IL-10 permits transient activation of dendritic cells to tolerize T cells and protect from central nervous system autoimmune disease

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IL-10 permits transient activation of dendritic cells to tolerize T cells and protect from central nervous system autoimmune disease

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Abstract

Dendritic cells (DCs) are key players in the development of immunity. They can direct both the size and the quality of an immune response and thus are attractive tools to mediate immunotherapy. DC function has been thought to reflect the cells' maturation, with immunosuppressive agents such as IL-10 understood to retain DCs in an immature and tolerogenic state. Here we report that DC activated in the presence of IL-10 do show functional and phenotypic maturation. Their activation is transient and occurs earlier and more briefly than in cells matured with LPS alone. Despite initially equivalent up-regulation of surface MHC and co-stimulation, the IL-10-treated DCs expressed little IL-12 and failed to stimulate T cell proliferation both *in vitro* and *in vivo*. Interaction with IL-10-treated DCs rendered antigen-specific T cells unresponsive to subsequent challenge and their injection reduced the severity of experimental autoimmune disease. Our data suggest that IL-10 acts not by inhibiting maturation but instead by controlling the kinetics and the quality of DC activation. This alternative pathway of DC differentiation offers significant therapeutic promise.

Introduction

The central role of dendritic cells (DCs) in activating naive T cells gives them a strategic position in the control of immunity. They present to T cells both antigen and information about the pathogen and tissue from which that antigen came, and so they direct both the strength and the quality of the T cell response (1). The outcome of DC action can be as different as immunity and tolerance (2) and DCs are being investigated as therapeutic agents both to boost anti-tumour responses (3, 4) and to alleviate autoimmunity or graft rejection (5, 6). The distinct functions of DCs have often been thought to reflect different stages of DC maturation, with immunogenicity a feature of activated or 'mature' DCs and tolerance induction restricted to resting, 'immature' cells (7). The use of DCs to curtail unwanted immune responses requires a stability of function, however, that is resistant to any maturation stimuli encountered later, *in vivo*. We demonstrate here that DCs can undergo a rapid and transient activation process and still elicit tolerance.

Classical understanding of DC biology pictures an immature cell stationed in the periphery, continuously sampling its environment through phagocytosis and pinocytosis (8). These cells migrate to the draining lymph node under steady-state conditions, presenting their antigenic load in the absence of adequate co-stimulation and thereby maintaining self-tolerance (9). DC maturation occurs in the context of infection or inflammation when signals from the host or the invading microorganism trigger pattern recognition receptors expressed by the DC and thus elicit a process involving up-regulation of MHC and co-stimulatory molecules and cytokine release that together transform the DC into a powerful T cell stimulator (10, 11).

This definition of DC maturation has been muddied recently by a growing appreciation of alternative patterns of DC development (12). DCs can stimulate both T_h1 and T_h2 immunity (13) and yet the phenotype required to drive a T_h2 response comprises little surface MHC or known co-stimulation and thus shares few of the characteristics of classically activated DCs (14–16). Lutz and Schuler (17) coined the term 'semi-mature' to describe a population of DCs that displayed high levels of surface MHCII, CD80 and CD86 but did not up-regulate cytokine production; despite their co-stimulatory capacity, these cells elicited T cell tolerance *in vivo* (18). Similar populations of 'paralysed' and 'exhausted' DCs have also been reported (19, 20).

Several studies have used chemical or cytokine modification of DC development in order to manipulate T cell

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responses (6). IL-10 is an immunosuppressive cytokine, originally described as a product of a polarized T_h2 clone (21) and now known to be released by a variety of cells including keratinocytes, B cells, macrophages and DCs (22, 23). Attempts to use IL-10 therapeutically have been difficult: systemic administration *in vivo* has been shown to ameliorate experimental arthritis (24) but to aggravate symptoms of experimental autoimmune encephalomyelitis (EAE) (25). Concentrating its action onto appropriate cells and with relevant timing appears critical for a suppressive outcome. The impact of IL-10 on DCs is described as an inhibition of maturation, preventing the up-regulation of activation markers such as MHC class II and CD86, causing the DCs to interact with T cells while still essentially immature (26). This gives rise to an anergic or regulatory T cell response (26–28).

Tolerance induction by immature DCs can be broken by concomitant DC activation, however (9). Here we investigate the ability of mature DCs to elicit tolerance by driving DC activation in the presence of IL-10. We reveal a transient and accelerated activation that is accompanied by terminal differentiation of the cells. Despite phenotypic equivalence to classically activated DC controls, these DCs, *in vivo*, render T cells profoundly unresponsive to subsequent challenge and protect against experimental induction of EAE, a mouse model of multiple sclerosis. They thus offer a significant and stable therapeutic potential in the prevention of autoimmune disease.

Methods

Mice

BALB/c, C57BL/6 and DO11.10 TCR transgenic [H-2A^d restricted, ovalbumin peptide (OVA) 323–339 specific] (29) mice were bred and maintained under specific pathogen-free conditions in the animal facilities of the Faculties of Medicine and Science and Engineering at the University of Edinburgh. Mice were used at 6–12 weeks of age.

DC preparation

DCs were generated from bone marrow precursors under the influence of granulocyte/macrophage colony-stimulating factor (GM-CSF) using a protocol based on that of Inaba *et al.* (30). Bone marrow was collected from murine femurs and tibias and cell clusters dispersed by passage through a 25-G needle. Red blood cells were removed using lysis buffer (Sigma–Aldrich, Poole, UK). Cells were seeded into 24-well tissue culture plates at 3.75×10^5 cells per ml and 1 ml per well. The culture medium was RPMI 1640 (Sigma) supplemented with 10% FCS (Labtech International, Lewes, UK), 2 mM L-glutamine (Gibco BRL, Life Technologies, Paisley, UK) and 50 U/ml penicillin plus 50 μg/ml streptomycin (Gibco). GM-CSF was added in the form of 5% supernatant from the transfected cell line X63-gmcsf (31). Cultures were washed at day 3 and day 6 to remove non-adherent granulocytes and lymphocytes. On day 7, loosely adherent DCs were harvested by more vigorous washing, leaving firmly adherent macrophages attached to the plate. DC preparations were 85–90% pure (CD11c+, MHCII+) with the remainder of cells predominantly Gr1+ granulocytes.

