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LABORATORY INVESTIGATION

$CD4^+CD25^+$ T regulatory cells induced by LPS-activated bone marrow dendritic cells suppress experimental autoimmune uveoretinitis in vivo

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Abstract
Background Tolerance-inducing DC are considered to be less mature than immunogenic DC, but the conditions promoting a less mature DC phenotype are not clear. We have previously shown that lipopolysaccharide (LPS) can have differential effects on DC function depending on the timing of DC exposure to LPS. Here, we show that early LPS-activated bone marrow derived DC (early DC, eDC), when administered subcutaneously to mice in vivo, promote tolerance to EAU induced via immunisation with interphotoreceptor retinol binding protein (IRBP) peptide 161–180. The effect correlates with the failure of eDC to secrete IL-12, and appears to be mediated in part via expansion of naturally occurring $CD4^+$ $CD25^+$ T regulatory cells (Tregs), which also mediate suppression of EAU on adoptive transfer to naive mice followed by immunization with autoantigen.
Methods Immature DC were prepared from BMDC cultures. Early DC (eDC) and late DC (lDC) for tolerance experiments were obtained by differential timing of LPS addition and their cytokine secretion profile was analyzed. eDC and lDC were subcutaneously injected into mice. From the dLN $CD4^+$ $CD25^+$ $GITR^+$ T regulatory cells found to express FoxP3 were isolated and transferred into mice prior to immunisation with IRBP. The immune response was scored by histopathology. Tregs were characterized in vitro by intracellular staining, cytokine secretion assay and transwell experiments.
Results eDC secrete IL-10 but no IL-12 or IFNγ. When injected subcutaneously into naive mice, they expand the population of $CD4^+CD25^{+high}$ $GITR^+$ T cells expressing FoxP3 in the dLN, thus increasing the total number of IL-10 producing cells. eDC induced Tregs inhibit $CD4^+$ $CD25^-$ T effector cell proliferation by a contact dependent process, and both eDC and Tregs suppress retinal damage when adoptively transferred.
Conclusions We suggest that DC maturation may be necessary for both tolerance and immunity, but differential levels of activation and/or cytokine production direct the outcome of DC-T cell interaction and this is determined by IL-12 production. T regulatory cells induced in vivo by contact with eDC are able to suppress disease in the EAU model by adoptive transfer.

Keywords Dendritic cells · Experimental autoimmune uveoretinitis · Lipopolysaccharide · T regulatory cells

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Introduction

Recent experimental evidence has challenged the view that dendritic cell (DC) function can be categorised in terms of mature immunogenic versus immature tolerogenic differentiation stages [25, 27]. Extensive studies have focused on different ways of ex vivo manipulation in order to generate DC that have the capacity to be tolerogenic. It has been shown that DC exposed to TNF-α induce IL-10 producing $CD4^+$ T cells that prevent autoimmune encephalomyelitis (EAE) in the murine model, leading to the hypothesis that suppression of disease by DC might depend not so much on

the surface phenotype of these cells, but rather on the cytokine environment they create [21].These phenomena have been confirmed in a model of experimental autoimmune thyroiditis where TNF-α treated semimature DC failed to produce pro-inflammatory cytokines and induced T regulatory cells (Tregs) which suppressed disease [36]. In addition, induction of immunity versus tolerance may depend not only on the functional status of the DC, but also on which T cell subset is induced [11].

We have previously shown that bone marrow DC cultured in GM-CSF express low levels of activation markers and, when administered by subcutaneous injection, reduce the severity of inflammation in mice with experimental autoimmune uveoretinitis (EAU), a CD4 T cell mediated disease model with close similarities to human sight-threatening uveitis. In contrast, DC exposed to the TLR4-agonist lipopolysaccharide (LPS), failed to inhibit EAU [14]. However, a previous study showed that under certain conditions, LPS treatment of DC induced an immature phenotype [22]. In further work, we also observed that DC cytokine production after LPS stimulation depended greatly on the timing of administration of the LPS, in that immediate treatment of purified DC in LPS led to high levels of IL-10 production and almost no IL-12 production, while treatment of DC with LPS after culture for 16–24 h post-purification led to IL-12 rather than IL-10 secretion [15]. This clearly demonstrated the effect of the microenvironment on DC function, and suggested that under certain conditions LPS stimulation could induce IL-10-secreting DC with the potential to promote tolerance. Thus, there are subtleties in the increasingly recognised link between innate and adaptive immune recognition which remain to be explored, and which may have significance for DC vaccination protocols.

