

Both exogenous and endogenous interleukin-10 affects the maturation of bone-marrow-derived dendritic cells *in vitro* and strongly influences T-cell priming *in vivo*

CLAUS HAASE, TRINE N. JØRGENSEN & BIRGITTE K. MICHELSEN

Hagedorn Research Institute, Gentofte, Denmark

SUMMARY

In order to avoid autoimmunity and excessive tissue destruction, the action of certain immunoinhibitory substances are very important for negative regulation of the immune system. Interleukin-10 (IL-10) is an important immunoregulatory cytokine which is thought to negatively affect both T cells and antigen-presenting cells *in vivo*. Adoptive transfer of IL-10-treated bone-marrow-derived dendritic cells (BMDCs) may be one therapeutic avenue to inhibit autoimmunity. In this study we present a comprehensive analysis of the effects of IL-10 on murine BMDC. We demonstrate that IL-10 can prevent BMDC maturation, as measured by both cytokine production and T-cell priming capacity *in vitro*. Furthermore, we show that IL-10 can inhibit DC maturation induced by strong stimulatory signals such as lipopolysaccharide or a mixture of cytokines (interferon- γ , tumour necrosis factor- α , IL-4). Interestingly, maturation of both T helper 1- and T helper 2-inducing DCs, characterized by the induction of high levels of interferon- γ and IL-4-production by responding T cells, respectively, was inhibited by IL-10 *in vitro*. Finally, our data suggest that both endogenous and exogenous IL-10 affect the T-cell stimulatory capacity of BMDCs after injection of *in vitro*-treated BMDCs into naïve mice. These data both support existing data as well as point towards a new understanding of the many aspects of IL-10-mediated immunosuppression.

INTRODUCTION

The importance of dendritic cells (DCs) during the induction of an immune response is well established, and DCs are thought to be the most powerful of all antigen-presenting cells (APC), capable of activating even naïve T cells.^{1,2} DCs exist in at least two different maturation stages: immature and mature. Immature DCs can be converted into mature cells by maturation signals such as bacterial lipopolysaccharide (LPS), tumour necrosis factor- α (TNF- α), unmethylated CpG-containing DNA, interferon- γ (IFN- γ) or cross-linking of CD40 by CD40 ligand.³

Received 10 June 2002; revised 7 August 2002; accepted 9 September 2002.

Abbreviations: BMDC, bone marrow-derived dendritic cell; DC, dendritic cell; ELISA, enzyme-linked immunosorbent assay; G6PDH, glucose-6-phosphate dehydrogenase; KLH, keyhole limpet haemocyanin; mAb, monoclonal antibody; Mix, 10 ng/ml IFN- γ , TNF- α , IL-4; MPX, multiplexed; TBP, TATA-binding protein.

Correspondence: Claus Haase, Hagedorn Research Institute, Niels Steensens Vej 6, DK-2820 Gentofte, Denmark. E-mail: csha@novonordisk.com

Interestingly, the origin and activation stage of the mature DC strongly affects the outcome of the immune response. Thus, differential production of cytokines – and especially of interleukin (IL)-12 – the levels of costimulatory molecules, the developmental origin of the DC and the dose of antigen, all play a role when deciding between the induction of a T helper (Th)1 or a Th2 response.^{4–8}

The immunoregulatory cytokine, IL-10, has been demonstrated to affect both human and mouse DC maturation *in vitro*, and the effects of IL-10 on DC maturation seem to depend upon the DC subset examined. In general, IL-10 treatment of DCs suppresses IL-12 production,^{9–11} but whereas IL-10-treated murine splenic DCs induce a Th2 response,¹¹ bone marrow-derived murine DCs (BMDCs) treated with IL-10 show generally impaired antigen presentation *in vitro*.¹² Likewise, IL-10 treatment of human DCs decreases the T-cell stimulatory capacity¹³ and induces tolerance in responding T cells.¹⁴ Also, it has been reported that IL-10 treatment of human DCs can promote differentiation into macrophages.^{15–17} Thus, IL-10 has multiple effects on the different subsets of DCs known at present.

The possibility of using IL-10 to prevent maturation of BMDCs was of interest to us, and we wanted to study whether IL-10 could affect the induction of an immune response after

adoptive transfer of antigen-pulsed BMDCs. One study has described the effects on *in vivo* priming by DCs treated with IL-10 *in vitro*.¹¹ This study showed that keyhole limpet haemocyanin (KLH)-pulsed IL-10-treated murine splenic DCs induced a Th2 response upon injection into naïve mice, whereas control DCs induced a Th1 response. No other studies on this therapeutically interesting aspect have been reported specifically regarding BMDCs. As DCs can be generated in large quantities from bone-marrow precursors, utilizing this DC subset seemed attractive to us; however, whether BMDCs would respond to IL-10 treatment, similarly to other subtypes of DCs, was uncertain (reviewed in ref. 18), given the described differences between, e.g. splenic DCs and BMDCs with respect to lineage origin, surface markers and cytokine production.^{19,20} Furthermore, some DC subsets have even been shown to be resistant to the immunoinhibitory effects of IL-10.²¹ In addition, other factors that might affect T-cell priming include both the antigen studied and the possibility that endogenous IL-10 produced by DCs could affect T-cell priming capacity, as recently demonstrated in human DCs *in vitro*.²²

Therefore, although the effects of IL-10 treatment of DCs have been studied *in vitro*, the *in vivo* effects induced upon injection of such IL-10-treated DCs are, to a large extent, unknown. Furthermore, the effects of IL-10 on the widely used BMDC subset have still not been fully described *in vitro* or *in vivo*. It was therefore necessary to test the effects of IL-10 on the BMDC subset.

Here, we present a comprehensive study of the effects of exogenous and endogenous IL-10 on the maturation and T-cell stimulatory capacity of murine BMDCs, both *in vitro* and *in vivo*. We demonstrate that exogenous IL-10 can neutralize the stimulatory capacity of either bacterial LPS or a cytokine mixture (IFN- γ , TNF- α , IL-4), with greatly impaired T-cell stimulation as a consequence. Both IFN- γ and IL-4 production by activated T cells is impaired as a result of IL-10 pretreatment of stimulatory BMDCs *in vitro*, suggesting that IL-10 can inhibit the development of both Th1- and Th2-inducing DCs. Furthermore, we demonstrate that both endogenous and exogenous IL-10 influence the magnitude of the immune response initiated after injection of antigen-pulsed BMDCs into naïve mice.

MATERIALS AND METHODS

Animals and reagents

All mice (C57BL/6J, BALB/c) were bred at M & B (Ry, Denmark). All animal experiments were carried out according to the Danish Law of Animal Welfare and the ethical guidelines of Novo Nordisk A/S. Recombinant cytokines were purchased from Pharmingen (Heidelberg, Germany).

Generation of BMDC

BMDCs were generated as described previously.²³ Briefly, femurs and tibiae of C57BL/6 mice were flushed and single cells prepared. Red blood cells were lysed and cells were washed in ice-cold Hanks' balanced salt solution (HBSS). T cells were lysed using baby rabbit complement (Harlan Seralabs, Leicestershire, UK) and antibodies towards mouse CD4 (RL172-4 hybridoma supernatant), CD8 (31M hybridoma supernatant) and Thy (AT83 hybridoma supernatant). The remaining cells

were plated in RPMI-1640 with 5% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μ g/ml streptomycin, 10 mM HEPES and 50 μ M 2-mercaptoethanol at 1×10^6 cells/well in a 24-well plate (Nunc, Roskilde, Denmark). Every other day, non-adherent cells were removed and fresh medium added. BMDCs developed in the presence of 20 ng/ml granulocyte-macrophage colony-stimulating factor (GM-CSF) (days 0–4) and 10 ng/ml