DC activation

Day 7 DCs were replated at 1×10^6 cells per ml and 1 ml per well in fresh 24-well plates using culture medium with GM-CSF. LPS (0.1 μg/ml; from *Escherichia coli* 055:B5, Sigma) and recombinant mouse IL-10 (50 ng/ml; R&D systems, Abingdon, UK) were added as appropriate.

Flow cytometry

DCs were harvested at various times after activation, as stated in the text, washed and stained for expression of MHCII (I-A^d/I-E^d specific, FITC labelled, clone 2G9), CD11c (PE labelled, HL3), CD80 (PE, 16-10A1) and CD86 (PE, GL1), all from PharMingen (San Diego, CA, USA). The membrane-impermeable dye ToPro3 (Molecular Probes, Leiden, The Netherlands) was included for the final 5 min of staining. Samples were analysed with a FACScalibur flow cytometer and CellQuest software (Becton Dickinson, Mountain View, CA, USA) using a live cell gate set by forward and side scatter characteristics and ToPro3 exclusion. To measure cell death, unstained cells were re-suspended in 1 μM ToPro3 immediately before acquisition. Analysis was then performed on an ungated population.

Antigen uptake

DCs were harvested after activation, washed and 2×10^5 cells re-suspended in 50 μl PBS or FITC–dextran (2 mg/ml in PBS; MW 70 000; Molecular Probes). Cells were incubated at 37°C or on ice for 45 min, flooded with ice-cold PBS for 5 min, washed and re-suspended in 1% paraformaldehyde for analysis by flow cytometry.

RNA extraction and reverse transcriptase–PCR

Total RNA was isolated from 1×10^6 DCs using Trizol® reagent (Gibco) and standard phenol–chloroform extraction. cDNA was synthesized with the Expand™ Reverse Transcriptase kit (Roche Diagnostics, Lewes, UK). PCR used *Taq* Supreme polymerase and buffers from Helena Biosciences (Sunderland, UK). Reactions were performed with an initial denaturation of 2 min at 94°C followed by cycles of denaturation for 30 s at 94°C, annealing for 30 s and extension for 30 s at 68°C. Primers and programmes were as follows: IL-12p40, sense primer 5′-ATGGCCATGTGGGAGCTGGAGAAAG-3′ and anti-sense primer 5′-GTGGAGCAGCAGATGTGAGTGGCT-3′, amplified product 255 bp, annealing at 62°C, 34 cycles; IL-12p35, 5′-GATCATGAAGACATCACACGG-3′ and 5′-AGAATGATCTGCTGATGGTTG-3′, 257 bp, annealing at 65°C, 34 cycles and β-actin, 5′-GCACCACACCTTCTACAATGAG-3′ and 5′-GTCTAGAGCAACAT AGCAC AGC-3′, 409 bp, annealing at 62°C, 25 cycles.

Quantitative real-time reverse transcriptase–PCR

RNA was extracted as for reverse transcriptase (RT)–PCR and treated with DNase1 (Gibco). cDNA was synthesized using random hexamers and the TaqMan Reverse Transcription kit (Perkin-Elmer Applied Biosystems, Foster City, CA, USA). Real-time RT–PCR was performed as described (32). Relative quantification was done on an ABI PRISM 7700 Sequence Detector (Perkin-Elmer) using pre-developed TaqMan reagents according to the manufacturer's instructions.

Each reaction involved multiplex amplification of 18S rRNA to account for differences in the quantity or quality of RNA present. Thermal cycling conditions were 2 min at 50°C and 10 min at 95°C followed by 40 cycles of 2-step PCR consisting of 15 s at 95°C and 1 min at 60°C. All samples were amplified in triplicate. For each sample, the amount of target mRNA was expressed as an *n*-fold difference relative to the amount of target mRNA expressed by unstimulated DCs at 0 h.

IL-12 protein quantification

Supernatants were harvested from DC cultures 6 h after stimulation and assessed for IL-12 p40 and p70 expression by ELISA using paired mAb purchased from PharMingen. Intracellular cytokine staining was performed 6 h after stimulation in the presence of GolgiStop™ (PharMingen) at a dilution of 1:1500. Cells were then stained for CD11c before being fixed and permeabilized (BD Cytofix/Cytoperm™ kit) and stained with a PE-conjugated antibody against IL-12p40 (all from PharMingen). Samples were analysed by flow cytometry using forward and side scatter characteristics to gate on live cells.

T cell stimulation in vitro

DCs were harvested after activation, washed and pulsed with graded doses of OVA (323-ISQAVHAAHAEINEAGR-339; Albachem Ltd, Edinburgh, UK) for 90 min at 37°C. Pulsed DCs were washed and plated with CD4+ DO11.10 T cells at 1×10^4 DCs plus 1×10^5 T cells per 200 μl well. Proliferation was measured as [^{3}H]thymidine incorporation during the last 16 h of a 3-day culture. CD4+ T cells were purified from pooled spleen and lymph nodes by removing MHCII- and CD8-positive contaminants with a MACS depletion column (Miltenyi Biotec Ltd, Bisley, UK), and were routinely >80% CD3+ CD4+. Culture medium was as for DCs but without GM-CSF. 2-Mercaptoethanol was added to 50 μM (BDH Merck, Poole, UK).

T cell stimulation in vivo

DO11.10 lymph node cells were injected intravenously into BALB/c recipients using 5×10^6 cells per mouse. One day later, mice received DCs that had been stimulated for 6 h and pulsed with 50 μg/ml OVA (323–339), as above, using 5×10^5 DCs per mouse. Some mice were later rechallenged *in vivo* using 25 μg OVA (323–339) emulsified in CFA containing heat-killed *Mycobacterium tuberculosis* (Sigma) injected subcutaneously into each hind leg. Responses were assessed by harvesting the draining inguinal lymph nodes. When a second cohort of DO11.10 cells were injected, these were labelled with 5,6 carboxyfluoroscein diacetate succinimidyl ester (CFSE) (Molecular Probes) at 10 nM for 8 min at room temperature. In these experiments, one group received 200 μg alum-precipitated ovalbumin subcutaneously as a cell-free alternative to DC immunization. Detection of DO11.10 cells used the clonotypic antibody KJ1.26 (33), purified and biotinylated in-house.

