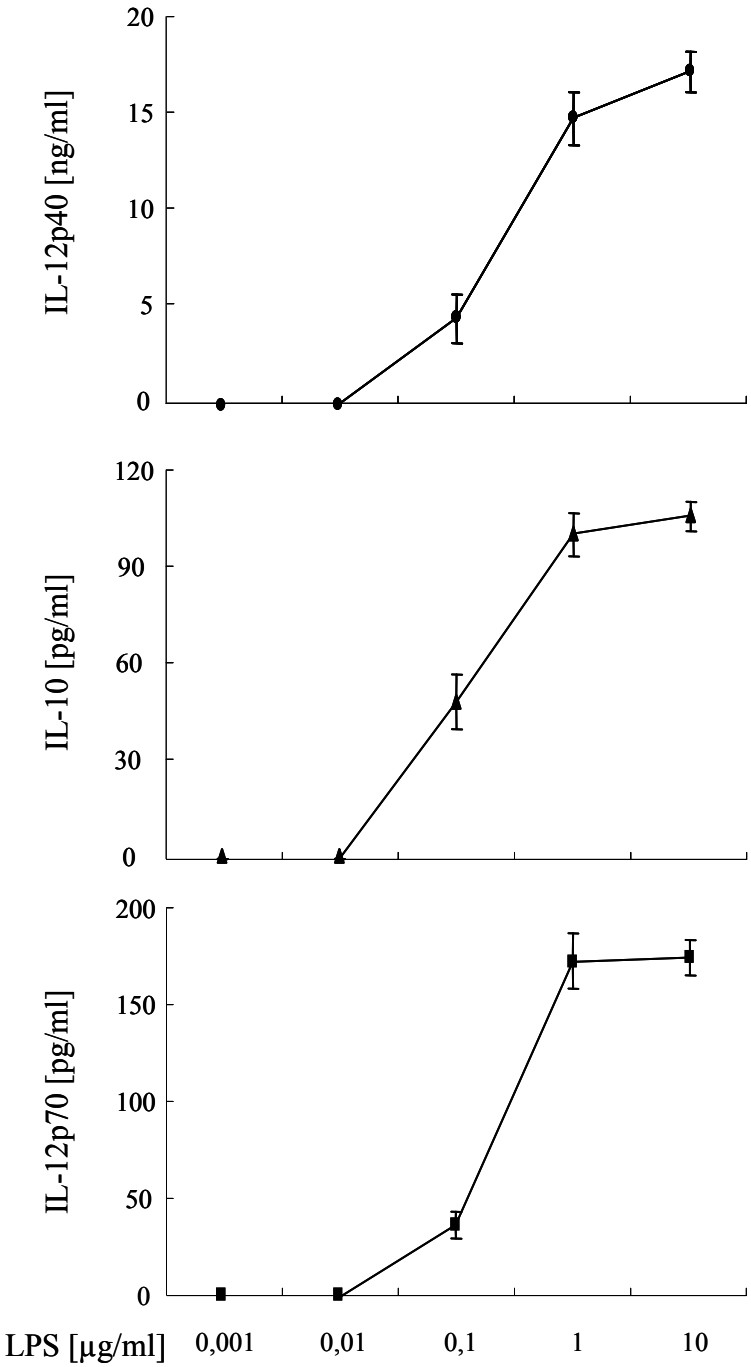

Figure 1. IL-4 dose-dependently regulates IL-12 producing capacity by human monocyte-derived dendritic cells.

DC generated in the presence of 50ng/ml IL-4 and 50ng/ml GM-CSF were stimulated with different doses of LPS and cytokine production was analyzed by ELISA technique. Experiments were repeated 3 times and results are expressed as mean ± SD.

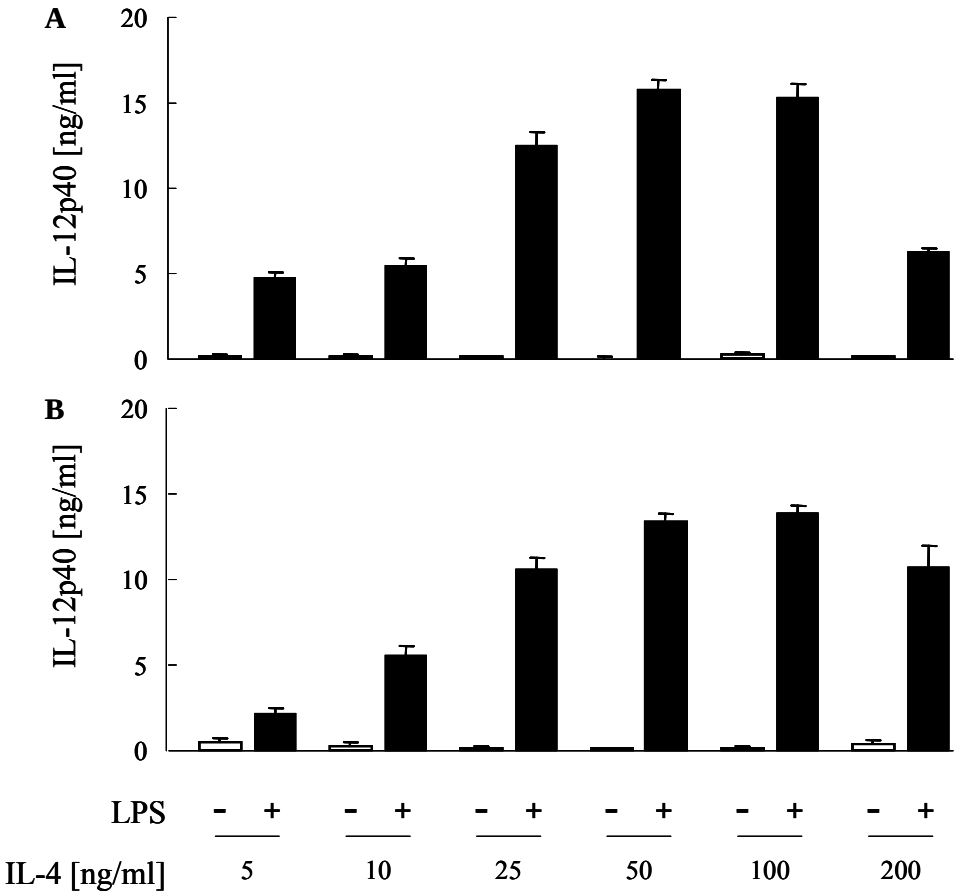

Figure 2. IL-4 dose-dependently regulates IL-12 producing capacity by human monocyte-derived dendritic cells.

A, DC were obtained in the presence of different concentrations of IL-4, stimulated with LPS and IL-12p40 secretion was analyzed by ELISA. Unstimulated immature DC served as negative control. Data are representative of 3 independent experiments and results are expressed as mean ± SD. B, $CD14^+$ monocytes were first cultured for 3 days with GM-CSF and low-dose IL-4 (5 ng/ml) followed by a three day culture with different doses of IL-4 (5 to 200 ng/ml). DC were stimulated with LPS and IL-12p40 production was analyzed by ELISA. Data are representative of 3 independent experiments and results are expressed as mean ± SD.

Importantly, the presence of exogenous IL-4 was needed during DC differentiation and not during TLR4 mediated IL-12 production as presence or

absence of IL-4 during DC stimulation with LPS did not affect the amount of released IL-12 (Fig. 3).