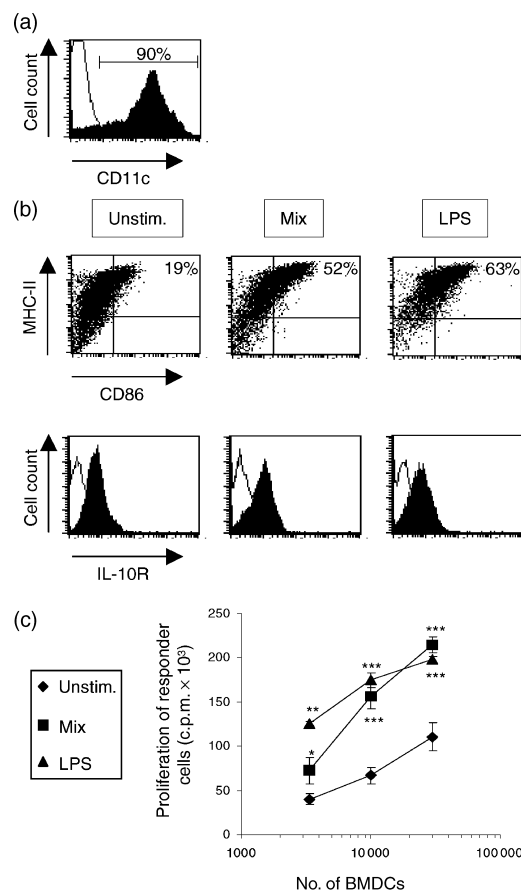


Figure 1. Day-8 bone marrow-derived dendritic cells (BMDCs) are semi-mature and can be matured further by incubation with lipopolysaccharide (LPS) or Mix [10 ng/ml interferon- γ (IFN- γ), tumour necrosis factor- α (TNF- α), interleukin (IL)-4]. BMDCs, generated as described in the Materials and methods, were stimulated with either Mix (10 ng/ml) or LPS (100 ng/ml) for 24 hr, or were not stimulated. (a) Flow cytometric analysis of BMDCs by staining with antibodies specific for CD11c, as described in the Materials and methods. (b) Flow cytometric analysis of BMDCs. Cells were stimulated as indicated, harvested and stained for CD11c, major histocompatibility complex (MHC)-II and CD86 or IL-10 receptor, as described in the Materials and methods. Diagrams shown are gated on CD11c⁺ cells. (c) Cells were harvested and used as stimulators in a mixed leucocyte reaction (MLR) against allogeneic T cells. Shown is the proliferation of T cells stimulated with unstimulated DCs (\blacklozenge), Mix-stimulated DCs (\blacksquare) or LPS-stimulated DCs (\blacktriangle), as measured by [³H]thymidine ([³H]TdR) incorporation. Error bars represent the standard deviation. Results were analysed using the Student's *t*-test. **P* < 0.05; ***P* < 0.01; ****P* < 0.005 between unstimulated and Mix or LPS, respectively. All experiments were performed at least three times with similar results obtained on each occasion. c.p.m., counts per minute; unstim., unstimulated.

GM-CSF (days 5–6). On day 6, non-adherent cells were transferred to new plates in media containing 5 ng/ml GM-CSF. On day 7, cells were stimulated with 100 ng/ml LPS (*Escherichia coli* K235, no. L2143; Sigma, Copenhagen, Denmark) or with a cytokine mixture (Mix: 10 ng/ml TNF- α , IFN- γ and IL-4) and/or 20 ng/ml IL-10, unless otherwise indicated. All stimulations were carried out at a cell density of 10^6 /ml. Cells were harvested on day 8. Phenotypic analysis revealed 90–95% CD11c⁺ cells, and no cells expressed CD8 α (see Fig. 1 and data not shown).

Antibodies

The monoclonal antibodies (mAbs) used in this study included phycoerythrin (PE)-conjugated rat anti-CD4 (GK1.5), PE-conjugated rat anti-IL-10 receptor (1B1.3a), PE-conjugated rat anti-major histocompatibility complex (MHC) class II, I-A^b (M5/114-15-2), fluorescein isothiocyanate (FITC)-conjugated rat anti-CD8 α (53-6.7), FITC-conjugated rat anti-CD86 (GL1) and biotin-conjugated hamster anti-CD11c (HL3) (all from Pharmingen). Biotin-coupled antibodies were revealed by streptavidin-allophycocyanin (APC). Isotype controls included: FITC-conjugated rat IgG2a, κ (R35-95); PE-conjugated rat IgG2b, κ (A95-1); and biotin-conjugated hamster immunoglobulin G (IgG), group 1 (G235-2356).

Multiplex reverse transcription–polymerase chain reaction analysis

Semi-quantitative multiplex (MPX) reverse transcription–polymerase chain reaction (RT–PCR) was carried out as previously described.²⁴ Briefly, RNA was isolated using the RNeasy method of Biotec Laboratories, Inc. (Veenendaal, The Netherlands). One microgram of denatured RNA was used for cDNA synthesis by incubation with 40 U of RNasin (Promega, Mannheim, Germany), 200 U of M-MLV Reverse Transcriptase (Gibco, Invitrogen, Paisley, UK), 3 μ g of random primers (Gibco), 10 mM dithiothreitol (DTT) (Gibco), 1 mM dNTPs (Gibco) and 1 \times reverse transcriptase buffer (50 mM Tris–HCl, pH 8.3, 75 mM KCl, 3 mM MgCl₂; Gibco) in a final volume of 25 μ l. The reaction was allowed to stand for 5–10 min at room temperature before incubation for 1 hr at 37°. PCR reactions were carried out in 25- μ l reactions using 0.5 μ l of cDNA, 1 \times

PCR buffer (Mg²⁺-free; Life Technologies, Invitrogen, Paisley, UK), 1.5 mM MgCl₂ (Life Technologies), 0.2 mM of each primer (see Table 1), 1.25 U of *Taq* DNA polymerase (Life Technologies), dNTPs (0.04 mM dATP, dTTP, dGTP plus 0.02 mM dCTP; Gibco) and 16.6 μ M [α -³²P]dCTP (Uppsala, Sweden). MPX RT–PCR was performed in sets of three to five genes per reaction, and the program was always: 96° for 30 seconds; followed by 22 cycles of 96° for 30 seconds, 55° for 30 seconds, and 73° for 30 seconds. The labelled PCR products were identified using standard polyacrylamide gels (6%, 7 M urea, UltraPure Sequagel[®] sequencing system; National Diagnostics, Yorkshire, UK), supplemented with 0.4 μ l/ml Temed (*N,N,N',N'*-tetramethyl-ethylenediamine; Bio-Rad, Hercules, CA) and 8 μ l/ml 10% ammonium persulphate (Sigma). Gels were exposed to a phosphorimager storage screen overnight and scanned on a Typhoon Imager (Molecular Dynamics, Sunnyvale, CA). All bands were standardized to the following internal controls: glucose-6-phosphate dehydrogenase (G6PDH); and TATA-binding protein (TBP) (see Table 1).

Enzyme-linked immunosorbent assay

Measurement of cytokine production by enzyme-linked immunosorbent assay (ELISA) was performed using the following ELISA sets: IL-1 α , IL-2, IL-4, IL-6, IL-10, IL-12 p70, IL-18, TNF- α and IFN- γ (OptEIA[™]; all from Pharmingen) or IL-1 β (R & D Systems, Abingdon, UK). All measurements were carried out according to the manufacturers' instructions. In all cases, the washing procedure was performed using a Labsystem Multi-wash plate-washing machine. Detection was carried out using a multiplate reader (Victor; Wallac, Turku, Finland) at 450 nm. Calculation of cytokine content was undertaken using the supplied recombinant standards.

Mixed leucocyte reaction (MLR)

BMDCs were pretreated as indicated and harvested on day 8 after setup. Cells were washed in phosphate-buffered saline (PBS), incubated with mitomycin C (6.3 μ g/ml, 20 min, 37°), washed again, and distributed at the indicated cell densities in U-bottomed 96-well plates. T cells were purified from the spleens of BALB/c mice by loading splenocytes onto a CD3

Table 1. Primers used for the reverse transcription–polymerase chain reaction (RT–PCR)

Cytokine	5' Primer	3' Primer	bp*	GB acc. no.†
IL-1 α	CAA GAT GGC CAA AGT TCC TG	GCT TGA CGT TGC TGA TAC TG	241	AF10237
IL-1 β	CTC CAC CTC AAT GGA CAG AA	ACC GCT TTT CCA TCT TCT TC	211	M15131
IL-6	CAA GAA AGA CAA AGC CAG AG	TTG GAT GGT CTT GGT CCT TA	172	X54542
IL-12 p35	AGA CCA CAG ATG ACA TGG TG	GTT GAT GGC CTG GAA CTC TG	290	M86672
IL-12 p40	AAG ACC CTG ACC ATC ACT GTC	CAG AGA CGC CAT TCC ACA TGT	300	M86671
IL-15	GAC AGT GAC TTT CAT CCC AG	TTC TCC TCC AGC TCC TCA CA	200	U14332
TNF- α	CTA CTC CCA GGT TCT CTT CA	TGA CTC CAA AGT AGA CCT GC	287	X02611
TGF- β 1	AGG TCA CCC GCG TGC TAA TG	TTC CAC ATG TTG CTC CAC AC	188	M13177
TBP	ACC CTT CAC CAA TGA CTC CTA TG	ATG ATG ACT GCA GCA AAT CGC	190	D01034
G6PDH	AGA ACC ACC TCC TGC AGA TG	TCC CAC CGT TCA TTC TCC AC	268	M26655

*Length of the PCR products, in base pairs.