Induction and assessment of EAE

C57BL/6 mice were immunized with 100 μg myelin oligodendrocyte glycoprotein ($MOG_{35–55}$) peptide in a total volume of 100 μl CFA to induce EAE disease, injecting 50 μl subcutaneously into each hind leg. Mice also received 200 ng pertussis toxin (Health Protection Agency, Porton Down, UK) intraperitoneally on the same day and 2 days later. Clinical signs of EAE were assessed daily using a discrete scoring system: 0, no signs; 1, flaccid tail; 2, impaired righting reflex and/or impaired gait; 3, partial hind limb paralysis; 4, total hind limb paralysis; 5, hind and fore leg paralysis; 6, moribund or dead. When given, 5×10^5 DCs were delivered intravenously 21 days before disease induction.

Statistical analyses

Unpaired Student's *t*-test was used to determine the statistical significance between two groups. Significant differences in total disease burden of mice with EAE disease were calculated using the Mann–Whitney *U*-test.

Results

DC activation is transient and occurs in the presence of IL-10

The cytokine signals received by DCs influence both their own phenotype and that of the T cells they stimulate (34). To obtain a population of DCs capable of suppressing pathological T cell responses, we activated bone marrow-derived DCs in the presence of the regulatory cytokine IL-10. LPS is an archetypal DC stimulus and induced a clear wave of phenotypic activation, with expression of MHC and co-stimulatory molecules increasing, peaking and falling again within a 24 h period (Fig. 1). The variation in expression was most striking for CD86; surface levels of CD80 were comparatively stable. DCs stimulated with LPS plus IL-10 achieved equivalently strong up-regulation of CD86 but their peak expression occurred earlier, around 3–6 h, and down-regulation was much more advanced by 24 h (Fig. 1). Throughout the time course, the expression of the DC marker CD11c was stable and similar for both populations of cells (not shown). Thus, the regulatory function of IL-10 appears not to be a simple inhibition of maturation; its influence is kinetic.

IL-10-treated DCs are fully differentiated

To confirm the activation status of DCs stimulated in the presence of IL-10, we assessed their ability to internalize a fluorescent antigen. The switch from antigen uptake to antigen presentation is a hallmark of classical DC activation (8, 35), allowing antigen uptake to be used as a measure of immaturity. Unstimulated, immature DC exposed to FITC–dextran actively internalized the antigen and became brightly fluorescent (Fig. 2A). Cells that had been matured in LPS for 24 h acquired less antigen: in a typical experiment, the mean fluorescence of this population fell from 89 to 39 units (Fig. 2A). DCs stimulated with LPS and IL-10 also showed reduced antigen uptake, their fluorescence matching that of LPS-treated DCs rather than unstimulated cells. The fully differentiated nature of the IL-10-treated DCs was further demonstrated by their limited lifespan in culture (Fig. 2B). The final consequence of DC activation is death (36), and while unstimulated cells lived longer than either population given LPS, IL-10-treated DCs again behaved as mature cells: their

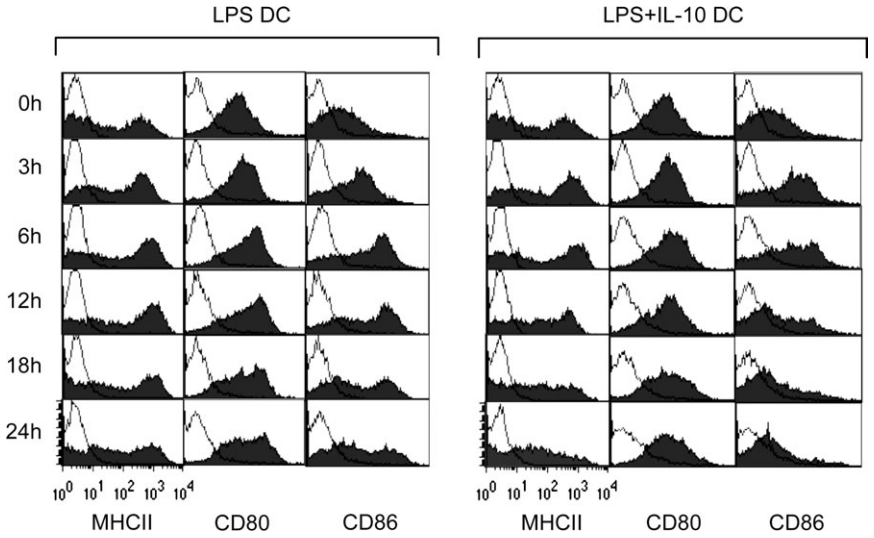

Fig. 1. DC phenotype after stimulation. DCs were harvested at day 7 of culture and stimulated with LPS or LPS plus IL-10. Samples were taken for analysis by flow cytometry at various times afterwards. Data shown is gated on CD11c+ cells and open peaks represent isotype controls. The data are representative of four separate experiments.

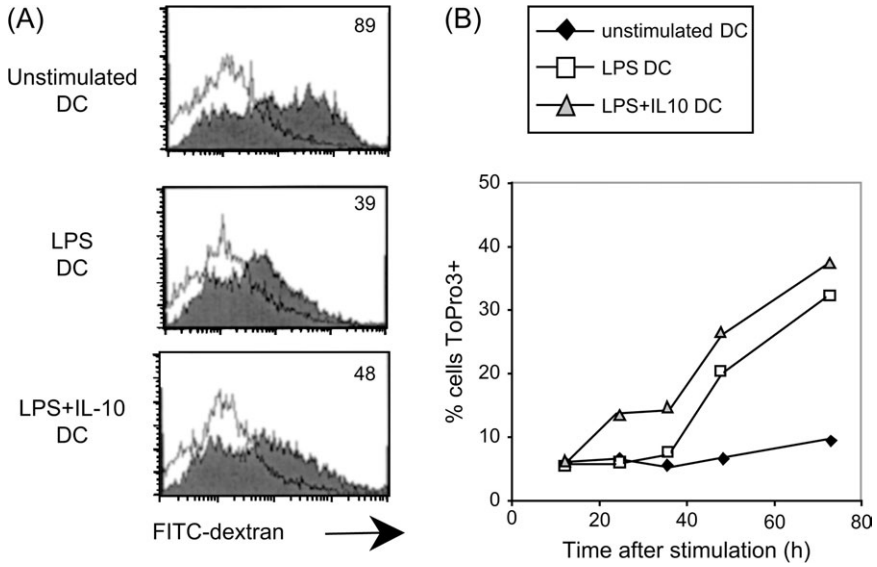

Fig. 2. Terminal differentiation of IL-10-treated DCs. (A) DCs were replated with or without LPS and IL-10 for 24 h before being washed and pulsed with FITC–dextran (2 mg/ml). Internalization was assessed by flow cytometry. Open peaks indicate background uptake when incubated on ice; filled peaks show specific uptake at 37°C. Numbers give the mean fluorescent intensity for all cells at 37°C. (B) DCs were stimulated with or without LPS and IL-10 and stained with the membrane impermeant dye ToPro3 at various times afterwards. The graph shows percentage cell death as a function of time. Both sets of data are representative of at least three independent experiments.

death rate matched that of cells in LPS alone, suggesting that IL-10 accelerates DCs through activation and on into death.