We have extended these observations in the present study by culturing mouse bone marrow derived DC (BMDC) with LPS at different times after isolation and purification, and directly tested their effect on inhibition of EAU in B10RIII mice. Our data reveal that early LPS- activated bone marrow derived DC (early DC, eDC), when administered subcutaneously to mice in vivo, promote tolerance to EAU induced via immunisation with interphotoreceptor retinol binding protein (IRBP) peptide 161–180 [32].

We show furthermore that these eDC appear to induce an increase of $CD4^+CD25^+$ T regulatory cells which also mediate suppression of EAU on adoptive transfer to naive mice.

Materials and methods

Animals

Inbred male B10RIII mice 8–12 weeks of age were provided by the Medical Research Facility at Aberdeen University Medical School. EAU was induced in mice by immunizing subcutaneously with IRBP peptide 161–180 (SGIPYIISYLHPGNTILHVD; purity > 95%) synthesized by Sigma Genosys Co. (Cambridge, UK) in CFA. The procedures adopted conformed to the regulations of the Animal License Act (UK) and to the ARVO statement for The Use Of Animals in Ophthalmic and Vision Research.

Generation of BMDC

Bone marrow (BM) cells were depleted of mature myeloid cells, B and T cells using a monoclonal antibody mixture [rat anti-B220 (clone RA3-6B2), anti-CD4 (clone GK 1.5), anti CD8 (clone 53–6.7) and anti-MHC-II (clone P7/7)] and sheep anti-rat dynabeads (Dynal Biotech, Bromborough, UK) for immunomagnetic separation. All antibodies were from BD Pharmingen UK, Ltd (Oxford, UK) except MHC-II, which was from Serotec (Oxford, UK). BM cell cultures were performed in supplemented RPMI 1640 (Gibco-BRL, Paisley, UK). 5% GM-CSF rich supernatant from the Ag8653 myeloma cell line transfected with murine GM-CSF cDNA (gift from G. Stockinger, NIMR, London) was added to the culture medium as previously described [14]. At the end of the 6-day culture, the contaminating granulocytes were depleted using anti-mouse Gr-1 mAb [(clone RB6-8C5), Pharmingen, San Diego, Calif., USA]. For each experiment, purity controls were performed: on average the DC stained between 91 and 95% positive for the surface marker CD11c and the percentage of CD3 or CD45/B220 positive cells in all experiments ranged between 0.5 and 1% only.

Generation of early LPS-activated DC (eDC)

Purified immature DC were prepared from BMDC cultures using a procedure described by Inaba et al. [13] and modified by Jiang et al. [14]. Early DC (eDC) for tolerance experiments were obtained by adding LPS (1 μg/ml) immediately after purification for 16 h. IRBP peptide (30 μg/ml) was added overnight.

Adoptive transfer experiments

10^6 eDC pulsed with IRBP were injected subcutaneously into the scruff of the neck of each mouse of a group of six per treatment and experiment. After 72 h the neck lymph nodes [superior cervical (SCLN), submandibular (SMLN) and internal jugular (IJLN)] were harvested and pooled within each group. T regulatory cells following eDC or PBS injection were isolated as the $CD4^+CD25^+$ fraction after MACS (Miltenyi Biotech, Bergisch-Gladbach, Ger-

many) separation. $CD4^+CD25^+$ cell purity was around 98% in each experiment, which equalled that claimed by the magnetic bead manufacturers. 5×10^4 Treg cells/mouse were injected intraperitoneally into B10RIII mice. Twenty-four hours later the mice were immunized with IRBP peptide for induction of EAU.

Disease evaluation

Mice were killed by asphyxiation in CO_2 and their eyes dissected and fixed in 2.5% buffered glutaraldehyde and embedded in resin for standard H-E staining. Disease severity was scored in a masked fashion in three sections of each globe cut at different levels. Severity of disease was graded on a scale of 0–4 in half-point increments according to a semi-quantitative system as described [1]. Non-parametric analysis of the EAU grades was performed by Mann-Whitney rank sum test (SPSS, Chicago, Ill., USA). $P<0.05$ was considered statistically significant.

$CD4^+CD25^+$ isolation and staining for intracellular IL-10 and surface GITR

B10RIII mice 8–12 weeks old were injected with 10^6 eDC each or PBS (as a control) subcutaneously in the scruff of the neck. After 3 days, the animals were killed and the draining neck lymph nodes harvested and pooled in the two groups and plated out in 24 well flat bottom plates (5×10^6 cells/well) in 1 ml of culture medium. Cells were cultured in medium alone or stimulated with either IRBP peptide 161–180 (3 μg/ml) or 5 μl/ml of anti-CD3 (purified, clone 145 2CII BD Biosciences Pharmingen, Oxford, UK) for 48 h and Golgi Stop at 0.67 μl/ml (BD Pharmingen) for the last 4 h of the culture. Cells were harvested and surface-stained for CD4 (anti-CD4 RM4-5), CD25 (anti-CD25 7D4) and CD3 (anti-CD3 17A2), then fixed and permeabilised with 0.1% Saponin and then incubated with anti-IL-10 antibody (JES5-16E3). All antibodies were used as colour-conjugates (BD Pharmingen) and four-colour FACS analysis was performed consecutively (FACS LSR; Becton Dickinson, UK). For surface staining purified polyclonal goat anti-GITR and normal goat IgG (control Ig) were purchased from R&D Systems (Minneapolis, Minn., USA) and FITC-labelled donkey anti-goat from Jackson ImmunoResearch Laboratories (West Grove, Pa., USA).