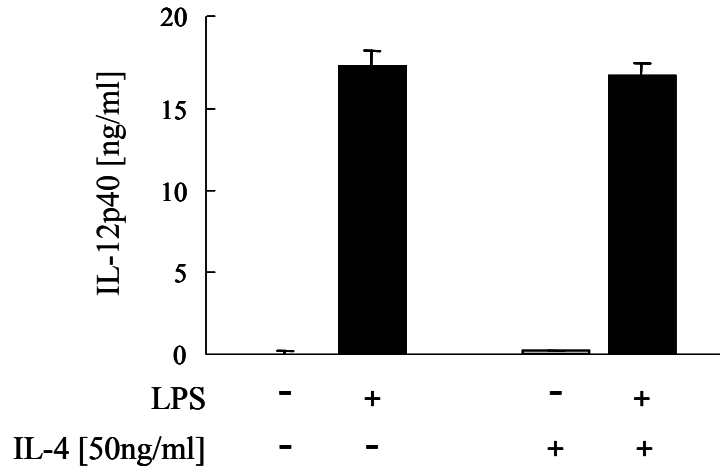

Figure 3. IL-4 dose-dependently regulates IL-12 producing capacity by human monocyte-derived dendritic cells.

$CD14^+$ PBMC were first cultured for 6 days with GM-CSF and high-dose IL-4 (50 ng/ml) followed by a 24 hours culture with or without IL-4 (50 ng/ml). DC were stimulated with LPS and IL-12p40 production was analyzed by ELISA. Data are representative of 5 independent experiments and results are expressed as mean ± SD.

DC differentiation from precursor cells under the influence of different doses of IL-4

As IL-4 significantly altered cytokine production by DC differentiated from monocytic precursor cells, we carefully analyzed whether IL-4 also had an effect on the differentiation of DC and DC surface marker expression. To this end, freshly isolated adherent $CD14^+$ human peripheral blood mononuclear cells (PBMC) were cultured in the presence of granulocyte-macrophage colony stimulating factor (GM-CSF) and IL-4 over 6 days. GM-CSF was applied in a concentration of 50ng/ml throughout all experiments. As IL-4 deficient culture conditions do not allow human monocytes to differentiate into dendritic cells [33], concentrations of IL-4 in the range from 5ng/ml to 50ng/ml were used in these experiments. This unequivocally resulted in the generation of immature

%	CD11c	CD14	MHCII	CD86	CD83
IL-4 5ng/ml					
Untreated	96 ± 4	4 ± 3	86 ± 13	79 ± 22	5 ± 3
+ LPS	95 ± 5	4 ± 3	91 ± 10	89 ± 10	53 ± 13
IL-4 50ng/ml					
Untreated	96 ± 3	4 ± 3	91 ± 8	83 ± 20	5 ± 3
+ LPS	97 ± 3	5 ± 2	89 ± 8	89 ± 10	51 ± 17

MFI	CD11c	CD14	MHCII	CD86	CD83
IL-4 5ng/ml					
Untreated	851 ± 205	7 ± 2	78 ± 19	86 ± 35	6 ± 3
+ LPS	962 ± 131	9 ± 4	210 ± 36	146 ± 28	24 ± 9
IL-4 50ng/ml					
Untreated	958 ± 257	7 ± 2	77 ± 13	93 ± 12	7 ± 3
+ LPS	1076 ± 226	10 ± 6	234 ± 33	153 ± 45	27 ± 9

Table 1. Analysis of marker expression of DC generated in the presence of IL-4 by flow cytometry.

DC were obtained from $CD14^+$ monocytes by culture with GM-CSF and different concentrations of IL-4 (5 to 50 ng/ml). After culture unstimulated or LPS-stimulated cells were analyzed for indicated marker expression by flow cytometry. Data are representative of 5 independent experiments and results are expressed as mean ± SD.

DC, characterized by a $CD11c^{high}/CD14^-$/ $MHCII^{low}/CD86^{low}$ phenotype (Fig. 4). No significant differences in the dynamic of DC generation and morphology were detected in terms of microscopic structure, cell viability under either low (5ng/ml) or high (50ng/ml) concentrations of IL-4. Analysis by flow cytometry

revealed that $CD11c^{+}$ cells with high forward and side scatter represented at least 94% of the population under all conditions (Tab. 1 and Fig. 4). All $CD11c^{high}$ dendritic cells unequivocally expressed $MHCII^{low}$ and $CD86^{low}$ irrespective of the different concentrations of IL-4. Moreover, CD83, a marker for dendritic cell maturation, remained negative during these different culture conditions (Fig. 4).

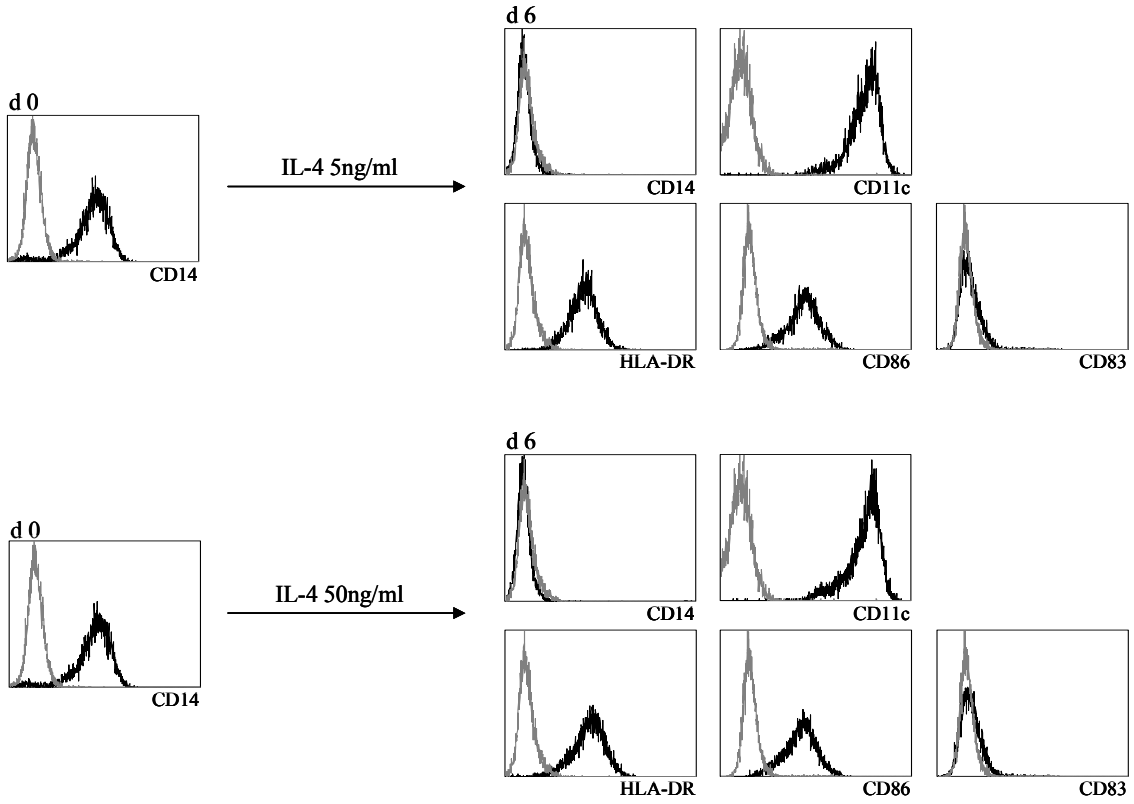

Figure 4. DC differentiation from precursor cells under the influence of different doses of IL-4.