Activation is cytokine deficient

To determine whether IL-10 affected the quality of DC activation as well as its kinetics, we examined IL-12 production by RT–PCR. Both *p40* and *p35* genes were strongly induced by LPS stimulation, with or without IL-10 (Fig. 3A). The response was short-lived, with down-regulation of both genes apparent by 14 h post-stimulation. Although the kinetics were affected, with *p40* expression peaking earlier in the presence of IL-10 (Fig. 3A), the dominant effect was one of scale. This was reinforced when the samples were quantified by real-time RT–PCR (Fig. 3B). DCs activated in the presence of IL-10 displayed a brief, early up-regulation of IL-12 *p40*. Expression was maximal 2 h after stimulation, 2000-fold above the pre-stimulation background and had returned to baseline by 6 h. This response was dwarfed by that seen with LPS alone, with peak expression then 10-fold higher. The *p35* subunit showed a similar pattern: although transcription was

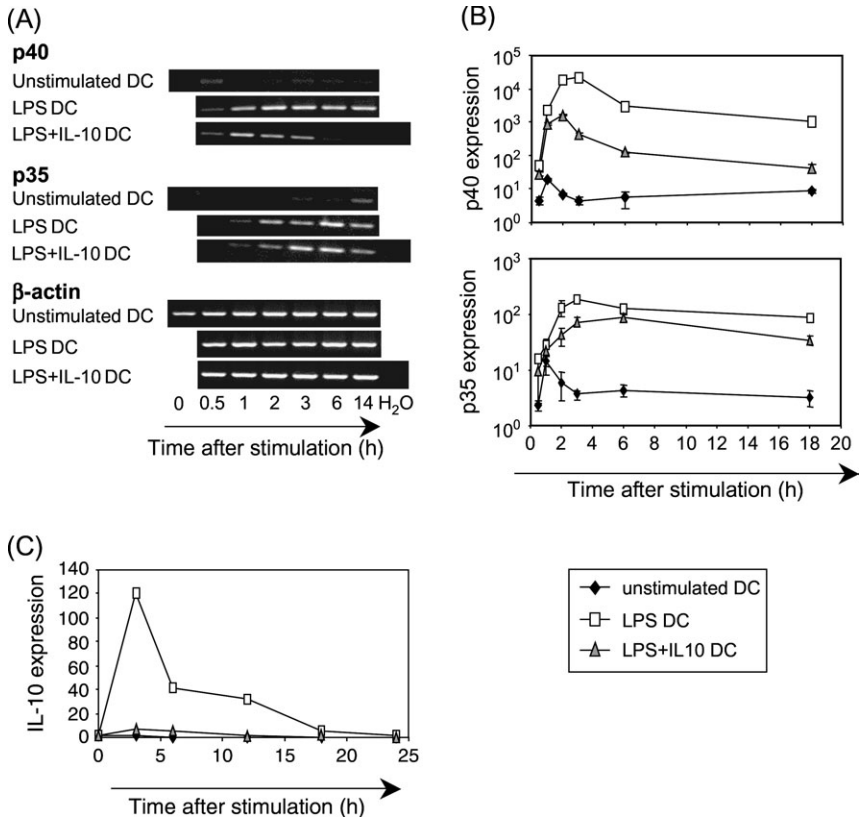

Fig. 3. Cytokine expression in IL-10-treated DCs. DCs were stimulated with LPS and IL-10 and samples taken for RNA extraction at various times afterwards. (A) Expression of the two IL-12 subunits assessed by RT–PCR using β-actin for comparison. H_2O, water blank. (B and C) Quantitative real-time RT–PCR. *Y*-axis scale indicates an *n*-fold increase above expression at 0 h. Data are representative of three independent experiments.

induced in the presence of IL-10, the effect was stronger in its absence. Notably, the decrease in IL-12 expression seen when IL-10 was included in the DC stimulation was not accompanied by any detectable increase in IL-10 production; the exogenous IL-10 in fact acted to suppress the IL-10 elicited by LPS alone (Fig. 3C).

To demonstrate that the transcriptional inhibition of *p40* and *p35* caused by IL-10 translated into a reduction in IL-12 protein expression, the cytokine content of DC cultures was assayed by ELISA 6 h after stimulation (Fig. 4A). The inhibition of LPS-elicited IL-12 in DCs activated in the presence of IL-10 was stark. Intracellular cytokine staining for the IL-12 p40 subunit confirmed that the impact of IL-10 was to reduce both the number of DCs producing IL-12 and the mean quantity of IL-12 generated per cell (Fig. 4B).

T cell stimulation is impaired

The defining feature of DC activation is their capacity to stimulate naive T cells. To assess this, we pulsed DCs with the 323–339 peptide of ovalbumin and cultured them with CD4+ cells taken from DO11.10 mice, engineered to express an OVA (323–339)-reactive transgenic TCR (29). When the DCs were used 24 h after stimulation, the presence of IL-10 during DC activation suppressed subsequent T cell proliferation to a level not equal to but consistently below that elicited by the unstimulated control DCs (Fig. 5A). This was in keeping with the limited expression of MHC and co-stimulatory molecules by IL-10-treated DCs at this time point (Fig. 1). Importantly, even at 6 h post-stimulation and despite equivalent surface levels of MHCII, CD80 and CD86, IL-10-treated DCs still did not prime T cells as effectively as their LPS-matured counterparts (Fig. 5B). This effect was as striking *in vivo* as *in vitro*: mice given a cohort of TCR transgenic, OVA (323–339)-reactive CD4+ cells showed a significantly smaller expansion of antigen-specific cells following administration of peptide-pulsed, IL-10-treated DCs harvested 6 h after stimulation than those injected with DCs activated in LPS alone (Fig. 5C).