†GenBank accession no. of the specified genes.

acc. no., accession number; G6PDH, glucose-6-phosphate dehydrogenase; IL, interleukin; TBP, TATA-binding protein; TGF- β 1, transforming growth factor- β 1; TNF- α , tumour necrosis factor- α .

selection column (R & D Systems), according to the manufacturer's instructions. Flow cytometric analysis showed that the eluted cells consisted of >95% CD3⁺ cells, of which two-thirds were CD4⁺ and one-third was CD8⁺. Eluted T cells were added to allogeneic stimulator cells at a concentration of 10⁵ cells/well in a total volume of 200 µl/well. T-cell proliferation was measured by the addition of 0.5 µCi of [³H]thymidine ([³H]TdR) (Amersham-Pharmacia, Uppsala, Sweden) after 96 hr of incubation. Cells were allowed to incorporate radioactivity for a further 16 hr. The incorporation of [³H]TdR was analysed by scintillation counting on a microbeta counter (Wallac) using the supplied software (Microbeta Trilux; Wallac). In some experiments, 100 µl of supernatant was removed for ELISA analysis at 24, 48 or 72 hr of incubation and replaced with fresh medium.

Flow cytometry

Cells were isolated, washed once in PBS and incubated with primary antibodies for 1–2 hr at 4°. When required, the cells were washed twice and incubated with streptavidin-APC for 30–45 min at 4°. All antibodies were used after dilution ×250–500 in PBS. Before analysis, cells were fixed in 1% paraformaldehyde for 30–60 min. The cells were analysed by flow cytometry on a fluorescence-activated cell sorter (FACS-Calibur; Becton-Dickinson, Heidelberg, Germany) with acquisition of 20 000 events. Viable cells were gated based on forward- and side-scatter properties, and all data was analysed using CellQuest software, version 3.1 (Becton-Dickinson).

Immunizations and isolation of T cells from lymph nodes

Female C57BL/6 mice were immunized subcutaneously at the base of the tail with 50 µg of the dust mite-derived *Der p* 1 peptide (RFGISNYCQIYPPNANKIREAL) in sterile PBS emulsified in 50 µl of complete Freund's adjuvant (CFA), yielding a final volume of 100 µl. After 5 days the mice were killed by cervical dislocation, and inguinal and lumbar lymph nodes were isolated. Single cells were obtained and red blood cells lysed by treatment with ACK-buffer for 5 min. Enrichment of CD3⁺ T cells was carried out using mouse T-cell enrichment columns (R & D Systems), giving >95% pure CD3⁺ cells. A total of 7.5 × 10⁴ T cells was added to 5 × 10³ mitomycin C-treated BMDCs that were pretreated as indicated above.

Injection of pulsed BMDCs

On day 7, BMDCs were pulsed with 25 µg/ml *Der p* 1 peptide for 24 hr. Where indicated, cells were also stimulated with 100 ng/ml LPS and/or 20 ng/ml IL-10 and, in some experiments, 10 µg/ml anti-IL-10 mAb (JES5-2A5) or a control mAb. The next day, cells were harvested by gentle pipetting and washed twice in PBS. Cells were counted, the cell density adjusted to 2.5 × 10⁶/ml in PBS and female C57BL/6 mice were injected subcutaneously at the base of the tail with 100 µl of the cell suspension, using two–three mice/group. Five days later, draining lymph nodes (inguinal and lumbar) were harvested, single cells prepared and red blood cells lysed. Cells were restimulated with peptide in medium containing 0.5% mouse serum *in vitro* for 56 hr, after which 0.5 µCi [³H]TdR (Amersham-Pharmacia) was added. Cells were incubated for a further 16 hr before harvesting, and thymidine incorporation was determined as for the MLR experiments. In some experiments, 100 µl of super-

natant was removed at 48 hr for ELISA and replaced with fresh medium.

Statistics

Statistical analysis of the data was carried out using a two-tailed Student's *t*-test.

RESULTS

Day-8 BMDCs can be further matured by either LPS or cytokines

DCs were generated from mouse bone marrow as previously described,²³ resulting in 90–95% CD11c-positive cells (Fig. 1a). To investigate the maturation stage of day-8 BMDCs, a flow cytometric analysis of the cells was performed. We found that unstimulated day-8 BMDCs expressed a wide range of MHC-II levels, whereas <20% of the cells expressed the costimulator CD86 (Fig. 1b, top row). Hence, only a small fraction of the cells were mature DCs, characterized by coexpression of high levels of MHC-II and CD86. However, after incubation for 24 hr with either a mixture of cytokines (Mix: TNF-α, IFN-γ, IL-4) or bacterial LPS as maturation stimuli, the cells matured with virtually all cells expressing MHC-II and demonstrating increased (but still variable) levels of CD86 (Fig. 1b, top row). The acquisition of a more mature phenotype after stimulation with either Mix or LPSs correlated with an increased stimulatory capacity towards allogeneic T cells (Fig. 1c).

Semi-mature BMDCs express functional IL-10 receptor, which is not down-regulated after maturation

IL-10 has previously been shown to have effects on immature DCs, whereas mature DCs in some cases are thought to be resistant to IL-10-mediated immunosuppression.²¹ We therefore tested, by examining the expression of IL-10 receptor, whether the semi-mature phenotype observed with our unstimulated DCs could respond to IL-10. Both unstimulated BMDCs and stimulated (Mix or LPS) BMDCs expressed low levels of IL-10 receptor and these levels did not change during differentiation or maturation (data not shown and Fig. 1b, bottom row). To determine whether the expressed IL-10 receptor was functional, the BMDCs were stimulated with LPS and increasing amounts of IL-10. It was found that LPS-induced IL-12 production in BMDCs was IL-10-sensitive in a dose-dependent manner (Fig. 2a, 2b). The down-regulation of IL-12 production by IL-10 correlated with a down-regulation of both IL-12 p40 and p35 mRNA, as well as a decrease in secreted IL-12 p70. Furthermore, preincubation of IL-10 with an IL-10-specific mAb, but not a control mAb, prevented the suppression of IL-12 production, demonstrating the specificity of IL-10 (Fig. 2c). As IL-12 production was completely suppressed by 20 ng/ml IL-10, we used this concentration in all subsequent experiments.