IL-4 does not affect the generation of human monocyte-derived DC from PBMC. $CD14^{+}$ PBMC were cultured with GM-CSF and different concentrations of IL-4 (5 to 50ng/ml) and DC phenotype was analyzed subsequently by flow cytometry. Representative data of 5 independent experiments with similar results are given.

Upon stimulation with LPS day 6 immature dendritic cells increased of cell volume and granularity (Fig. 5), vigorously up-regulated CD86 and MHCII (Fig. 6) and acquired CD83 expression independent of the amount of IL-4 added (Fig. 7).

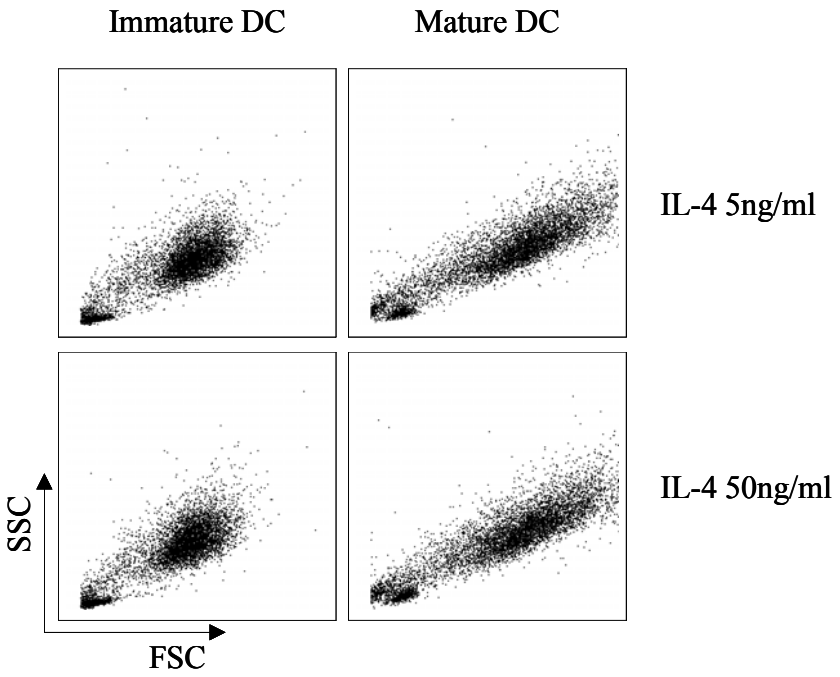

Figure 5. Maturation capacity of human DC differentiated under the influence of different doses of IL-4.

DC were obtained from $CD14^+$ monocytes by culture with GM-CSF and different concentrations of IL-4 (5 to 50ng/ml). On day 6 DC were stimulated with LPS and analyzed by flow cytometry for volume (FCS), granularity (SSC). Representative data out of 5 experiments are shown.

These data demonstrate that different concentrations of IL-4 present during differentiation from monocyte precursors to DC have no apparent impact on morphology and maturation of human monocyte-derived DC.

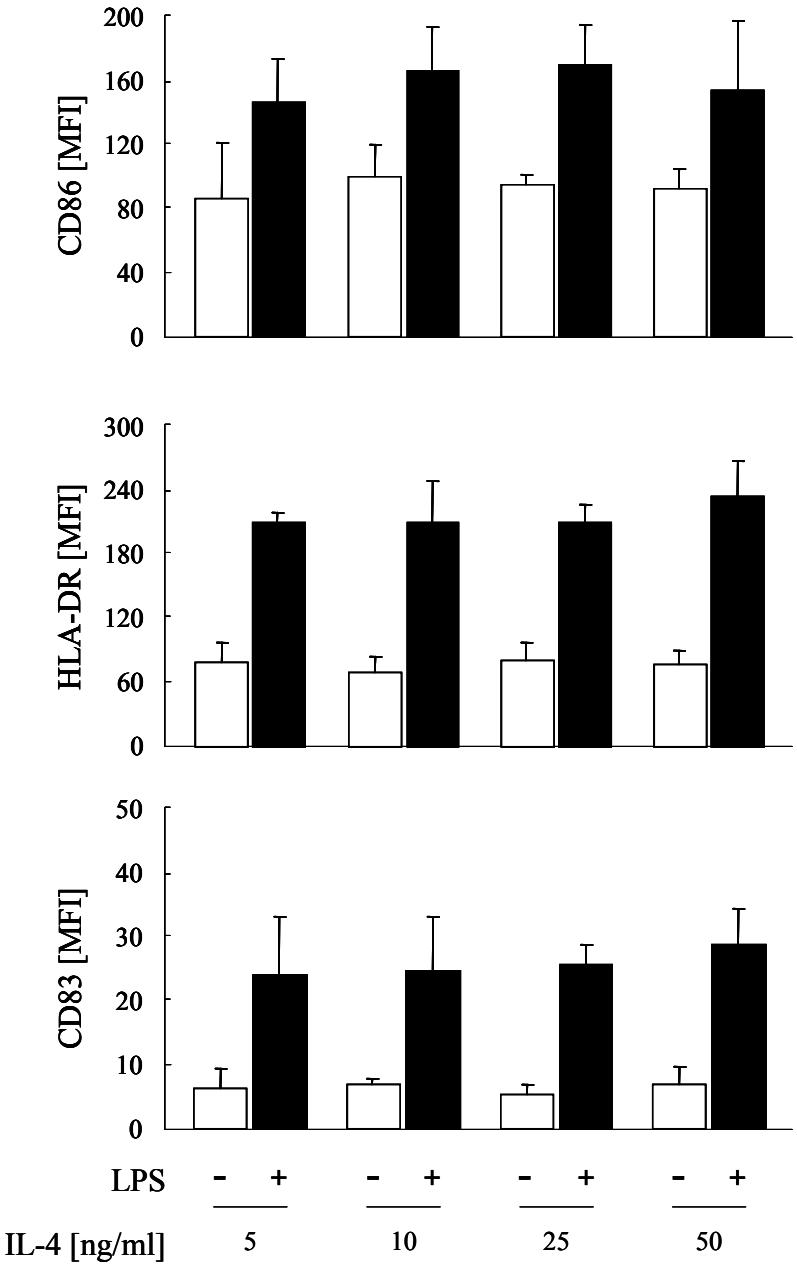

Figure 6. Maturation capacity of human DC differentiated under the influence of different doses of IL-4.

DC were obtained from $CD14^+$ monocytes by culture with GM-CSF and different concentrations of IL-4 (5 to 50ng/ml). On day 6 DC were stimulated with LPS and analyzed by flow cytometry for MHC II and maturation markers. Representative data out of 5 experiments are shown.

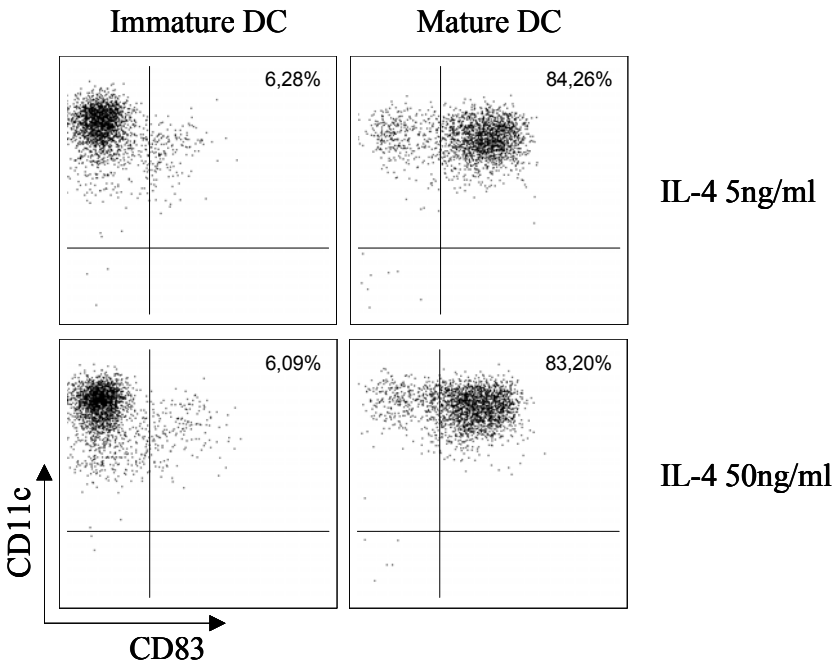

Figure 7. Maturation capacity of human DC differentiated under the influence of different doses of IL-4.