Tolerance induction

The reduced T cell response seen after *in vivo* stimulation of DO11.10 cells by IL-10-treated DCs (Fig. 5C) could represent either a deficit in T cell priming or an active induction of non-responsiveness. To distinguish the two possibilities, recipients of transferred OVA (323–339)-specific CD4+ T cells were then immunized with OVA (323–339)-pulsed DCs and later rechallenged with the same peptide in CFA. When the draining lymph nodes were cultured *ex vivo* with antigen, those from mice first injected with LPS-stimulated DCs showed robust proliferation (Fig. 6). In contrast, when the initial immunization was with IL-10-treated DCs, proliferation after secondary challenge was completely absent.

To test whether the lack of secondary response after administration of DCs activated in the presence of IL-10 (Fig. 6)

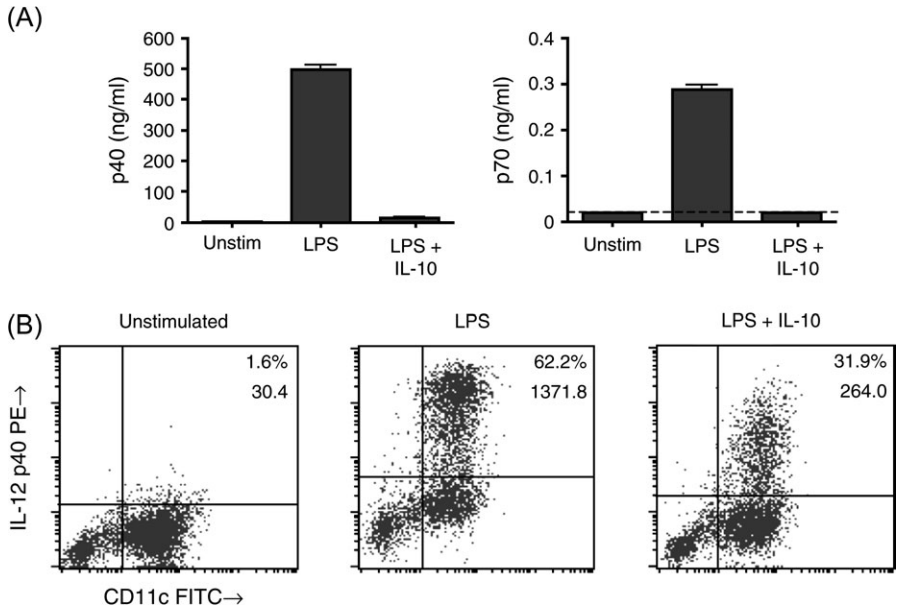

Fig. 4. IL-12 production by DCs. (A) Expression of IL-12 protein measured in DC supernatants was assessed 6 h after stimulation by ELISA. Dashed line indicates limit of detection. (B) Intracellular cytokine staining for IL-12p40 was performed after 6 h stimulation in the presence of monensin. Numbers give the percentage of live CD11c+ cells positive for IL-12p40 and the *y*-axis mean fluorescent intensity of these cells. Quadrant gates were drawn using isotype control staining in each case. Data are representative of (A) two and (B) three independent experiments.

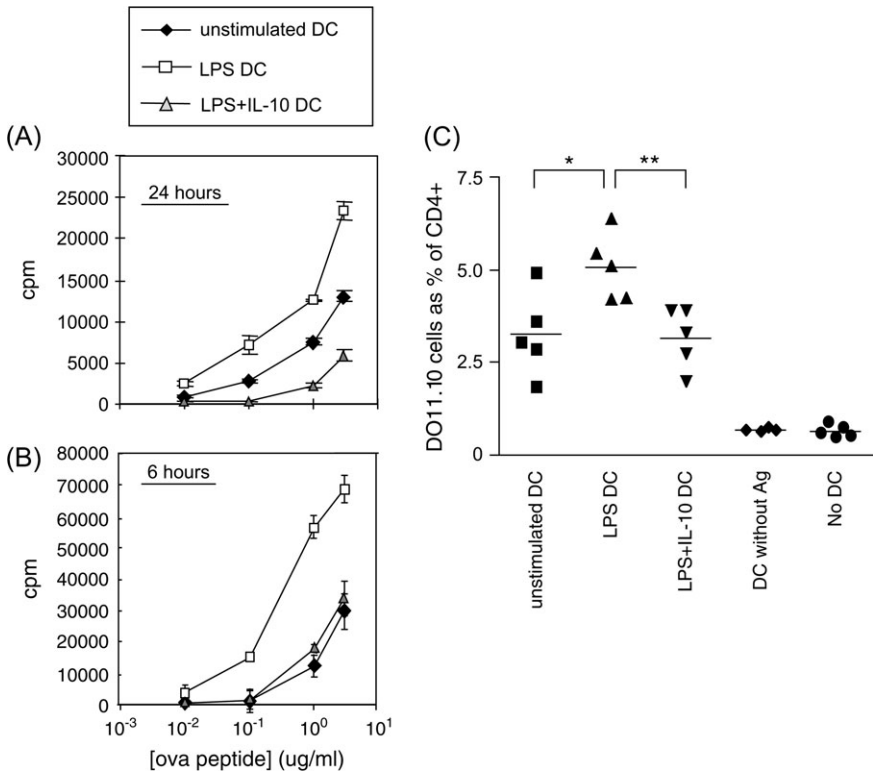

Fig. 5. IL-10-treated DCs stimulate a limited T cell response. DCs were harvested, replated with or without LPS and IL-10 for either (A) 24 h or (B) 6 h, pulsed with OVA and co-cultured with DO11.10 T cells. Proliferation was measured during the last 16 h of a 72-h culture. Data are shown as the mean of triplicate cultures ± standard error of mean, and is representative of four independent experiments. (C) Mice received an adoptive transfer of DO11.10 LNC 24 h before being immunized with DCs-given medium alone (unstimulated), LPS or LPS plus IL-10 for 6 h before being pulsed with OVA. Mice were sacrificed 5 days after immunization and the percentage of DO11.10 cells in CD4+ splenocytes was measured by flow cytometry. Each point represents an individual mouse and the graph is representative of seven independent experiments, each using 4–5 mice per group. **P* = 0.011 and ***P* = 0.004.