Production of proinflammatory cytokines is inhibited by IL-10

DCs are known to produce proinflammatory cytokines upon activation.²⁵ As Mix-induced maturation of BMDCs was prob-

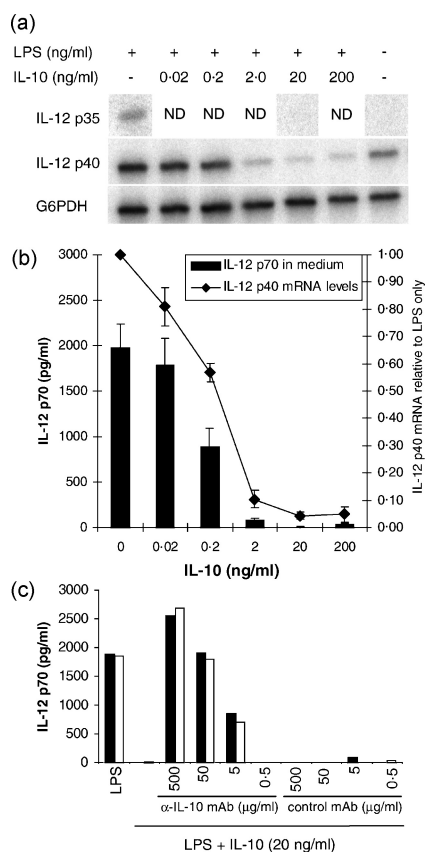


Figure 2. Interleukin (IL)-10 suppresses lipopolysaccharide (LPS)-induced IL-12 production by bone marrow-derived dendritic cells (BMDCs). BMDCs were stimulated on day 7 after set-up with 100 ng/ml LPS, either alone or in combination with increasing amounts of IL-10. After 24 hr, RNA was purified as described in the Materials and methods. mRNA levels of IL-12 p40 and p35 were determined by semi-quantitative multiplexed (MPX) reverse transcription-polymerase chain reaction (RT-PCR). Supernatants were analysed for secreted IL-12 p70 by enzyme-linked immunosorbent assay (ELISA). (a) RT-PCR data showing specific gel bands for IL-12 p35, p40 and glucose-6-phosphate dehydrogenase (G6PDH). (b) Quantification of RT-PCR data and ELISA data. RT-PCR data represent the average \pm SD of five independent experiments, and IL-12 p40 mRNA levels relative to stimulation with LPS alone are shown. ELISA data shown represent the average \pm SD of four independent experiments performed. Unstimulated cells did not secrete any IL-12 p70 (data not shown). (c) IL-10-mediated inhibition of IL-12 production is cytokine specific. BMDCs were stimulated with LPS or LPS + IL-10, as described above, except that IL-10 was preincubated for 1 hr at 37° with an IL-10-specific monoclonal antibody (mAb), with a control antibody, or with no antibody before addition to the cells. One experiment of two performed in duplicate is shown.

ably different from LPS-mediated maturation, we tested whether LPS or Mix induced the production of proinflammatory cytokines and whether the production of cytokines could be inhibited by simultaneous IL-10 addition. We found that Mix induced a small up-regulation of IL-1 α , IL-1 β , IL-6 and TNF- α mRNA levels, whereas the levels of IL-12 p40 and p35, IL-15 and transforming growth factor- β (TGF- β) mRNA were unchanged (Fig. 3a). In contrast, LPS induced high levels of

IL-1 α , IL-1 β , IL-6, IL-12 p40 and p35, and TNF- α mRNA, whereas TGF- β mRNA was down-regulated. Interestingly, only LPS could mediate the secretion of IL-1 α , IL-1 β , IL-6, IL-10 and TNF- α , whereas Mix could not, despite the up-regulation of mRNA. Adding IL-10 showed that IL-10 was capable of blocking the up-regulation of cytokine mRNA and the secretion of functional cytokines induced by both LPS and Mix. In conclusion, LPS but not Mix was able to induce high levels of cytokine production from BMDCs, and this production could be blocked by simultaneous incubation with IL-10.

IL-10 suppresses BMDC-mediated T-cell activation *in vitro* and affects the cytokine production by responding T cells

As IL-10 showed an inhibitory effect on cytokine production by DCs, we wanted to test how the T-cell priming capacity of the BMDCs was affected after treatment with IL-10. BMDCs were pretreated with Mix or LPS, with or without additional IL-10, and used as stimulators of allogeneic T cells. Both Mix and LPS-induced maturation of BMDCs was inhibited by the inclusion of IL-10 during pretreatment of the cells, and T-cell proliferation and T-cell-dependent IL-2 secretion was decreased to levels comparable to those induced by unstimulated BMDCs (Fig. 4a, 4b). Importantly, we did not observe any toxic effects of IL-10 on BMDCs when measured by propidium iodide incorporation or by visual inspection (data not shown).

The cytokine secretion of primed T cells was also affected by IL-10 treatment of the stimulators (Fig. 4c). Unstimulated BMDCs primed T cells for both IFN- γ and IL-4 production. In contrast, LPS-treated BMDCs induced a type 1 response with high production of IFN- γ , whereas Mix-treated BMDCs induced a type 2 response with high production of IL-4. However, this skewing towards type 1 or type 2 was completely abrogated when IL-10 was present during pretreatment of the BMDCs. Therefore, we suggest that IL-10 blocks the final maturation of BMDCs, which in turn affects the differentiation of naïve T cells and thus the cytokine profile of primed immune responses.

IL-10 inhibits BMDC-mediated reactivation of T cells

To investigate whether the observed inhibitory effects on BMDC maturation could also be demonstrated when activating *in vivo*-primed T cells, we studied T-cell activation in a T-cell restimulation assay. Consequently, naïve mice were immunized with the dust mite major allergen peptide, Der p I, in CFA and draining lymph nodes were isolated 5 days later. The level of proliferation of primed T cells isolated from these lymph nodes was found to depend on the maturation state of the BMDCs used as stimulators during T-cell restimulation *in vitro*. Including IL-10 during pretreatment of BMDCs inhibited the increased T-cell proliferation induced by Mix or LPS-treatment (Fig. 5).

Exogenous IL-10 can inhibit the stimulatory capacity of BMDCs *in vivo*

As very few studies have described the implications of IL-10 treatment of BMDCs *in vitro* upon subsequent *in vivo* priming,