FoxP3 analysis

Neck lymph nodes were harvested from B10RIII animals that had received eDCs pulsed with IRBP peptide 161–180 4 days prior to harvest. Cells were isolated by MACS separation and total RNA was extracted using TRIZOL (Invitrogen Ltd, Paisley, UK). 4–5 μg RNA was reverse transcribed using M- MLV RT and oligo dT primers.

Obtained cDNA was taken as the template for PCR, using the following primers for GAPDH: 5′ GAA GGG CTC ATG ACC ACA GTC CAT G-3′ and 5′ TGT-TGC-TGT-AGC-CGT-ATT-CAT-TGT-C 3′, for FoxP3: 5′- CAG CTG CCT ACA GTG CCC CTA G- 3′ and 5′- CAT TTG CCA GCA GTG GGT AG- 3′. The PCR consisted of a denaturation step of 2.5 min at 94°C, followed by 32 cycles each of 30 s at 94°C, 57°C and 72°C. The PCR products were run on a 1.8% agarose gel and analyzed using Gene Tool and Gene Snap from Syngene (Cambridge, UK). For real time PCR, the PCR product was purified using QIAquick PCR Gel extraction columns and quantified prior to real time PCR. PCR product was used for standard curve, PCR was then performed with DNA Engine Opticon 2 Continuous Fluorescence Detector (MJ Research), using 10 μl Master Mix from Finnzyme DyNAmo SYBRGreen qPCR Kit (Finnzymes Oy), 5 μl 1.2 μM primer and 5 μl cDNA. PCR consisted of a 10-min denaturation step at 94°C, followed by 40 cycles of 94°C for 30 s, 57°C for 30 s, 72°C for 30 s and 76°C for 10 s for HPRT, an additional step at 79°C for 10 s was added for FoxP3. Primers for FoxP3 were: 5′- CCC AGG AAA GAC AGC AAC CTT 3′ and 5′ TTC TCA CAA CCA GGC CAC TTG 3′, primers for HPRT were 5′ TGA AGA GCT ACT GTA ATG ATC AGT CAA C 3′ and 5′ AGC AAG CTT GCA ACC TTA ACC A 3′. All samples were run in triplicates.

Cytokine secretion analyses

Cytokine profiles in culture supernatants were evaluated using the Becton Dickinson Cytometric bead multiplex array system for IL-12, TNF-α, IFNγ, IL-10 and IL-6 according to the manufacturer's instructions.

In vitro $CD4^+CD25^+$ T cell transwell experiments

For transwell analysis of the requirement for cell-cell contact, $CD4^+CD25^-$ cells were obtained from draining lymph nodes at day 6 after IRPB immunization. $CD4^+CD25^+$ cells were obtained from neck lymph nodes (=dLN) at day 3 after eDC transfer. Spleen APCs were prepared from splenocytes depleted of $CD4^+$ and $CD8^+$ cells with immunomagnetic beads, treated with mitomycin C (Sigma, 25 μg/ml for 40 min) and extensively washed before adding to T cells. For transwell culture 1×10^5 $CD4^+CD25^-$, 1×10^5 APCs and 5×10^4 $CD4^+CD25^+$ and 1 μg/ml anti-CD3 antibody were added as indicated in the bottom well or insert (Falcon, 0.4 μm) (see Results). After 3 days, cultured cells were transferred into a 96-well plate and pulsed with 0,5 μCi ^{3}[H]Thymidine for a further 16 h.