DC were obtained from $CD14^+$ monocytes by culture with GM-CSF and different concentrations of IL-4 (5 to 50ng/ml). On day 6 DC were stimulated with LPS and analyzed by flow cytometry for CD83. Representative data out of 5 experiments are shown.

IL-4 inversely regulates IL-12p70 and IL-10 production by human DC

To further elucidate the role of IL-4 on DC differentiation we next examined the effect of various doses of IL-4 on IL-12p70 and IL-10 production by LPS-stimulated DC. Unstimulated DC served as negative control and produced neither IL-12p70 nor IL-10. As suggested from the analyses of IL-12p40, DC

differentiated from monocytic precursors at 5 ng/ml IL-4 produced significantly less IL-12p70 than DC differentiated at 50 ng/ml, the optimal dose for the induction of IL-12p40 production (Fig. 8).

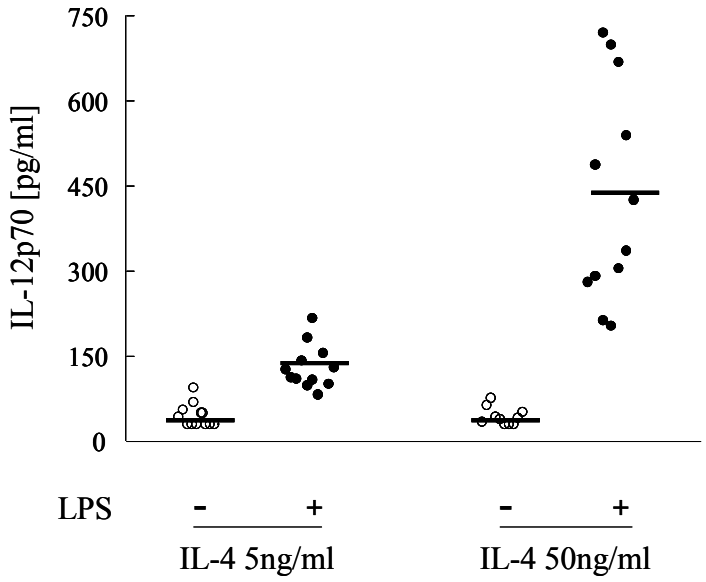

Figure 8. IL-4 inversely regulates IL-12p70 and IL-10 production by human DC.

DC were generated from human PBMC in the presence of either low or high dose IL-4, stimulated with LPS and IL-12p70 production was analyzed by ELISA. Results are obtained from blood samples of 5 different donors.

The capacity of DC to produce IL-10 was regulated inversely to IL-12p70 (Fig. 9), showing a critical regulatory role of IL-4 during DC differentiation on the

balance between pro-inflammatory IL-12p70 and anti-inflammatory IL-10 production after stimulation.

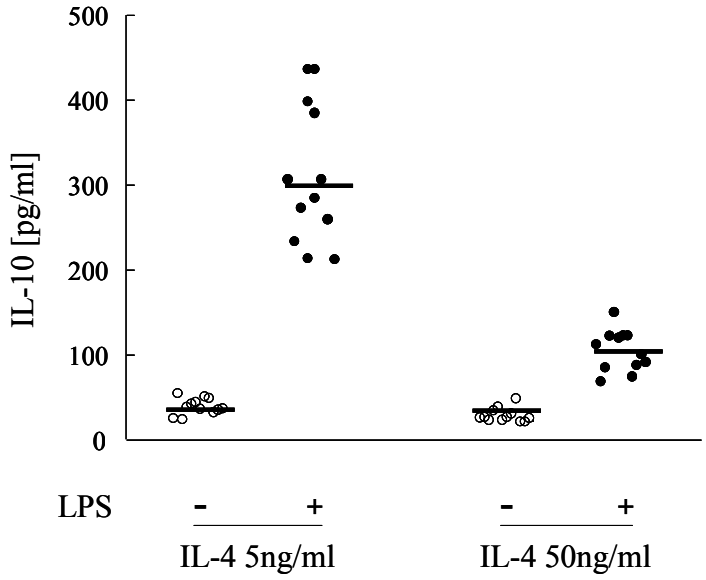

Figure 9. IL-4 inversely regulates IL-12p70 and IL-10 production by human DC.

DC were generated from human PBMC in the presence of either low or high dose IL-4, stimulated with LPS and IL-10 production was analyzed by ELISA. Results are obtained from blood samples of 5 different donors.

In DC differentiated with low IL-4 doses, the dominating cytokine was IL-10 leading to an IL-12 to IL-10 ratio <1. In contrast, DC differentiated at a dose of 50 ng/ml IL-4, the IL-12 to IL-10 ratio was close to 10, resulting in an IL-12p70 dominated cytokine milieu (Fig. 10).

Thus, low doses of IL-4 allow the differentiation of a predominantly IL-10 producing DC phenotype – even though the surface markers of the two types of DC were indistinguishable.

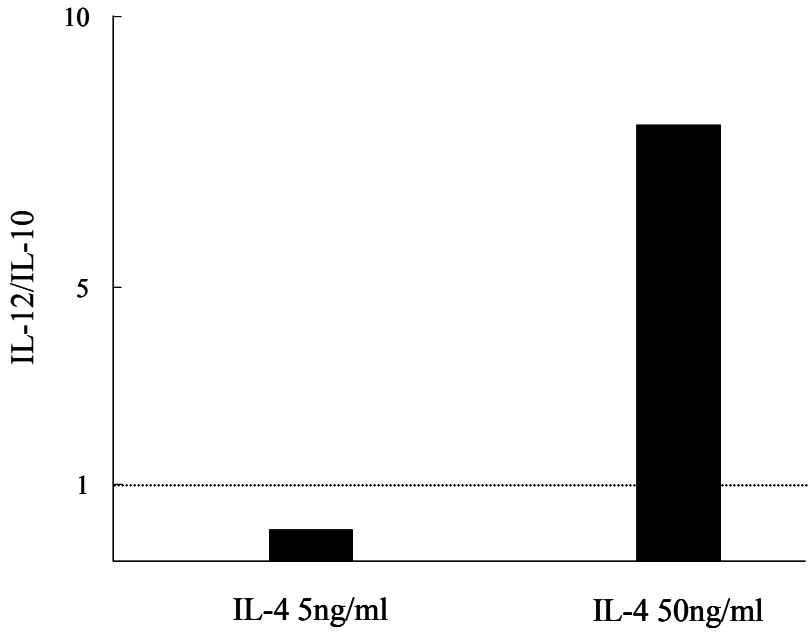

Figure 10. IL-4 inversely regulates IL-12p70 and IL-10 production by human DC.