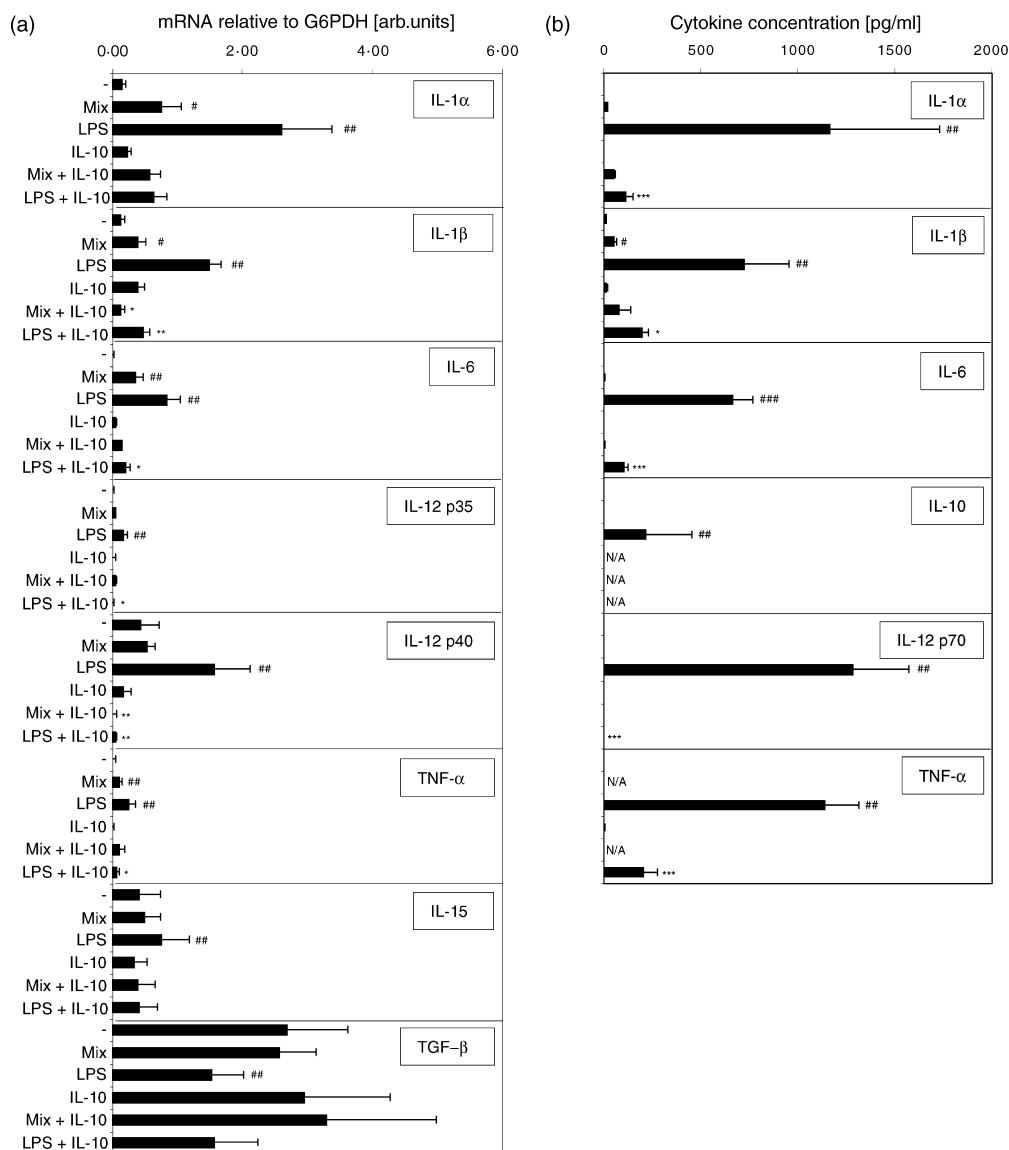


Figure 3. Cytokine production in bone marrow-derived dendritic cells (BMDCs) after stimulation. BMDCs were stimulated, as indicated, for 24 hr. Cells and supernatants were harvested for analysis by (a) multiplexed (MPX) reverse transcription-polymerase chain reaction (RT-PCR), or (b) sandwich enzyme-linked immunosorbent assay (ELISA), as outlined in the Materials and methods. (a) MPX RT-PCR. All data are normalized to glucose-6-phosphate dehydrogenase (G6PDH) mRNA levels, and the mean values \pm SD of three to five independent experiments are shown. Similar results were obtained after normalization to another internal standard, TATA-binding protein (TBP) (data not shown). Results were analysed using the Student's *t*-test. #*P* < 0.05; ##*P* < 0.01 between unstimulated and Mix [10 ng/ml interferon- γ (IFN- γ), tumour necrosis factor- α (TNF- α), interleukin (IL)-4] or lipopolysaccharide (LPS), respectively; **P* < 0.05; ***P* < 0.01 between Mix and Mix + IL-10 or LPS and LPS + IL-10, respectively. (b) ELISA. The mean values \pm SD (pg/ml) of three to four independent experiments are shown. Results were analysed using the Student's *t*-test. #*P* < 0.05; ##*P* < 0.01; ###*P* < 0.005 between unstimulated and Mix or LPS, respectively; **P* < 0.05; ***P* < 0.01; ****P* < 0.005 between LPS and LPS + IL-10. N/A, not applicable. No IFN- γ or IL-4 was produced from these cells (data not shown).

we examined whether IL-10 could affect T-cell activation *in vivo* after injection of antigen-pulsed BMDCs. BMDCs were pulsed with the *Der p* I antigen peptide and injected subcutaneously into naïve syngeneic mice. Upon restimulation of draining lymph node cells *in vitro*, we found that T cells primed by untreated, as well as IL-10-treated BMDCs, responded by low but measurable levels of antigen-specific proliferation, whereas LPS-treated BMDCs had induced a much more powerful

immune response, as measured by both proliferation and IFN- γ production (Fig. 6b, 6c). Interestingly, BMDCs pretreated with both LPS and IL-10 induced comparable levels of T-cell priming *in vivo*, as did unstimulated or IL-10-treated BMDCs (Fig. 6b, 6c). We could not detect any significant production of IL-4 from *Der p* I-primed T cells, which is in accordance with data suggesting that *Der p* I is predominantly an IFN- γ -inducing antigen when pulsed to DCs (ref. 26, and data not shown). These

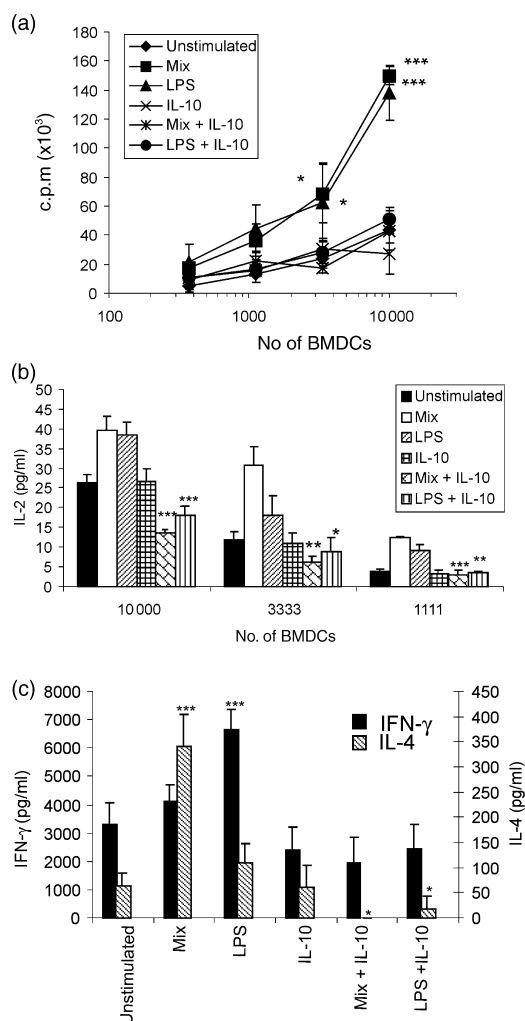


Figure 4. Interleukin (IL)-10 suppresses activation of bone marrow-derived dendritic cells (BMDCs) *in vitro*. BMDCs were pretreated, as indicated, for 24 hr. Cells were harvested and used as stimulators in a mixed leucocyte reaction (MLR), as described in the Materials and methods. (a) Proliferation of T cells after [3 H]thymidine ([3 H]TdR) incorporation. One representative experiment is shown of more than eight performed, with similar results obtained on each occasion. c.p.m., counts per minute. (b) IL-2 secretion was measured by enzyme-linked immunosorbent assay (ELISA) after 24 hr of incubation with BMDCs. Shown is one representative experiment out of two performed. No IL-2 was produced from T cells alone (data not shown). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.005$, Student's *t*-test between Mix [10 ng/ml interferon- γ (IFN- γ), tumour necrosis factor- α (TNF- α), interleukin (IL)-4] and Mix + IL-10, or lipopolysaccharide (LPS) and LPS + IL-10, respectively. (c) Mix and LPS mature DCs differently. The cytokine production of CD3 $^+$ T cells primed by differentially pretreated BMDCs (T cell:BMDC ratio = 10:1) was measured for IFN- γ and IL-4 by sandwich ELISA after 4 days of incubation. No IL-10 was detected in these cultures (data not shown). Results shown are representative of two or three independent experiments. * $P < 0.05$; *** $P < 0.001$, by the Student's *t*-test compared to T cells stimulated with unstimulated BMDCs.

data demonstrate that IL-10 can inhibit the LPS-induced maturation of BMDCs *in vitro*, strongly affecting the stimulatory capacity of the cells *in vivo*. Furthermore, the induction of

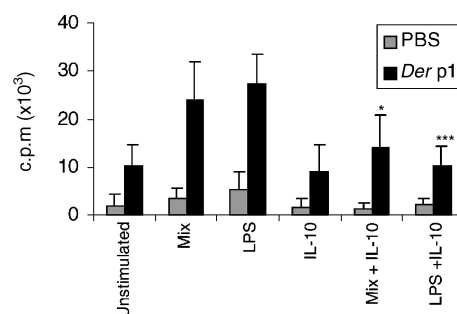


Figure 5. The inhibitory effect of interleukin (IL)-10 is observable in the reactivation of T cells. C57BL/6 mice were immunized subcutaneously with phosphate-buffered saline (PBS) or Der p I peptide in complete Freund's adjuvant (CFA) at the base of the tail, as described in the Materials and methods. Five days later, draining lymph nodes (lumbar and inguinal) were removed and CD3 $^+$ T cells were purified. T cells (7.5×10^4) were then restimulated with Der p I peptide *in vitro* using BMDCs (5×10^3) that were stimulated, as indicated, for 24 hr. Proliferation was evaluated after 72 hr by [3 H]thymidine incorporation. Results are shown as average value \pm SD from three independent experiments. * $P < 0.05$; *** $P < 0.001$, by the Student's *t*-test between Mix and Mix + IL-10 and lipopolysaccharide (LPS) and LPS + IL-10, respectively.

high levels of IFN- γ production during restimulation of *in vivo*-primed T cells correlated with the production of IL-12 by the injected BMDCs (Fig. 6a), emphasizing the role of IL-12 in the induction of type 1 immune responses.