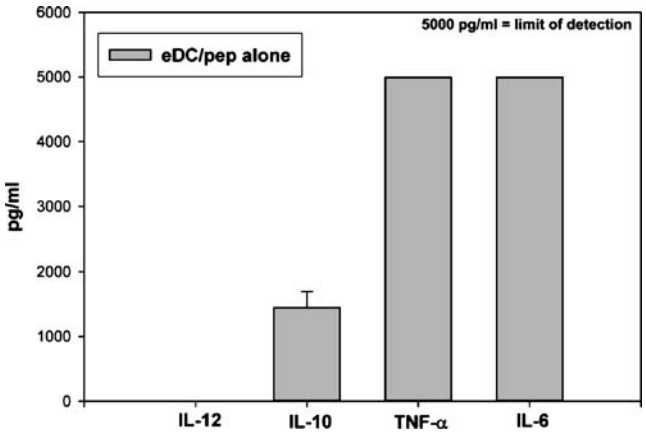


Fig. 1 Early DC (*eDC*) in medium alone secrete high amounts of IL-10, IL-6 and TNF-α and only little IL-12. A cytometric bead array assay by flow cytometry was performed with 24 h-culture supernatants of early DC (1×10^6). The MFI is connverted to pg cytokine. The eDC had been pulsed with IRBP peptide for 12 h. The assay was set up in triplicates. 100 µl of supernatant of each well was harvested at 24 h and FACS analysis performed. Results are shown mean±1 SD (*n*=three wells) from one experiment of three

Results

LPS activated bone marrow derived dendritic cells (eDC) secrete IL-10 but no IL-12

We have previously shown [14, 15] that time-dependent maturation signals in the life cycle of cultured DC could be involved in polarizing their function toward tolerogenicity or immunogenicity. Using this method, we collected clusters of BM cells after a 6-day period of culture in medium supplemented with GM-CSF, depleted them of Gr-1-positive cells and obtained $CD11c^{low}$-positive, CD8-α negative DC that were low expressors of MHC II, and moderate expressors of CD40 and CD86 on their surface (data not shown). To these we added LPS and interphotoreceptor retinoid-binding protein (IRBP) 161–180 peptide immediately after cell harvesting from the clusters, and cultured them for a further 16 h. In previous work, we have shown that eDC secrete IL-10 but no IL-12 [15], and have extended this work to include other pro-inflammatory cytokines. DC were cultured in medium alone and the IL-10 content was assessed in the supernatants after 24 h by Cytometric Bead Assay (Fig. 1). We found that there was no IL-12 production, but that there was significant IL-10 secretion as well as high levels of TNF-α and IL-6 secretion. IL-10 secretion persisted for some days but TNF-α production decreased after 3 days of culture (data not shown).

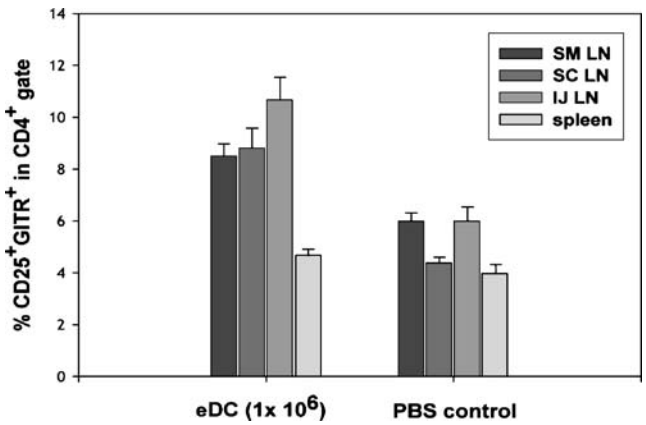
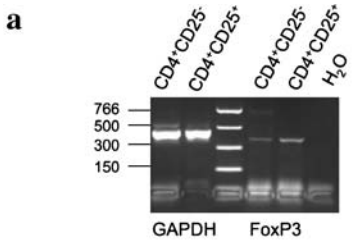


Fig. 2 Percentage of $CD4^+CD25^+GITR^+$ T cells in submandibular (*SMLN*), superficial cervical (*SCLN*), internal jugular (*IJLN*) lymph nodes and spleen after immunisation with early DC (*eDC*) as measured by flow cytometry

$CD4^+CD25^+$ $GITR^+$ T cells found to express FoxP3 are upregulated in the draining lymph node following subcutaneous injection of LPS activated eDC