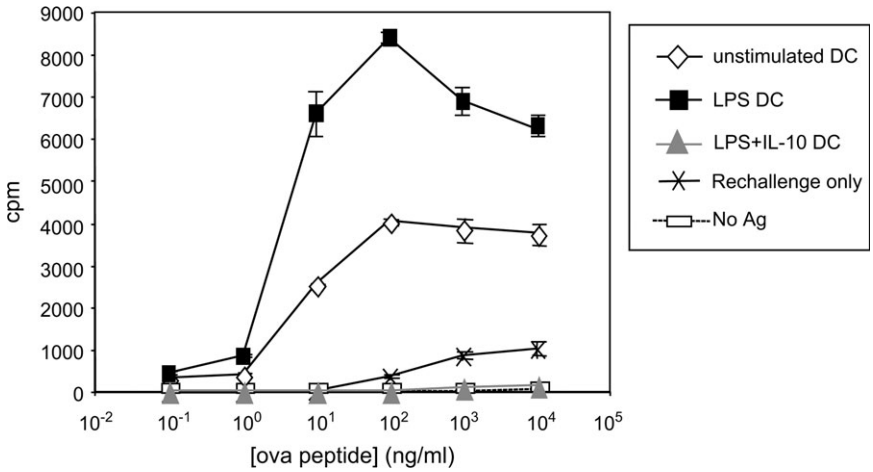

Fig. 6. Secondary challenge of T cells *in vivo*. Mice received an adoptive transfer of DO11.10 LNC prior to immunization with DCs-given medium alone, LPS or LPS plus IL-10 for 6 h and pulsed with OVA. Seven days later, all mice were challenged with OVA in CFA. Positive controls (rechallenge only) received an adoptive transfer of DO11.10 cells 24 h before immunization with the same OVA in CFA as the experimental mice. Negative controls (No Ag) were given LPS DCs without antigen at immunization and PBS in CFA at rechallenge. Draining LNs were collected 10 days after rechallenge, pooled within groups and plated for *ex vivo* culture with graded doses of OVA peptide. Proliferation was measured by [^{3}H]thymidine incorporation and data are shown as the mean of triplicate wells ± standard error of mean. The graph is representative of two independent experiments, each using 4–6 mice per group.

was caused by the induction of a suppressive or regulatory cell population, we injected a second cohort of naive TCR transgenic T cells into mice that had earlier received both DO11.10 T cells and DC immunization (Fig. 7A). While the proliferation of the second delivery of TCR transgenic T cells in response to CFA challenge was diminished in mice previously administered IL-10-treated DCs (Fig. 7B), this reduction was no more marked than in mice originally injected with either control DCs or indeed peptide in alum (Fig. 7C). The ability of any immunization to inhibit the proliferative capacity of the second cohort of transgenic cells may then reflect competition for access to antigen or antigen presenting cell following strong expansion of the original population of antigen-specific cells (37) and so, using this model, we failed to detect any activity of suppressive cells *in vivo* after administration of IL-10-treated DCs.

DCs activated in the presence of IL-10 suppress central nervous system autoimmune disease

To assess the therapeutic potential of the profound hyporesponsiveness induced in antigen-specific T cells by DCs activated in the presence of IL-10, we used an EAE model in which paralysis was initiated by immunization of C57BL/6 mice with the p35–55 peptide of MOG (38). When $MOG_{35–55}$-pulsed DCs were administered to mice before induction of EAE, and despite their phenotypic equivalence to LPS-activated cells, only the IL-10-treated DCs significantly reduced the severity of disease (Fig. 8). Interestingly, this effect was partially independent of peptide antigen: IL-10-treated DCs that were not loaded with $MOG_{35–55}$ also inhibited disease, although to a lesser extent than their peptide-pulsed equivalents. Thus, activating DCs in the presence of IL-10 drives a tolerogenic phenotype capable of limiting central nervous system autoimmune disease.

Discussion

DCs can direct both the size and character of an immune response and so are attractive candidates as immunotherapy in a range of clinical disorders (39). The immunogenic and tolerogenic functions of DCs have been understood to represent distinct stages of DC maturation (9, 40). Here we stimulate DCs in the presence of the regulatory cytokine IL-10 and demonstrate the ability of a fully differentiated DC phenotype to curtail T cell responses *in vivo*. We reveal a kinetic and qualitative influence of IL-10 on the process of DC activation, suggesting that a simple functional separation of immature and mature DCs may not be sufficient to describe the biology of these cells, and offering significant potential for a stable DC-mediated therapy against autoimmune disease, allergy and transplant rejection.

IL-10 is one of a number of factors reported to modulate DC function by holding the DC in immaturity (41–43). Other examples include apoptotic cells (35, 44), malaria-infected erythrocytes (45) and steroidal anti-inflammatories (46), so the mechanism of regulation is relevant to immunity, tolerance, infection and therapy. We have shown that DCs activated in the presence of IL-10 do respond to an LPS stimulus, down-regulating antigen uptake and increasing expression of MHC and CD80/86, but that this activation is transient. The apparent immature phenotype reported by others describing DCs 48 h or more after addition of IL-10 (26, 47–49) is therefore consistent with the observations presented here, even if the kinetics of DC activation in human monocyte-derived DCs are slower than in murine bone marrow equivalents (50). The distinction of our data is that the DCs here are phenotypically activated when they meet T cells, and yet the T cell outcome remains tolerance. This regulatory function could indicate the presence of tolerogenic molecules on the surface of IL-10-treated DCs (51, 52), although our analysis