DC were generated from human PBMC in the presence of either low or high dose IL-4, stimulated with LPS and IL-12p70/IL-10 production was analyzed by ELISA. Results are obtained from blood samples of 5 different donors and IL-12p70/IL-10 ratio is displayed.

Mutual negative regulation of IL-4 and IL-10 in the differentiation of human IL-12 secreting DC

It has been proposed that IL-4 promotes IL-12p70 production through activation of negatively regulatory promoter sites of the IL-10 promotor [34, 35]. Therefore,

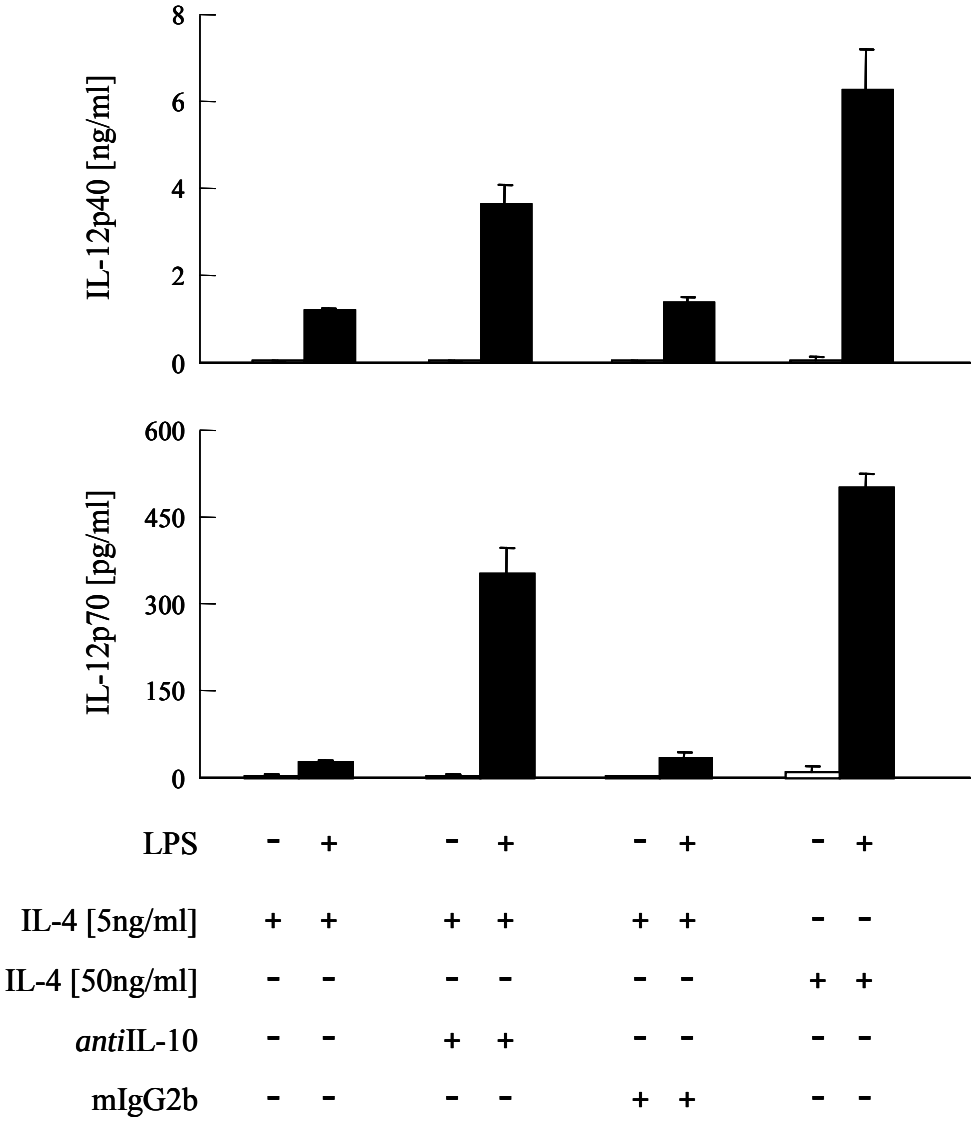

Figure 11. Mutual negative regulation of IL-4 and IL-10 in the differentiation of human IL-12 secreting DC.

Monocyte-derived DC were generated in the presence of low-dose (5 ng/ml) or high dose IL-4 (50 ng/ml). Additionally, IL-10 was blocked by neutralizing antibodies. After culture cells were stimulated with LPS and IL-12p40 and IL-12p70 secretion capacity was analyzed by ELISA.

we postulated that IL-10 might dominate at low concentrations and thus negatively regulate IL-12p70 induction in differentiating DC. To test this hypothesis, we neutralized IL-10 in the culture of low dose IL-4 treated DC. Blocking IL-10 with monoclonal antibodies resulted in a three-fold upregulation of both IL-12p40 and IL-12p70 in DC cultured with 5 ng/ml IL-4 (Fig. 11). On the other hand, supplementation of IL-10 to the culture of DC differentiated at high IL-4 doses reduced their capacity to produce either IL-12p40 or IL-12p70 (Fig. 12).

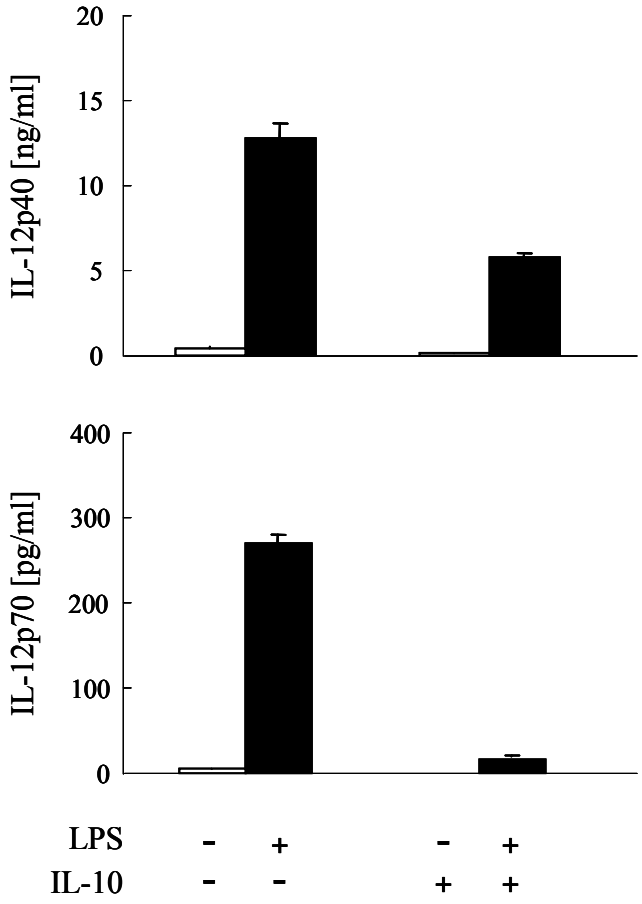

Figure 12. Mutual negative regulation of IL-4 and IL-10 in the differentiation of human IL-12 secreting DC.

Monocyte-derived DC were generated in the presence of low-dose (5 ng/ml) or high dose IL-4 (50 ng/ml). Additionally, IL-10 was added. After culture cells were stimulated with LPS and IL-12p40 and IL-12p70 secretion capacity was analyzed by ELISA.