Endogenous IL-10 regulates BMDC-mediated T-cell priming *in vivo*

Because our studies showed that LPS induced high levels of IL-10 secretion by BMDCs (Fig. 3b), we analysed the effects of endogenous IL-10 on BMDC-mediated T-cell priming *in vivo*. BMDCs were stimulated with LPS and either an anti-IL-10 mAb or a control mAb. Based on flow cytometric analysis, elimination of IL-10 did not affect the LPS-mediated maturation of the cells (Fig. 7a). However, neutralization of endogenous IL-10 caused a significantly higher production of IL-12 by the BMDCs in response to LPS (Fig. 7b) and resulted in a higher stimulatory capacity *in vivo*, as measured by both proliferation and IFN- γ production (Fig. 7c, 7d) by *in vivo*-primed T cells during restimulation *in vitro*. Therefore, we conclude that the production of endogenous IL-10 affects BMDC-mediated T-cell priming *in vivo*.

DISCUSSION

The present study demonstrates the effects of IL-10 on murine BMDC both *in vitro* and *in vivo*. We demonstrate that *in vitro*-generated day-8 BMDCs express low levels of functional IL-10 receptor and that the expression of IL-10 receptor is unchanged after maturation. We show that IL-10 can block the maturation of both type 1-inducing DCs (induced by LPS) and type 2-inducing DCs (induced by Mix), as measured by the cytokine production of allogeneic T cells *in vitro*, and we show that both exogenously and endogenously derived IL-10 affect T-cell

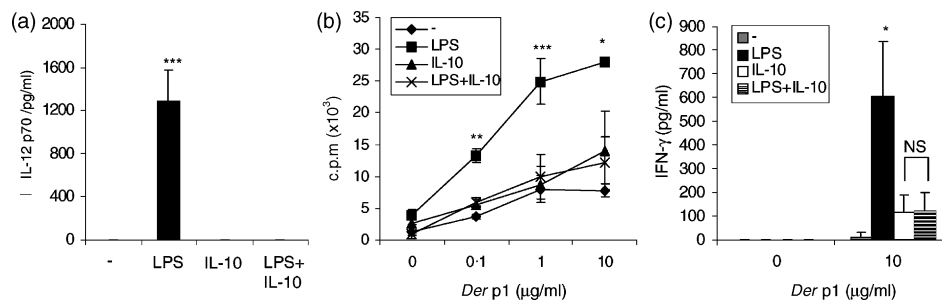


Figure 6. Interleukin (IL)-10 pretreatment influences the stimulatory capacity of bone marrow-derived dendritic cells (BMDCs) *in vivo*. BMDCs were stimulated on day 7, as indicated, and simultaneously pulsed with 25 μ g/ml Der p I peptide. After 24 hr, cells were harvested, washed extensively and counted. (a) IL-12 production of BMDCs before injection, as measured by using sandwich enzyme-linked immunosorbent assay (ELISA). Mice were immunized subcutaneously at the base of the tail with 2.5×10^5 BMDCs. After 5 days, recall experiments of draining lymph node cells (lumbar and inguinal) to the Der p I peptide were carried out *in vitro*, as described in the Materials and methods. (b) Proliferation of lymph node cells, as measured by [3 H]thymidine incorporation. (c) Interferon- γ (IFN- γ) production by lymph node cells after 48 hr, as measured by using ELISA. One representative experiment out of three is shown, performed each with three mice per group. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.005$, by the Student's *t*-test, comparing BMDCs pretreated with lipopolysaccharide (LPS) and all other groups.

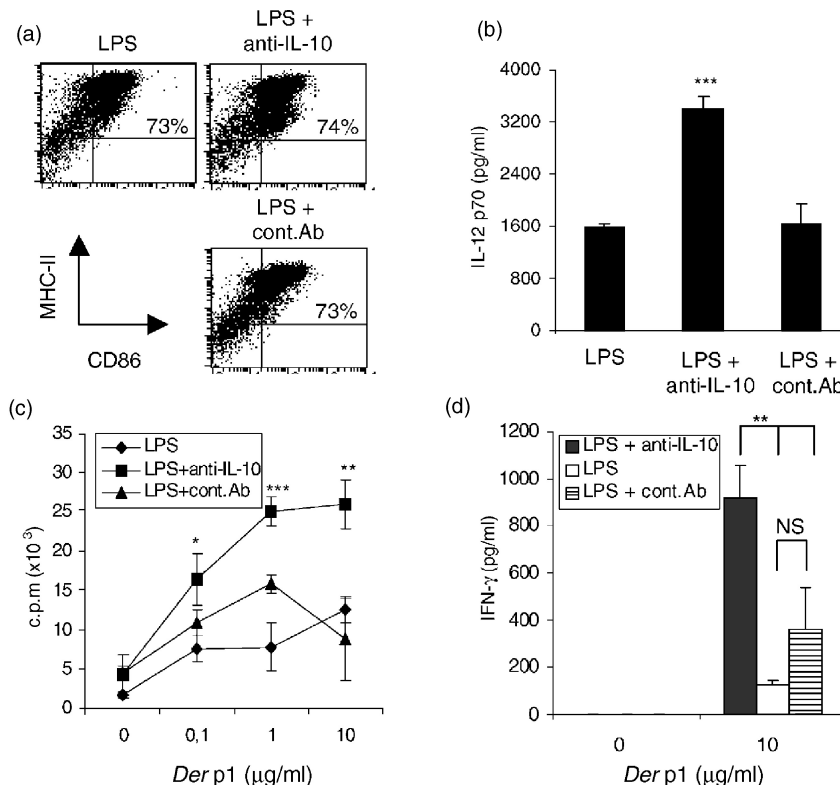


Figure 7. Neutralization of endogenous interleukin (IL)-10 *in vitro* improves T-cell priming and interferon- γ (IFN- γ) production *in vivo*. Bone marrow-derived dendritic cells (BMDCs) were stimulated on day 7, as indicated, and simultaneously pulsed with 25 μ g/ml Der p I peptide. After 24 hr, cells were harvested, washed extensively and counted. Inclusion of anti-IL-10 monoclonal antibody (mAb) did not influence the maturation state of BMDCs (a) but significantly increased the level of IL-12 production by the cells, as measured by enzyme-linked immunosorbent assay (ELISA) (b). Mice were immunized subcutaneously at the base of the tail with 2.5×10^5 BMDCs. After 5 days, recall experiments of draining lymph node cells (lumbar and inguinal) to the Der p I peptide were carried out *in vitro*, as described in the Materials and methods. (c) Proliferation of lymph node cells, as measured by [3 H]thymidine incorporation. (d) IFN- γ production of lymph node cells after 48 hr, as measured by ELISA. One representative experiment out of three performed is shown, each with three mice per group and with similar results obtained on each occasion. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.005$ (Student's *t*-test), comparing BMDCs pretreated with lipopolysaccharide (LPS) + anti-IL-10 and all other groups.

priming *in vivo* after injection of antigen-pulsed BMDCs into naïve mice.

Our data demonstrate that IL-12 production by murine BMDCs similarly to murine splenic DCs can be regulated by IL-10,^{10,11} and we extend these studies by demonstrating that even in the presence of a strong IL-12-inducing signal such as bacterial LPS, IL-10 is capable of suppressing this signal. Hence, in the presence of IL-10, induction of IFN- γ -producing T cells should be inhibited, as these are dependent on IL-12 production by the DC.⁴ Indeed, we found that pretreatment of BMDCs with IL-10 was capable of inhibiting the induction of IFN- γ -producing T cells *in vitro* in an allogeneic MLR (Fig. 4c) as well as *in vivo* using antigen-pulsed BMDCs (Fig. 6). The impaired IL-12 production by IL-10-treated BMDCs is probably the cause of the observed reduction in IFN- γ -production by T cells, as previously shown in other systems.^{4,27} We did not observe a shift from Th1 to Th2 when treating cells with IL-10 *in vitro*, as previously reported,¹¹ but rather a general decrease in the T-cell stimulatory capacity of IL-10- BMDCs. The reason for this is probably the use of different subsets and maturation stages of DCs, as well as different antigens.