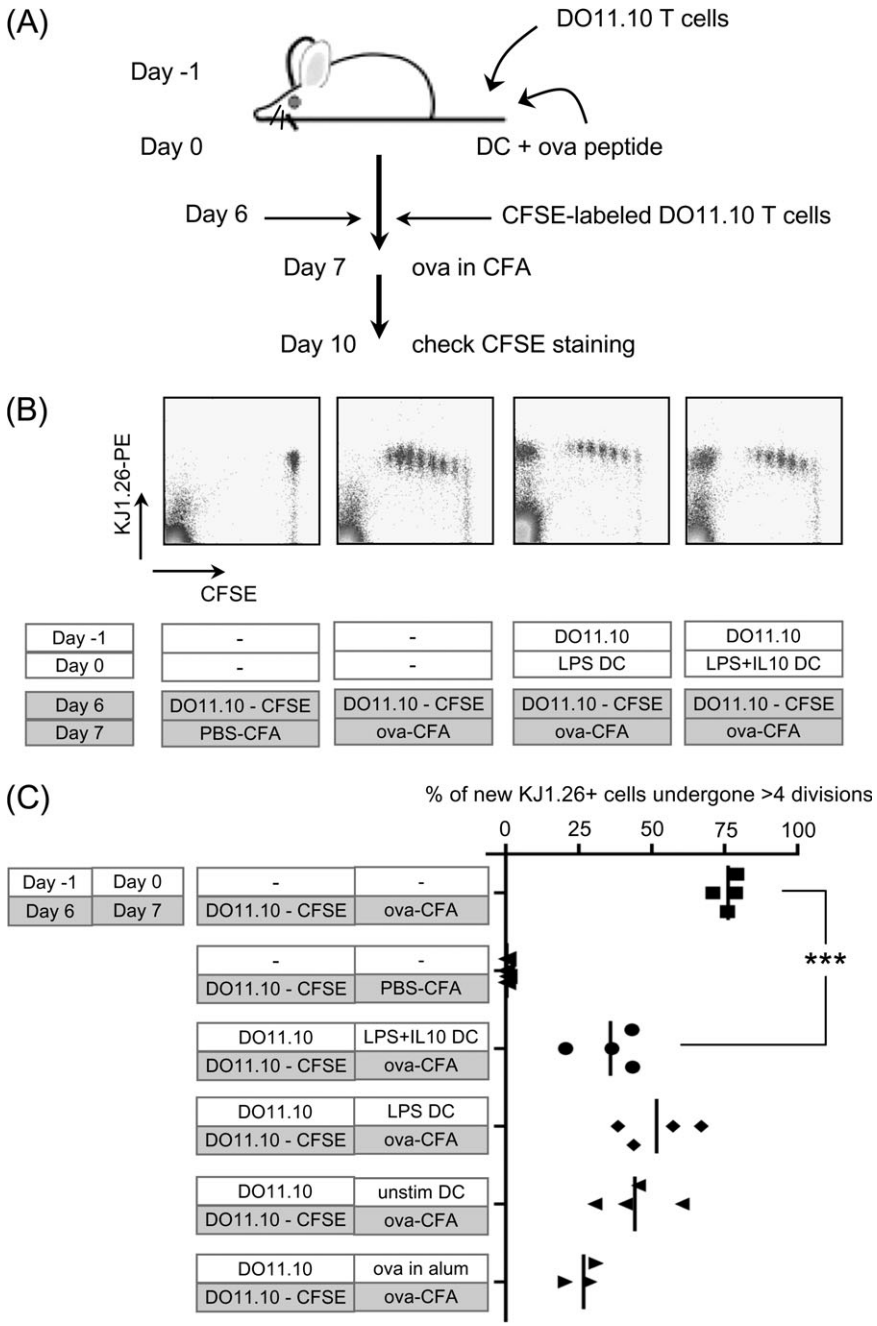

Fig. 7. Competitive suppression of antigen-specific T cells *in vivo*. (A) Experimental outline: Mice received an adoptive transfer of DO11.10 LNC prior to immunization with DCs given medium alone, LPS or LPS plus IL-10 for 6 h and pulsed with OVA. Twenty-four hours before all mice were later challenged with OVA in CFA, a second cohort of DO11.10 cells was administered, these labelled with CFSE. Proliferation of this group of T cells was measured by CFSE fluorescence and flow cytometry. (B) Example plots showing clear division of CFSE-labelled cells. (C) Data are shown as the percentage of the labelled cohort of DO11.10 cells that had undergone 4 or more cell divisions when analysed on day 10. Each point represents an individual mouse and the graph is representative of three independent experiments, each using 3–4 mice per group. While the proliferation seen in all three groups immunized with DCs was significantly reduced below that in mice receiving only the OVA–CFA rechallenge (***$P = 0.0002$), there was a significant difference neither between them nor between the IL-10-treated DC group and control mice in which alum-precipitated antigen was used to drive strong proliferation of the original DO11.10 cells (OVA–alum).

revealed no significant expression of PDL-1 or RANK (not shown). A kinetic explanation is also possible. Our data show that the high levels of MHC and CD80/86 on our IL-10-treated DCs are not maintained. Their decline is rapid, with down-regulation almost complete within 24 h in the presence of IL-10. The surface of mature DCs is considered relatively stable: the half-life of MHCII has been measured at over 100 h (53). This implies that the reduction in marker expression is an active process, not a passive loss. If the interaction between DCs and responding T cells is normally a lengthy

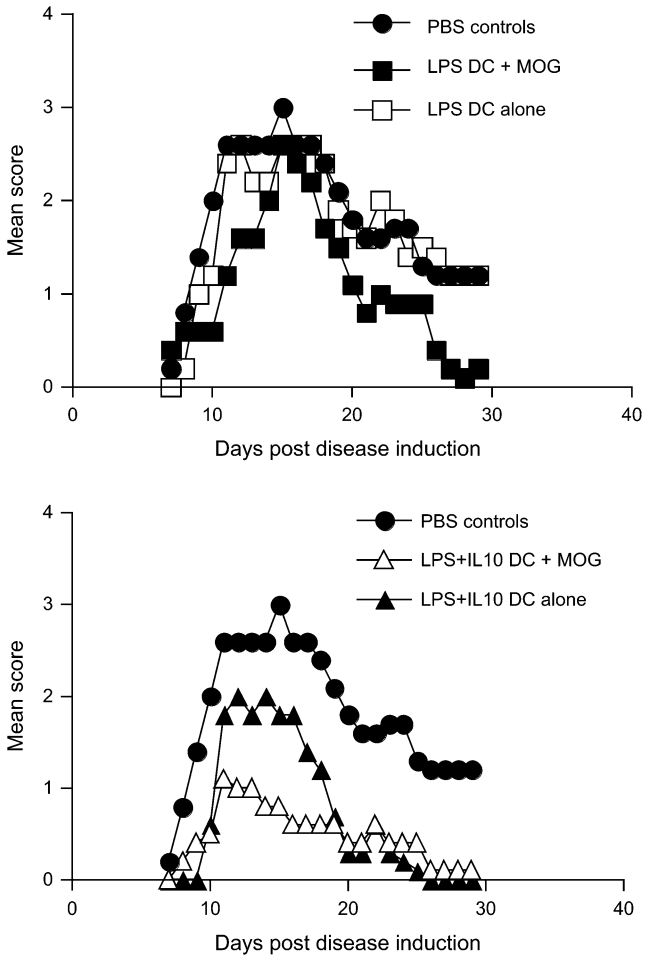

Fig. 8. IL-10-treated DCs protect from autoimmunity. DCs were stimulated with LPS or LPS plus IL-10 for 6 h and pulsed with $MOG_{35–55}$ peptide. These DCs were injected into mice 21 days before immunization with $MOG_{35–55}$ in CFA to induce EAE disease. Data are shown as the mean disease score for the 5 mice in each group and the graphs are representative of two independent experiments. Mice receiving IL-10 DCs + MOG showed a significantly lower disease burden than that of the PBS controls ($P < 0.0001$).

conversation (54), then the premature down-regulation of key DC molecules may be sufficient to limit T cell proliferation. Their activation would literally be aborted (55).