IL-4 promotes a functional DC phenotype driving Th1 immune responses

One main function of IL-12 is to bridge innate immunity with the differentiation of adaptive immune responses. The priming of naïve T cells toward an IFN-γ producing Th1 phenotype *in vivo* reflects its pro-inflammatory role for adaptive immune responses [36]. To test whether IL-4 also effects the differentiation of functional Th1 promoting DC, we first analysed the capacity of DC differentiated under either low or high concentrations of IL-4 to induce a Th1 phenotype in normal peripheral T cells.

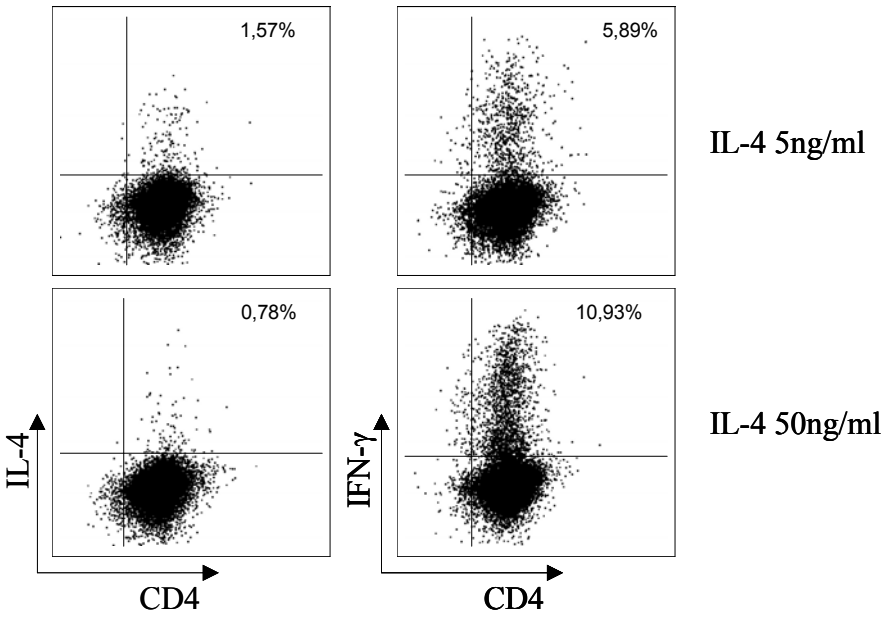

Figure 13. IL-4 promotes a functional DC phenotype driving Th1 immune responses.

DC were generated in the presence of low-dose (5 ng/ml) or high dose IL-4 (50 ng/ml), stimulated with LPS and cultured with allogeneic T cells in the presence of SEB for 12 days. T cells were restimulated with PMA/ionomycine or plate-bound CD3/CD28 and cytokine production was analyzed by flow cytometry Data are representative of 3 independent experiments and results are expressed as mean ± SD.

These DC were stimulated with LPS and activated autologous $CD4^+$ T-helper cells with SEB. After 12 days of co-culture, the $CD4^+$ T cells were harvested and stimulated either with PMA and ionomycine or with anti-CD3 and anti-CD28

antibodies for another 24 or 48 hours respectively for cytokine production. IC-FACS-analysis revealed that the percentage of IFN-γ expressing T cells was 1,8-times higher (exclusively enhanced) in T cells activated by DC differentiated at 50 ng/ml IL-4 than in those primed by DC differentiated at 5 ng/ml IL-4 (Fig. 13).

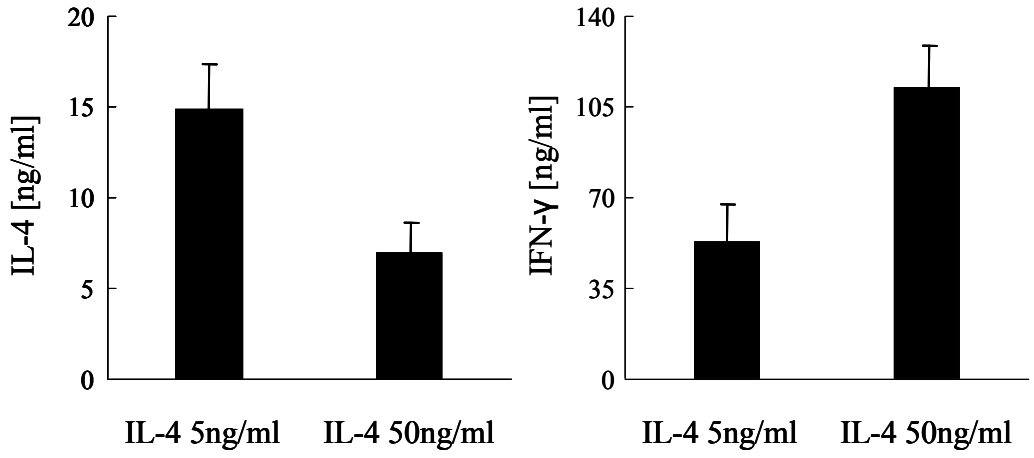

Figure 14. IL-4 promotes a functional DC phenotype driving Th1 immune responses.

DC were generated in the presence of low-dose (5 ng/ml) or high dose IL-4 (50 ng/ml), stimulated with LPS and cultured with allogeneic T cells in the presence of SEB for 12 days. T cells were restimulated with PMA/ionomycine or plate-bound CD3/CD28 and cytokine production was analyzed by ELISA. Data are representative of 3 independent experiments and results are expressed as mean ± SD.

ELISA of the 48 hours T cell culture supernatants provided even more pronounced differences with 2,1-fold enhanced IFN-γ production and 1,9-fold reduced IL-4 production in $CD4^+$ T cells activated by high-dose IL-4 differentiated DC compared to T cells activated by low dose IL-4 DC (Fig. 14).

IL-4 determines the capacity of DC to prime naïve $CD4^+CD45RA^+$ T cells for IFN-γ production

To differentiate between expansion of already polarized $CD4^+$ Th1 cells and *de novo*-induction of Th1 cells from naïve precursors, we sorted naive autologous $CD4^+CD45RA^+$ T cells and primed these naïve $CD4^+$ T cells with DC differentiated at low or high IL-4 concentrations. In agreement with the detailed analysis of the DC phenotype, DC differentiated at 5 ng/ml and those differentiated at 50 ng/ml induced similar proliferation in T cells confirming their equivalence in costimulation. In sharp contrast, immature DC induced only marginal T cell proliferation (Fig. 15).