Overall, we observed a strong correlation between IL-12 production in the BMDCs and IFN- γ production by the responding T cells, both in the MLR (Fig. 4) and after injection of antigen-pulsed BMDCs into naïve mice (Fig. 6, Fig. 7). These results are in agreement with other studies showing that IL-12 production by the APC is critical for the induction of type 1 immune responses.^{4,27,28} Furthermore, our data demonstrates that BMDCs – like splenic CD8⁺ DCs⁴ – are capable of producing IL-12 in biologically significant amounts and of activating a type 1 response *in vivo*. Thus, BMDCs, as well as splenic CD8⁺ DCs, are capable of inducing a type 1 response *in vivo*, whereas to date, splenic CD8⁻ DCs have been found to preferentially induce type 2 responses. It should be noted, however, that not only the type of DC, but also the nature of the antigen studied, may influence the type of immune responses generated by a given subtype of DCs, and therefore further studies using different subtypes of DCs and different antigens are required to clarify what role the different DC subsets play in the induction of an immune response. In our system, the *Der p* I peptide has indeed previously been shown to result in the priming of IFN- γ -producing T cells both after immunization in CFA and after pulsing of DCs (ref. 26, and C.H. T.N.J. & B.K.M., unpublished).

Our study suggests that IL-10 production by the BMDCs themselves influences their IL-12 production and T-cell stimulatory capacity, as neutralization of autocrine IL-10 production by specific mAb could enhance IL-12 production *in vitro* and T-cell priming *in vivo*. These data support recently published reports, demonstrating that autocrine IL-10 production affects both maturation of DCs *in vitro*²² and induction of a type 1 response *in vivo*.^{29,30} The results are intriguing and suggest that DCs might control the nature of the immune response by regulating autocrine IL-10 production. In correlation, IL-10-producing DCs appear to be induced after oral or nasal exposure to antigen.^{31–33} Furthermore, freshly isolated DCs from Peyer's patches have been found to produce IL-10.³⁴ Therefore, it seems probable that autocrine IL-10 production by DCs plays an important role in preventing excessive activation of the immune

system in response to external antigens being inhaled or ingested. Furthermore, it is possible that the differences in IL-12 production observed between splenic CD8⁺ and CD8⁻ DCs⁴ could be caused by an enhanced IL-10 production in the CD8⁻ subset, but this has yet to be demonstrated. However, such an inverse relationship was suggested by studying the kinetics of IL-12 and IL-10 secretion in human DCs,³⁵ as IL-10 production in this case was found to be initiated concomitantly with a decrease in IL-12 production.

The results presented in this study show that IL-10 can suppress DC maturation, also in the presence of strong activation and maturation signals such as Mix or LPS. This suggests that the immunoinhibitory capacity of IL-10 is superior to the immunostimulatory functions of LPS or certain cytokines. We propose that this represents a general mechanism of controlling DC maturation *in vivo*. By regulating IL-10 production, the immune system can prevent DC-mediated priming of naïve T cells and avoid excessive tissue destruction and possibly autoimmunity. In the presence of proinflammatory signals, such as bacterial compounds or virus, the balance between endogenous IL-10 and inflammation-induced IL-12 will eventually decide the outcome of immune activation. Thus, inhibition of DC maturation can be an important supplement to other IL-10-mediated effects such as inhibition of T-cell proliferation, T-cell cytokine production and macrophage deactivation.^{36–43}

A probable source of IL-10 *in vivo* is regulatory T cells. Recently, Dhodapkar *et al.* have shown that immature DCs prime IL-10-producing, regulatory-like T cells *in vivo*,⁴⁴ and others have shown the existence of IL-10-secreting regulatory T cells in various *in vitro* and *in vivo* assays.^{45–48} Thus, regulatory T cells might be a source of IL-10 production *in vivo* and, as such, regulatory T cells could control the immune response by suppressing DC maturation. This scenario suggests a feedback loop: before and after activation of the immune system and clearance of pathogen by mature DCs, immature DCs will dominate, resulting in the induction of regulatory T cells producing IL-10 and a high IL-10:IL-12 ratio. In contrast, during acute infection the IL-10:IL-12 ratio will decrease as a result of the induction of IL-12 production and, when reaching a certain level, will allow full action of the immune system. Therefore, the production of IL-10 will ensure steady-state immunosuppression at both the T-cell and the DC level, and will prevent activation of an immune response in the absence of infections. Furthermore, the fact that IL-10 can suppress DC activation, even in the presence of immunostimulatory substances such as LPS or cytokines, points towards the existence of a 'short-circuit' mechanism by which the immune system can be shut down, even though an infection has not been cleared, e.g. if tolerating the infection is more beneficial to the host than excessive inflammation and possible organ damage.

ACKNOWLEDGMENTS

The authors wish to thank Drs Nina Brenden, Kresten Skak and Helle Markholst for valuable comments and suggestions and for critical reading of the manuscript. Hagedorn Research Institute is a basic research component of Novo Nordisk A/S. This work was supported by a scholarship from the University of Copenhagen to C.H. (grant no. 501-601-2/99) and by a Freja research grant from the Danish Research Agency to B.K.M. (grant no. 5008-01-0003).