Immature DC have been implicated as the candidate APC for the induction of suppressor T cell function [16]. Mahnke et al. [23] have shown that by targeting immature DC with antigen coupled to a-DEC-205 mAb, $CD25^+$ regulatory T cells are induced in vivo that suppress proliferation of $CD4^+$ T cells in an MLR assay in a dose-dependent manner. Although CD25 is a useful marker for the identification of Treg cells, its expression upon activation of naive $CD4^+CD25^+$ T cells limits its usefulness for determining an exclusive Treg population. A functional role for the TNF receptor family member GITR (glucocorticoid-induced TNF receptor) was suggested after comparative gene expression analyses [26] of $CD4^+CD25^+$ and $CD25^-$ cells. In our experimental setting, we found that subcutaneous injection in the nape of the neck with IRBP-pulsed eDC led to an increase in the resident population of $CD4^+CD25^+GITR^+$ cells (which we presume represent the regulatory cell fraction within the total of $CD4^+$ $CD25^+$ T cell population) in all three cervical (=draining) lymph nodes but not in the spleen (Fig. 2). The expression appears to peak between the 48- and 72-h time point following DC injection (data not shown) and reaches >11% in the dLN compared with a maximum of 6% in the PBS control group). However, relying solely on the expression of GITR was not informative enough, as GITR is expressed also by non-regulatory $CD25^+$ cells that have responded to antigen. In contrast, FoxP3 is a key transcription factor for the development and function of Tregs [6, 10, 33] and is critical for both development and function of Tregs in the murine system. We show that our $CD4^+CD25^+$ cells induced by eDC express FoxP3, both by semi-quantitative PCR (Fig. 3a) and by quantitative RT-PCR (Fig. 3b), are consistent with the properties of naturally occurring $CD4^+$ $CD25^+$ T regulatory cells. In contrast, the relative levels of FoxP3 expression induced by late DC (lDC, i.e. DCs treated with LPS after a period of culture in vitro, see [15]) is less than in eDC (Fig. 3b).



		FoxP3		GAPDH		FoxP3/GAPDH
		MW	raw volume	MW	raw volume	
eDC	$CD4^+CD25^-$	375	7093.4	433	96629.59	0.073
	$CD4^+CD25^+$	368	13550.95	451	99719.59	0.136

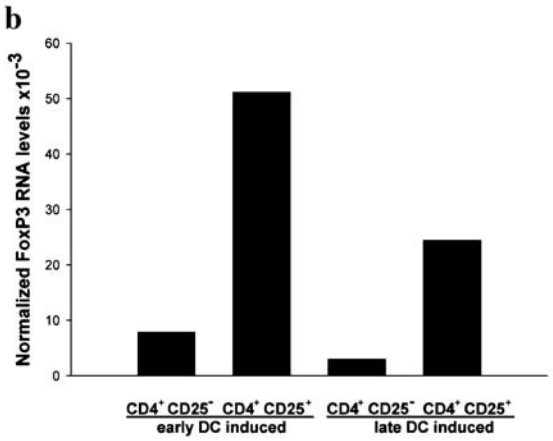


Fig. 3 **a** Semiquantitative PCR analysis of FoxP3 expression in $CD4^+CD25^+$ T cells compared to $CD4^+CD25^-$ T cells. Relative quantities of FoxP3 expression by $CD4^+25^+$ and $CD4^+CD25^-$ populations of T cells are shown as measured by densitometry. **b** Quantitative RT-PCR analysis of mRNA from eDC-induced $CD4^+CD25^+$ showing a high expression of FoxP3 in $CD25^+$ T cells compared with CD 25^- T cells. Late DC (*lDC*) were incubated with LPS 16h after purification form bone marrow precursors, while early DC (*eDC*) were incubated with LPS immediately after purification (see [14])

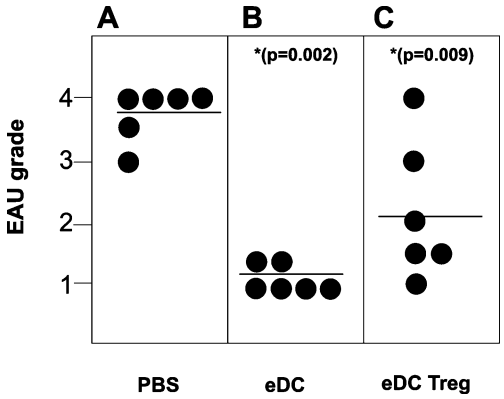


Fig. 4 Adoptively transferred eDC-induced Treg downregulate EAU. B10RIII mice received a single injection of Treg induced by eDC intraperitoneally 48 h prior to immunization, or eDC subcutaneously 10 days prior to immunization. Eyes were harvested for histopathology on day 16 and were graded on a scale of 0 (no disease) to 4 (maximum disease). Each *full circle* represents one mouse. *Horizontal bar*: average score of each group. **a** A single intraperitoneal injection of PBS before immunization in the control group showed a high grade of EAU. **b** A single subcutaneous injection of eDC/pep before immunization leads to suppression of disease as shown previously. **c** A single injection of eDC/pep-induced Treg significantly reduced EAU

The $CD4^+CD25^+$ T regulatory cell population in the dLN after IRBP-pulsed eDC injection is significantly expanded, but the percentage of IL-10 producing T Tregs remains unchanged

The mechanism of suppression of $CD4^+CD25^+$ T cells remains controversial. In most in vitro studies, cell contact was required for suppression and this seemed independent of cytokines such as IL-10 or TGF-β [31]. When we cultured the purified eDC-induced $CD4^+CD25^+$ population from the dLN in the presence of IRBP peptide (Table 1), we detected an overall increase in $CD4^+CD25^+$ cells. Interestingly, this increase was mostly due to a population of $CD25^{+high}$ expressing cells. Although the percentage of IL10 producing cells remained low and similar to control values (Table 1), the total number of $CD4^+CD25^{+high}$ IL-10-producing cells would be proportionately increased, leading to an overall increase in IL10 production in situ.