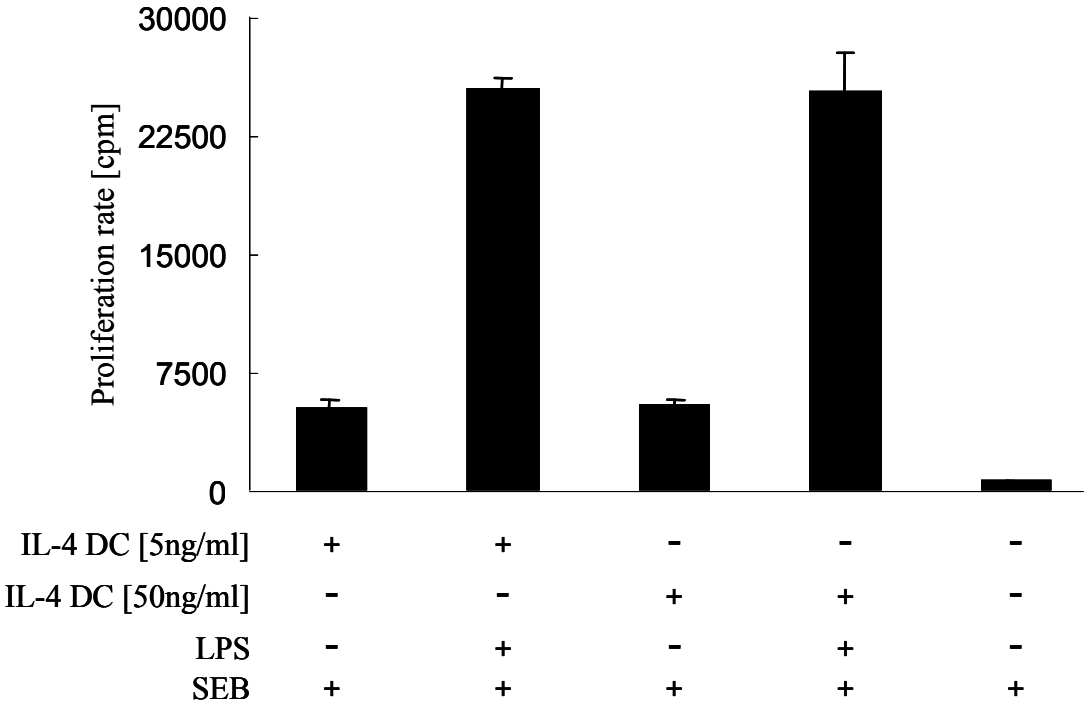

Figure 15. IL-4 promotes a functional DC phenotype driving Th1 immune responses.

IL-4 generated DC (low and high dose) were cultured with sorted naïve, autologous, $CD4^+CD45RA^+$ T cells. Proliferation of cells was determined by ^{3}[H]-Thymidin uptake. Data are representative of 3 independent experiments and results are expressed as mean ± SD.

The cytokine pattern of these T cells demonstrated that DC differentiated under high-dose IL-4, primed naïve human $CD4^+$ T cells toward an IFN-γ^{high}IL-4^{neg} Th1 cell phenotype. In contrast, naïve human $CD4^+$ T cells primed with DC differentiated at 5 ng/ml IL-4 produced 1,5 times more IL-4 and 2 times less IFN-γ confirming that these DC may allow the promotion of Th2 cells (Fig. 16).

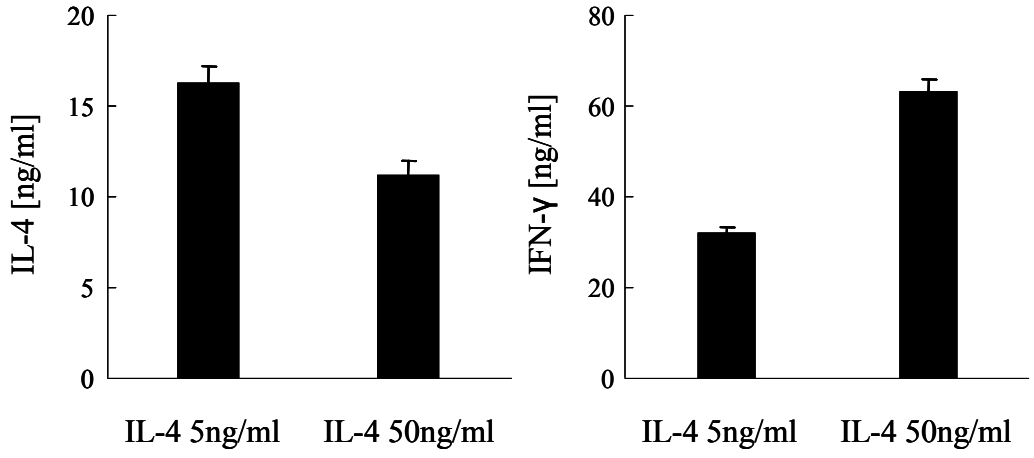

Figure 16. IL-4 promotes a functional DC phenotype driving Th1 immune responses.

IL-4 generated DC (low and high dose) were cultured with sorted naïve, autologous, CD4+CD45RA+ T cells. Cytokine production was analyzed by ELISA. Data are representative of 3 independent experiments and results are expressed as mean ± SD.

Together, these data clearly demonstrate that the concentration of IL-4 present during differentiation of DC precursors is crucial for the phenotype and quality of the resulting immune response. IL-12p70 producing DC result from an IL-4 dominated inflammatory cytokine milieu and are instructive for Th1 immune responses.

Discussion

In this study we demonstrate that IL-4 profoundly influences the functional DC phenotype in respect to T cell polarization in humans. DC constantly exit peripheral tissues, migrate to draining lymph nodes, and are replaced by newly recruited DC-precursors that differentiate into tissue resident DC [5, 37-39]. Using monocyte derived DC as DC precursor we show that high levels of IL-4 present during transformation determine IL-12p70 and IL-10 production by DC. Moreover, development of an IL-12p70 producing DC phenotype depends on IL-4 mediated suppression of IL-10 secretion during DC transformation. Consequently, DC derived from monocytic DC-precursors under high IL-4 conditions effectively primed naïve human autologous $CD4^{+}CD45RA^{+}$ T cells to become IFN-γ^{high}IL-4^{neg} Th cells, while DC differentiated under low IL-4 conditions allows the development of IL-4 producing Th cells. These data uncover the role of IL-4 in an inflammatory milieu for the differentiation of immigrated DC-precursors. Based on a regulatory balance between IL-12 and IL-10, IL-4 orchestrates the development of IL-12p70 producing DC and, consequently, IFN-γ dominated Th1 immune responses, a T cell phenotype abundant in chronic atopic dermatitis or chronic asthma.

The origin of tissue resident DC and the role of DC repopulating inflamed tissues is not yet completely understood. However, it is believed that i) newly immigrating haematopoietic precursors give rise to tissue resident DC [14, 37] and that ii) tissue-derived DC act as DC stem cells [5, 38, 39]. In the case of newly recruited human DC, it is widely accepted that cells of the monocytic lineage develop into tissue resident DC under appropriate conditions [11-13, 40-43]. Recently, it was proven experimentally that monocytes can transform into tissue resident DC *in vivo* and significantly contribute to the inflammatory milieu [14]. Yet, the exact factors that guide monocyte-derived DC precursor transformation into tissue resident DC are still unknown and, most importantly, the factors that orchestrate the functional phenotype of tissue resident DC are still enigmatic. On the other hand, the cytokine milieu present in skin diseases

such as atopic dermatitis is well characterized and IL-4 is known to play an important role in many inflammatory conditions, including allergic inflammation and atopic dermatitis [22-24, 27, 44-48]. As indicated by our studies, IL-4 is also crucial for the functional phenotype of fully differentiated DC providing the appropriate signals to stimulate, mature, and orchestrate Th cells.