A kinetic mechanism of tolerance induction, in which DCs stimulated in the presence of IL-10 undergo early activation, rapid down-regulation and subsequent death is supported by the recorded ability of IL-10 to promote DC apoptosis (56–58). Our data demonstrate that the action of IL-10 is not mediated solely by apoptotic removal of mature, immunogenic cells, since the phenotypically immature DCs present 24 h after addition of LPS and IL-10 show reduced antigen acquisition quite distinct from that of unstimulated cells. Instead, IL-10 appears to influence DC function by dictating the timing of progression into terminal maturation. The apoptotic death that marks the end point of this maturation process may offer an explanation for the degree of inhibition of autoimmune disease mediated by IL-10-treated DCs not loaded with specific peptide antigen: DCs in the recipient mouse could capture both dying, IL-10-treated DCs and the $MOG_{35–55}$ peptide later delivered in CFA. The expected outcome would be $MOG_{35–55}$-specific tolerance (59). That peptide-loaded DCs retain a more potent influence on disease progression than equivalents without antigen suggests that specific contact between an IL-10-treated DC and responding T cells permits additional, kinetic mechanisms of tolerance induction.

If DC regulation was purely kinetic, the T cell outcome would be dictated by the stage of differentiation that the DC had reached as it arrived in the lymph node. If it met a specific T cell at the height of activation, then strong immunity would result; if it was past its peak, the consequent immune response would be different, perhaps even tolerant. This is an extension of Lanzavecchia and colleagues' description of DC exhaustion (20, 60). Simplistically, it predicts that the same activation should occur in the presence and absence of IL-10 with the only difference being one of timing. This is indeed what happens in terms of surface phenotype, but analysis of the IL-12 expression of the two DC populations also revealed qualitative differences. Bioactive IL-12 is a p70 heterodimer. In DCs regulation appears to focus on the p35 subunit (61, 62) and an excess of p40 can form inhibitory homodimers (63). Our data show that the presence of IL-10 during DC stimulation inhibits the release of both p40 and p70 proteins. Temporal regulation and alternative activation may thus be complementary. The kinetic profiles of the individual co-stimulatory molecules differ: the variation in expression is more pronounced for CD86 than CD80, for example. The balance of co-stimulatory signals provided by DCs will consequently change over time, influencing the T cell outcome (52, 64, 65).

Interestingly, even at the peak of their activation, the IL-10-treated DCs here did not themselves produce IL-10. Several reports have associated IL-10 secretion by DCs with either T_h2 (66–68) or Tr1 induction (69, 70). DC-derived cytokines may contribute to T cell activation as well as polarization (71). Two descriptions of DCs that appeared phenotypically activated but secreted little cytokine both recorded a consequent suppression of T cell responses (18, 72). Lutz and Schuler (17) termed such DCs semi-mature, a description that befits their poor performance in both cytokine release and T cell stimulation, and the concept that the process of DC maturation can be separated into distinct stages has persisted (73–75). Our data add a kinetic perspective and thus further challenge the association between DC maturation and a functional conversion from tolerogenic to immunogenic cells. In support, Rea *et al.* (76) have demonstrated that suppression of DC function by the steroid dexamethasone was concomitant with maturation and Albert *et al.* (77) reported that both cross-priming and cross-tolerance require mature DCs. Reis e Sousa (12) has recently argued that the present model or semantics of DC maturation may need to be revised. Certainly, the impact of activating DCs in the presence of IL-10 currently appears paradoxical, with interpretations of resulting immaturity (42, 43) alongside evidence of positive gene induction and biological effects (78, 79).

Activation of IL-10-treated DCs may have a physiological importance. Preliminary data suggest that, while DCs given LPS for 24 h or more are resistant to the effect of IL-10, at 6 h post-stimulation, they remain responsive (not shown).

B cells have been shown to react to IL-12 by releasing IL-10, which implies that activated B cells in secondary lymphoid tissues could down-regulate T_h1 responses by dampening the stimulatory capacity of DCs (80). Since activation of B cells is a relatively late event in an immune response, any such B cell regulation would have to act on mature or maturing DCs as they arrive from the periphery as part of a sustained influx. This fits more easily with a kinetic or qualitative regulation of DC activation than with a strict inhibition of maturation, which would require the presence of IL-10 in the peripheral tissues. This type of regulation could explain recent data from an EAE model in which an IL-10 deficiency was restricted to B cells (38) and exacerbated disease was shown to correlate with increased levels of IFNγ. Importantly, the early response of these animals was similar to that of controls, but they then failed to recover: the regulation occurred late in the immune response. This may reflect the action of B cell-released IL-10 on DCs.

In infection, an uncoupling of DC maturation and strong immunity could be detrimental: pathogens able to subvert the process could activate DCs to create an immunosuppressive environment that favours microbial replication rather than host survival (81). As tools to counter unwanted or excessive immune responses, however, the potential of activated and tolerogenic DCs is significant. The use of immature DCs to drive tolerance *in vivo* is tempered by the need to preserve that immaturity (82, 83) even in the face of ongoing infection or inflammation. Indeed, a recent report has demonstrated that the tumour necrosis factor α-treated DCs which originated the term semi-mature (18) remain open to stimulation and convert into classically activated, immunogenic DCs upon subcutaneous injection (84). The fully differentiated, tolerogenic phenotype that we describe implies a functional stability (9, 85) and hence reduced risk of unwanted exacerbation of the T cell response.

In summary, IL-10 has a powerful influence on DC function. We show that this is achieved not by preventing DC maturation, but by directing a rapid and transient activation that leaves the DC fully differentiated and able both to elicit T cell tolerance *in vivo* and protect against autoimmune disease. We suggest that these DCs have significant therapeutic potential.

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Abbreviations

CFSE	5,6 carboxyfluoroscein diacetate succinimidyl ester
DC	dendritic cell
EAE	experimental autoimmune encephalomyelitis
GM-CSF	granulocyte/macrophage colony-stimulating factor
LNC	lymph node cells
MOG	myelin oligodendrocyte glycoprotein
RT	reverse transcriptase

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