Pre-treatment of B10.RIII mice with LPS activated IL-10-secreting, eDC leads to marked inhibition of EAU induced by IRBP peptide, and the effect is mediated by T regulatory cells

Typical histopathological findings in IRBP-induced EAU include vasculitis, granuloma formation throughout the

Table 1 Percentage of $CD4^+CD25^+$ T regs of dLN cells and IL-10 expression after eDC transfer

	CD3/ CD4	CD3/CD4/ CD25	CD3/CD4/ CD25high	CD3/CD4/ CD25low	CD3/CD4 CD25high IL-10 exp	CD3/CD4 CD25low IL-10 exp
LN cells after early eDC transfer	29.57	19.95	10.08	10.11	1.63	2.35
LN cells after PBS transfer	27.12	12.76	1.88	11.39	1.85	0.87

Draining lymph nodes were harvested at day 3 after eDC or PBS transfer and cultured in the presence of IRPB 161–180 for 48 h. FACS analysis shows an increase in $CD4^+$ $CD25^+$ cells which is mostly due to $CD25^{+high}$ cells

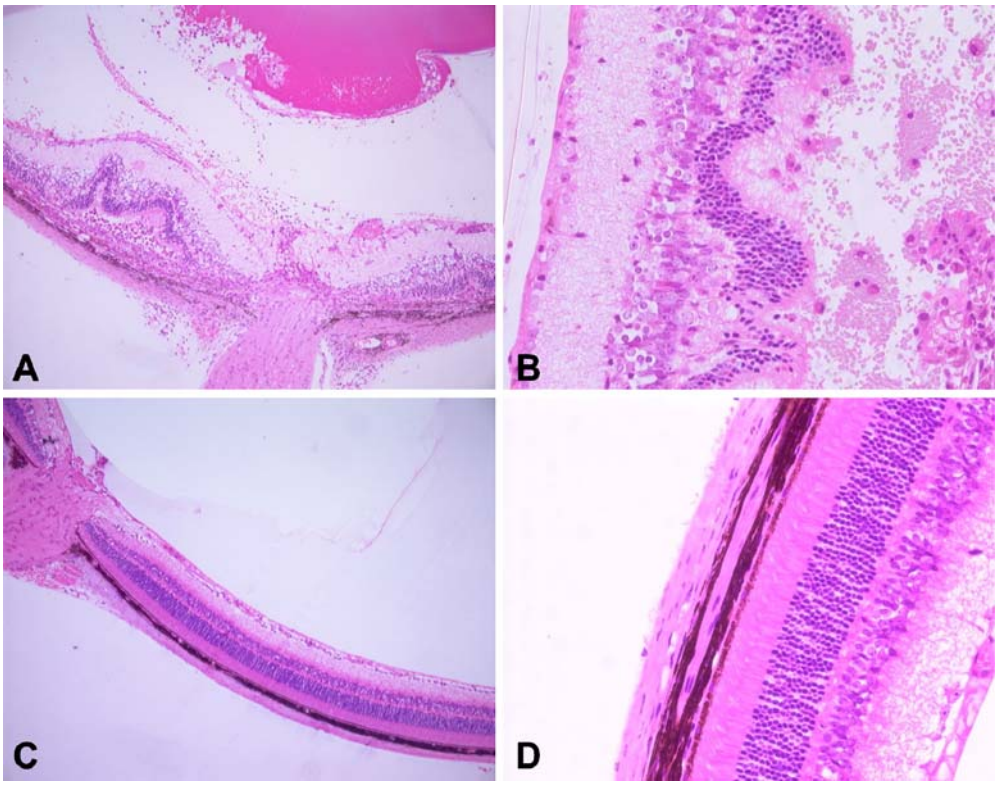


Fig. 5 Histopathology of eyes in mice pre-treated with PBS (**a**, **b**), adoptively transferred $CD4^+CD25^+$ T cells induced by peptide-pulsed eDC (**c**, **d**) prior to immunization: **a** and **c** show a 20× enlargement and **b**, **d** a 40× enlargement respectively, featuring the same eye. In **a** and **b** (PBS control) there is extensive retinal damage showing retinal folds subretinal exudates, granuloma formation, rod outer segment layer destruction and vitritis, whereas in **c** and **d** there is complete absence of disease and intact retina throughout. The results shown are representative of six mice in each group and two independent experiments