REFERENCES

- Banchereau J, Steinman RM. Dendritic cells and the control of immunity. *Nature* 1998; **392**:245–52.
- Steinman RM. The dendritic cell system and its role in immunogenicity. *Annu Rev Immunol* 1991; **9**:271–96.
- Banchereau J, Briere F, Caux C, Davoust J, Lebecque S, Liu YJ, Pulendran B, Palucka K. Immunobiology of dendritic cells. *Annu Rev Immunol* 2000; **18**:767–811.
- Maldonado-Lopez R, De Smedt T, Michel P *et al*. CD8alpha+ and CD8alpha- subclasses of dendritic cells direct the development of distinct T helper cells *in vivo*. *J Exp Med* 1999; **189**:587–92.
- Constant S, Pfeiffer C, Woodard A, Pasqualini T, Bottomly K. Extent of T cell receptor ligation can determine the functional differentiation of naive CD4+ T cells. *J Exp Med* 1995; **182**:1591–6.
- Hosken NA, Shibuya K, Heath AW, Murphy KM, O'Garra A. The effect of antigen dose on CD4+ T helper cell phenotype development in a T cell receptor-alpha beta-transgenic model. *J Exp Med* 1995; **182**:1579–84.
- Freeman GJ, Boussiotis VA, Anumanthan A *et al*. B7-1 and B7-2 do not deliver identical costimulatory signals, since B7-2 but not B7-1 preferentially costimulates the initial production of IL-4. *Immunity* 1995; **2**:523–32.
- Jonuleit H, Schmitt E, Schuler G, Knop J, Enk AH. Induction of interleukin 10-producing, nonproliferating CD4+ T cells with regulatory properties by repetitive stimulation with allogeneic immature human dendritic cells. *J Exp Med* 2000; **192**:1213–22.
- Koch F, Stanzl U, Jennewein P, Janke K, Heufler C, Kaempgen E, Romani N, Schuler G. High level IL-12 production by murine dendritic cells: upregulation via MHC class II and CD40 molecules and downregulation by IL-4 and IL-10. *J Exp Med* 1996; **184**:741–6.
- Huang LY, Sousa CR, Itoh Y, Inman J, Scott DE. IL-12 induction by a Th1-inducing adjuvant *in vivo*: dendritic cell subsets and regulation by IL-10. *J Immunol* 2001; **167**:1423–30.
- De Smedt T, Van Mechelen M, De Becker G, Urbain J, Leo O, Moser M. Effect of interleukin-10 on dendritic cell maturation and function. *Eur J Immunol* 1997; **27**:1229–35.
- Faulkner L, Buchan G, Baird M. Interleukin-10 does not affect phagocytosis of particulate antigen by bone marrow-derived dendritic cells but does impair antigen presentation. *Immunology* 2000; **99**:523–31.
- Buelens C, Willems F, Delvaux A, Pierard G, Delville JP, Velu T, Goldman M. Interleukin-10 differentially regulates B7-1 (CD80) and B7-2 (CD86) expression on human peripheral blood dendritic cells. *Eur J Immunol* 1995; **25**:2668–72.
- Steinbrink K, Wolf M, Jonuleit H, Knop J, Enk AH. Induction of tolerance by IL-10-treated dendritic cells. *J Immunol* 1997; **159**:4772–80.
- Morel AS, Quarantino S, Douek DC, Londei M. Split activity of interleukin-10 on antigen capture and antigen presentation by human dendritic cells: definition of a maturative step. *Eur J Immunol* 1997; **27**:26–34.
- Rieser C, Ramoner R, Bock G, Deo YM, Holt L, Bartsch G, Thurnher M. Human monocyte-derived dendritic cells produce macrophage colony-stimulating factor: enhancement of c-fms expression by interleukin-10. *Eur J Immunol* 1998; **28**:2283–8.
- Allavena P, Piemonti L, Longoni D, Bernasconi S, Stoppacciaro A, Ruco L, Mantovani A. IL-10 prevents the differentiation of monocytes to dendritic cells but promotes their maturation to macrophages. *Eur J Immunol* 1998; **28**:359–69.
- Moore KW, Malefyt RD, Coffman RL, O'Garra A. Interleukin-10 and the interleukin-10 receptor. *Annu Rev Immunol* 2001; **19**:683–765.
- Hochrein H, Shortman K, Vremec D, Scott B, Hertzog P, O'Keefe M. Differential production of IL-12, IFN-alpha, and IFN-gamma by mouse dendritic cell subsets. *J Immunol* 2001; **166**:5448–55.
- Vremec D, Pooley J, Hochrein H, Wu L, Shortman K. CD4 and CD8 expression by dendritic cell subtypes in mouse thymus and spleen. *J Immunol* 2000; **164**:2978–86.
- MacDonald KP, Pettit AR, Quinn C, Thomas GJ, Thomas R. Resistance of rheumatoid synovial dendritic cells to the immunosuppressive effects of IL-10. *J Immunol* 1999; **163**:5599–607.
- Corinti S, Albanesi C, La Sala A, Pastore S, Girolomoni G. Regulatory activity of autocrine IL-10 on dendritic cell functions. *J Immunol* 2001; **166**:4312–8.
- Inaba K, Inaba M, Romani N, Aya H, Deguchi M, Ikehara S, Muramatsu S, Steinman RM. Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. *J Exp Med* 1992; **176**:1693–702.
- Jensen J, Serup P, Karlsen C, Nielsen TF, Madsen OD. mRNA profiling of rat islet tumors reveals nkx 6.1 as a beta-cell-specific homeodomain transcription factor. *J Biol Chem* 1996; **271**:18749–58.
- Morelli AE, Zahorchak AF, Larregina AT *et al*. Cytokine production by mouse myeloid dendritic cells in relation to differentiation and terminal maturation induced by lipopolysaccharide or CD40 ligation. *Blood* 2001; **98**:1512–23.
- Hoynes GF, Le Roux I, Corsin-Jimenez M *et al*. Serrate1-induced notch signalling regulates the decision between immunity and tolerance made by peripheral CD4(+) T cells. *Int Immunol* 2000; **12**:177–85.
- Macatonia SE, Hosken NA, Litton M *et al*. Dendritic cells produce IL-12 and direct the development of Th1 cells from naive CD4+ T cells. *J Immunol* 1995; **154**:5071–9.
- Maldonado-Lopez R, De Smedt T, Pajak B *et al*. Role of CD8alpha+ and CD8alpha- dendritic cells in the induction of primary immune responses *in vivo*. *J Leukoc Biol* 1999; **66**:242–6.
- Igietsme JU, Ananaba GA, Bolier J *et al*. Suppression of endogenous IL-10 gene expression in dendritic cells enhances antigen presentation for specific Th1 induction: potential for cellular vaccine development. *J Immunol* 2000; **164**:4212–9.
- Demangel C, Bertolino P, Britton WJ. Autocrine IL-10 impairs dendritic cell (DC)-derived immune responses to mycobacterial infection by suppressing DC trafficking to draining lymph nodes and local IL-12 production. *Eur J Immunol* 2002; **32**:994–1002.
- Alpan O, Rudomen G, Matzinger P. The role of dendritic cells, B cells, and M cells in gut-oriented immune responses. *J Immunol* 2001; **166**:4843–52.
- McGuirk P, McCann C, Mills KHG. Pathogen-specific T regulatory 1 cells induced in the respiratory tract by a bacterial molecule that stimulates interleukin 10 production by dendritic cells: a novel strategy for evasion of protective T helper type 1 responses by *Bordetella pertussis*. *J Exp Med* 2002; **195**:221–31.
- Akbari O, DeKruyff RH, Umetsu DT. Pulmonary dendritic cells producing IL-10 mediate tolerance induced by respiratory exposure to antigen. *Nat Immunol* 2001; **2**:725–31.
- Iwasaki A, Kelsall BL. Freshly isolated Peyer's patch, but not spleen, dendritic cells produce interleukin 10 and induce the differentiation of T helper type 2 cells. *J Exp Med* 1999; **190**:229–39.
- Langenkamp A, Messi M, Lanzavecchia A, Sallusto F. Kinetics of dendritic cell activation: impact on priming of T(H)1, T(H)2 and nonpolarized T cells. *Nat Immunol* 2000; **1**:311–6.
- Fiorentino DF, Zlotnik A, Mosmann TR, Howard M, O'Garra A. IL-10 inhibits cytokine production by activated macrophages. *J Immunol* 1991; **147**:3815–22.

- 37 Fiorentino DF, Zlotnik A, Vieira P, Mosmann TR, Howard M, Moore KW, O'Garra A. IL-10 acts on the antigen-presenting cell to inhibit cytokine production by Th1 cells. *J Immunol* 1991; **146**:3444–51.
- 38 Bogdan C, Vodovotz Y, Nathan C. Macrophage deactivation by interleukin 10. *J Exp Med* 1991; **174**:1549–55.
- 39 Del Prete G, De Carli M, Almerigogna F, Giudizi MG, Biagiotti R, Romagnani S. Human IL-10 is produced by both type 1 helper (Th1) and type 2 helper (Th2) T cell clones and inhibits their antigen-specific proliferation and cytokine production. *J Immunol* 1993; **150**:353–60.
- 40 Taga K, Mostowski H, Tosato G. Human interleukin-10 can directly inhibit T-cell growth. *Blood* 1993; **81**:2964–71.
- 41 de Waal M, Yssel H, De Vries JE. Direct effects of IL-10 on subsets of human CD4+ T cell clones and resting T cells. Specific inhibition of IL-2 production and proliferation. *J Immunol* 1993; **150**:4754–65.
- 42 Fiorentino DF, Bond MW, Mosmann TR. Two types of mouse T helper cell. IV. Th2 clones secrete a factor that inhibits cytokine production by Th1 clones. *J Exp Med* 1989; **170**:2081–95.
- 43 Ding L, Linsley PS, Huang LY, Germain RN, Shevach EM. IL-10 inhibits macrophage costimulatory activity by selectively inhibiting the up-regulation of B7 expression. *J Immunol* 1993; **151**:1224–34.
- 44 Dhodapkar MV, Steinman RM, Krasovsky J, Munz C, Bhardwaj N. Antigen-specific inhibition of effector T cell function in humans after injection of immature dendritic cells. *J Exp Med* 2001; **193**:233–8.
- 45 Groux H, O'Garra A, Bigler M, Rouleau M, Antonenko S, De Vries JE, Roncarolo MG. A CD4+ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. *Nature* 1997; **389**:737–42.
- 46 Asseman C, Mauze S, Leach MW, Coffman RL, Powrie F. An essential role for interleukin 10 in the function of regulatory T cells that inhibit intestinal inflammation. *J Exp Med* 1999; **190**:995–1003.
- 47 Barrat FJ, Cua DJ, Boonstra A *et al.* *In vitro* generation of interleukin 10-producing regulatory CD4(+) T cells is induced by immunosuppressive drugs and inhibited by T helper type 1 (Th1)- and Th2-inducing cytokines. *J Exp Med* 2002; **195**:603–16.
- 48 Cottrez F, Hurst SD, Coffman RL, Groux H. T regulatory cells 1 inhibit a Th2-specific response *in vivo*. *J Immunol* 2000; **165**:4848–53.