Naïve Th cells differentiate into different and phenotypically distinct memory Th cell populations, among them Th1 and Th2 cells [49, 50]. This process is determined by the cytokine microenvironment present during T cell stimulation (IL-12 versus IL-4 respectively), the nature and strength of the pattern-recognition- and T cell- receptor- mediated signal, the genetic background and, most importantly, the type of the activation state of DC [30, 51, 52]. DC are the most potent antigen presenting cells that act as essential elements in the initiation and guiding of adaptive immunity. DC orchestrate Th cell polarization by expression of surface costimulatory molecules, by production of cytokines, and by modulating the cell-to-cell contact [53-56]. Thereby, DC determine the outcome and quality of immune responses allowing clearance of intracellular microbes such as Leishmania or the development of chronic inflammatory diseases such as inflammatory bowel disease or psoriasis by inducing Th1 cells through the secretion of IFN-γ. In atopic dermatitis or allergic asthma early phases of immune responses are characterized by high IL-4 levels [25]. On a T cell level, IL-4 is largely known for its capacity to initiate Th2 cell differentiation and to suppress Th1 responses, protective immunity against intracellular pathogens [21], and Th1 associated autoimmune diseases [20, 28, 57]. In humans, atopic dermatitis or allergic asthma display an early IL-4 dominated allergic inflammation that is followed by massive IFN-γ expression in the chronic lesions, a phenomenon referred to as “Th2 to Th1 cytokine switch” [22-24, 58]. Similar findings have been reported in animal models for AD [25, 45, 59]. The underlying mechanism of this `Th2 to Th1 cytokine switch´ in chronic asthma or atopic dermatitis is still not understood. The data reported here suggest that the high levels of IL-4 that dominate the initial phase of the allergic diseases, already determine the dynamics of the immune responses by conditioning DC

precursors to become Th1 inducing IL-12p70 producing DC cells. IL-4 is known to be a classical Th2 driving cytokine. Our results point toward a remarkable negative feed-back mechanism, as IL-12 is the most important Th1 and IFN-γ inducing cytokine [36, 60-62].

Culturing DC from precursor cells in a large scale is also important to develop cellular vaccination strategies for cancer or chronic infections [63]. The in vivo generation of cancer-antigen or microbe specific T cells, primed by adoptively transferred DC, aims to eradicate cancer cells and microbes. In these settings, $CD4^+$ T cells optimize the capacity of memory $CD8^+$ T cells to eradicate cancer cells [64-66]. Moreover, the therapeutic efficacy of $CD4^+$ T cells critically depends on a highly polarized, IL-4-deficient Th1 phenotype [67]. Some murine studies already showed that IL-4 can enhance the development of protective T cell-mediated immunity directed against tumor [29, 68, 69] or intracellular infection, when present during DC differentiation and activation [20]. Furthermore, IL-$4^{-/-}$ mice have a weakness in generating T-cell dependent protective immune reactions. This can be compensated by transplanting IL-4 producing tumor cells [70]. Taken together, these data from mouse studies already indicated that the presence of IL-4 during the differentiation of DC precursors can enhance delayed- type hypersensitivity reactions. In the light of our studies, it is likely that IL-4, present during DC precursor differentiation *in vivo* augments their capacity to effectively prime Th1 cell development. Whether application of IL-4 will allow improved vaccination strategies against cancer or microbes remains to be investigated.

Analyses of tissue factors that regulate DC phenotypes are of great importance for the understanding of inflammatory diseases especially how these exacerbate and clear and may help to improve tumor vaccination. Our study shows that one single T cell cytokine present in an inflammatory tissue critically determines the immune phenotype by orchestrating DC differentiation. It may

serve as “proof of principle” of how a tissue milieu in general can regulate DC phenotypes and complete immune responses.

Summary

During chronic inflammation, activated dendritic cells (DC), constantly migrate from peripheral tissues to draining lymph nodes and are replaced by newly recruited DC-precursors that differentiate into tissue resident DC. It is well documented that interleukin (IL)-4 expression dominates the early phases of multiple acquired immune responses, especially inflammation in atopic diseases such as atopic dermatitis and that DC are abundant at this early stage of inflammation. However, the precise role of IL-4 during early inflammation and its impact on the differentiation of newly recruited DC precursors remained elusive. To characterize the functional impact of IL-4 on the differentiation of human DC, we investigated in detail the role of IL-4 on the differentiation of monocytes into DC. As described, IL-4 is an inevitable prerequisite for the generation of DC and concentrations between 5 and 50ng/ml induced phenotypically indistinguishable DC upon activation with LPS. However, DC populations, differentiated under higher but physiological concentrations of IL-4, revealed a distinct polarized functional phenotype. High IL-4 concentrations during DC-precursor development resulted in DC that produced large amounts of IL-12p70, low IL-10 and primed naïve $CD4^+$ T cells to become Th1 cells. In contrast, DC generated under low dose IL-4 produced IL-10 instead of IL-12p70 and primed naïve $CD4^+$ T cells allowing IL-4 secretion and possibly Th2 induction. Thus, the amount of IL-4 released into the inflammatory milieu during early phases of immune responses might influence the differentiation of immigrating DC-precursors and the type of immune responses in response to infections or other stimuli as in atopic dermatitis or asthma.

Zusammenfassung

Während einer chronischen Entzündung wandern dendritische Zellen (DZ) kontinuierlich vom Ort des Geschehens (z.B. Haut) in die drainierenden Lymphknoten ein. Die fehlenden DZ werden dabei von Vorläuferzellen aus dem Blut rekrutiert, welche sich dann im Gewebe in DZ umwandeln. Das Interleukin 4 (IL-4) ist als dominierendes Zytokin in der Frühphase verschiedener erworbener Abwehrprozesse bekannt und vor allem in der atopischen Dermatitis. Zusätzlich findet man in der frühen Phase einer atopischen Hauterkrankung eine gehäufte Anzahl kutaner DZ. Ob nun eine Zusammenhang zwischen IL-4 und der gesteigerten Anzahl kutaner DZ in der atopischen Dermatitis besteht, und wie IL-4 auf die einwandernden Vorläuferzellen der DZ wirkt, ist bis heute nicht genau bekannt. Um dies zu untersuchen, studierten wir die Rolle von IL-4 auf das Differenzierungsverhalten von Monozyten in DZ. Hierbei zeigte sich, dass IL-4 für den Differenzierungsprozess notwendig ist. Zusätzlich fanden wir, dass verschiedene Konzentrationen von IL-4 (5 bis 50 ng/ml) keinen Effekt auf den Phänotyp von LPS-stimulierten DZ hat. Es zeigte sich jedoch, dass hohe IL-4 Konzentrationen die Funktionalität der DZ im Bereich der T-Zellantwort beeinflussten. Hohe IL-4 Spiegel während der Differenzierungsphase von DZ führten zu eine vermehrten Produktion von IL-12p70, hemmten aber gleichzeitig die IL-10 Sekrektion der DZ. Zusätzlich waren diese DZ in der Lage naive T-Zellen in einen Th-1 Phänotyp zu überführen. Im Gegensatz dazu produzierten DZ, welche in einem niedrigen IL-4 Milieu aus Monozyten generiert wurden, hohe IL-10 und niedrige IL-12p70 Spiegel und förderten die Entwicklung eines Th-2 Phänotyps in naiven T-Zellen. Unserer Experimente zeigen somit, dass erhöhte Mengen von IL-4, wie sie in der frühen Phase einer atopischen Dermatitis gefunden werden, die Entwicklung der DZ in der Haut maßgeblich beeinflussen und somit eine entscheidende Rolle in der weitere Pathogenese dieser chronischen Hautkrankheit spielen könnten.

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Curriculum Vitae

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EDUCATION AND PROFESSIONAL EXPERIENCE

1998	graduated at the Sofia English-Language-School with distinction
1998-2005	Study of Medicine: Eberhard-Karls-Universität Tübingen, Germany; Université de Genève, Switzerland; Medical University of Sofia, Bulgaria;
2005- 2006	Research fellowship, Eberhard-Karls-Universität Tübingen
since 2006	Resident at the Department of Dermatology, Eberhard-Karls-Universität Tübingen

MEMBERSHIPS AND ORGANISATIONS

2000-2004	International Federation of Medical Students' Organisations (IFMSA)
2005-	European Academy of Allergy and Clinical Immunology (EAACI)

GRANTS

2003	Grant of the Deutsche Akademische Auslandsdienst (DAAD)
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List of Publications

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