retina, subretinal exudates, retinal folds, retinal detachment and rod outer segment loss (for review, see [7]. We have previously shown that immature non-LPS activated DC inhibit induction of EAU, while LPS-activated mature DC augment disease severity [14]. Mature DC in those experiments synthesised high levels of IL-12. Here we show that LPS-activated, IRBP-pulsed, IL-10 secreting eDC are even more potent in inhibiting EAU induction (Figs 4, 5). In addition, adoptive transfer of Tregs harvested from dLN targeted by subcutaneous injection of eDC, from mice which had been previously immunised with LPS activated eDC, also led to a significant reduction in EAU, although not so potently as eDC treatment itself (Figs 4, 5).

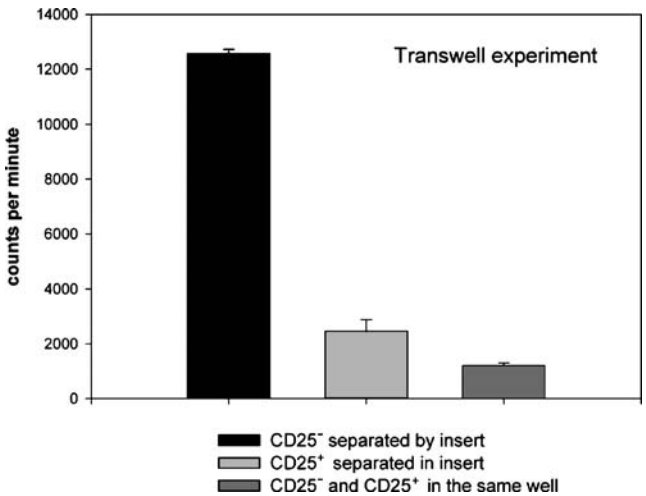


Fig. 6 Cell-cell contact is needed for functional activity of the $CD4^+CD25^+$ Tregs $CD4^+CD25^-$(isolated from dLN after IRBP immunization) and $CD4^+CD25^+$ (isolated from neck LN after eDC treatment) were either separated or not by a semipermeable membrane (Falcon 0.4 μm) and stimulated with aCD3 and MMC-treated APC on each side. In the presence of $CD4^+CD25^+$ proliferation of $CD4^+CD25^-$ effector cells is markedly inhibited

eDC induced $CD4^+CD25^+$ T regulatory cells inhibit $CD4^+CD25^-$ T effector cell proliferation by a contact dependent process

In order to test the in vitro capability of eDC induced Tregs to inhibit IRBP activation of T cells, a transwell experiment was performed as indicated in Materials and methods. In brief, $CD4^+$ $CD25^-$ cells from dLN of IRBP-immunised mice were prepared by immunomagnetic bead separation to over 95% purity and cultured in the lower wells of the transwell chambers with anti-CD3. Anti-CD3 was chosen stimulus for the T cells as an antigen-specific stimulus like IRBP would have to be presented by the APC in the system and it would therefore not be possible to exclude that any "suppressive" cytokine is derived from those cells.

In absence of $CD4^+CD25^+$ T cells or when $CD4^+CD25^+$ T cells were cultured in the upper wells of the transwell chambers, strong proliferative responses were induced. However, when the $CD4^+CD25^+$ T cells were co-cultured with the $CD4^+CD25^-$ T cells in the lower wells of the chambers, marked inhibition of proliferation was

observed, indicating the requirement for cell-cell contact for functional activity of the $CD4^+CD25^+$ Tregs (Fig. 6).

Discussion

The data in this report show for the first time that LPS-activated DC have the potential to induce tolerance to autoimmune disease and that they do so by expansion of naturally occurring $CD4^+CD25^+$ T regulatory cells. When adoptively transferred prior to immunisation with an autoantigen, these T cells inhibit disease of the target organ on a histological level. The eDC-induced Tregs appear to have the characteristics of classical $CD4^+CD25^+$ Tregs in that they express high levels of FoxP3, are $GITR^+$, variably express intracellular IL-10, and suppress $CD25^-$ T cells from proliferation via a contact mediated mechanism.

A question arising from these results is how does LPS mediate these effects in DC? LPS activation of DC is mediated via TLR4 and is classically described as leading to the induction of a mature phenotype [12] (upregulated expression of co-stimulatory molecules) and release of pro-inflammatory cytokines. However, recent studies have shown that these two processes are not causally linked. Inflammatory cytokine production is intrinsically linked to signalling via the adaptor proteins Myd 88 and TIRAP [11, 18, 37, 38], whereas expression of co-stimulatory molecules is Myd 88-independent [17]. Priming of CD4 Th1 cells by LPS activated Myd 88 $^{-/-}$ DC is impaired despite normal DC maturation and migration to the draining lymph node, but can be restored if $CD25^+$ Treg cells are transiently depleted [28]. This suggests that, in addition to co-stimulation, production of pro-inflammatory cytokines by DC is essential for Th1 cell priming. Whether this is a direct effect on the naive T cell is not clear, but it is possible also that the mechanism of action of cytokines such as IL-12 may be to remove the inhibitory effects of Treg cells during naive Th1 cell priming, for optimal induction of the immune response. Indeed this has already been directly demonstrated [29, 30].

This notion is supported by the data in the present report. We have previously shown that IL-12 production by in vitro LPS-activated DC is markedly affected by the timing of administration of the LPS. Early exposure of DC (eDC) to LPS prevents IL-12 production, while later administration after overnight culture (lDC) leads to high levels of IL-12 [15]. These effects are only seen when the initial culture conditions for DC generation consist of medium containing GM-CSF alone. When DC are generated in the presence of IL-4, high levels of IL-12 are produced while IL-10 production ceases [15]. These unpredicted effects of IL-4 on DC function have recently been confirmed in LPS-activated DC by others and are specific for DC, since IL-4 did not have the same effect on cytokine production by LPS-activated B cells [39]. Low IL-12 production with reciprocal high IL-10 production by LPS-activated eDC have been further confirmed in the present study, but interestingly was associated with high levels of other pro-inflammatory cytokines, such as TNF-α and IL-6. This suggests that the requirement for DC-cytokine associated inhibition of $CD25^+$ Tregs is restricted to IL-12.

The roles of TNF-α and IL-6 in DC maturation/activation are also somewhat contentious. While TNF-α is a recognised pro-inflammatory cytokine, several studies have demonstrated that TNF-α treated DC adopt a semi-mature phenotype and induce tolerance rather than immunity [8, 9, 27, 36]. In the present study, LPS activation of DC was accompanied by very high levels of TNF-α secretion which, if the above evidence withstands scrutiny in the context of T regulatory cells, may assist in down-regulating the immune response. In contrast, IL-6 is recognised as a pro-inflammatory cytokine which is required to render Th1 cells refractory to Treg cells [30]. In the current study, IL-6 was produced in significant amounts but was shown previously not to be different in tolerising versus immunising DC [14, 15]. However, it is also likely that IL-6 action on Th1 cells is dependent on having sufficient IL-12 for T cell priming [30], and therefore any IL-6 present in the milieu would have been ineffective. Taken together with the failure of eDCs in the present study to produce IL-12, the scenario is reminiscent of pulmonary DC, which are also CD8-α-negative, secrete IL-10 and appear to actively downregulate the production of IL-12 through IL-6. In the IL-6 $^{-/-}$ mouse, this effect is abrogated [4]. IL-6 was found to inhibit T cell-independent production of IL-12 when added to LPS/IFNγ-stimulated DC cultures [34] and was also found to downmodulate IL-12 levels in response to *Schistosoma mansoni* in a model using IL-6 $^{-/-}$ mice [20]. In addition, IL-6 has been found to inhibit Th1 differentiation directly, in an IL-12-independent manner, by upregulating SOCS1 expression in activated $CD4^+$ T cells [3].

The present study has therefore provided support for the view, as recently shown, that exposure of murine bone marrow derived dendritic cells to the TLR4 ligand, LPS preferentially induces a tolerising, IL-10-secreting phenotype and these IL-10 secreting DC favour tolerance induction by promoting expansion of $CD4^+CD25^+$ T regulatory cells (Fig. 6). In contrast, maturation of DC either by prolonged culture, prior to activation by LPS leads to an IL-12-secreting DC phenotype which promotes disease induction, partly through suppression of Treg [29, 30]. This may be an important distinction, since it suggests that Treg induction by DC vaccination procedures may be dependent not so much on production of inhibitory cytokines such as TGF-β and IL-10 (for review, ref [24]), for which there is conflicting evidence, but instead the

absence of IL-12 production by DC may be the key factor in failing to oppose the suppressive activity of naturally occurring and now expanded populations of Tregs. The notion that DC maturation is required for both tolerance induction and immunogenicity, but that the outcome depends on the state of activation of the DC, is not new [19, 35] and may explain the recognised activation-inhibiting properties of IL-10 [5] as well as the recently described tolerogenic effects of vasoactive-intestinal peptide-treated (VIP) dendritic cells [2]. If our approach is to have a future clinical application it is obvious that autologous DC must be used and that these are carefully and reproducibly activated in vitro.

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