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Lipopolysaccharide-primed heterotolerant dendritic cells suppress experimental autoimmune uveoretinitis by multiple mechanisms

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Summary

Exposure of bone-marrow-derived dendritic cells (BMDC) to high-dose ultrapure lipopolysaccharide for 24 hr (LPS-primed BMDC) enhances their potency in preventing inter-photoreceptor retinoid binding protein: complete Freund's adjuvant-induced experimental autoimmune uveoretinitis (EAU). LPS-primed BMDC are refractory to further exposure to LPS (= endotoxin tolerance), evidenced here by decreased phosphorylation of TANK-binding kinase 1, interferon regulatory factor 3 (IRF3), c-Jun N-terminal kinase and p38 mitogen-activated protein kinase as well as impaired nuclear translocation of nuclear factor κB (NF-κB) and IRF3, resulting in reduced tumour necrosis factor-α (TNF-α), interleukin-6 (IL-6), IL-12 and interferon-β secretion. LPS-primed BMDC also show reduced surface expression of Toll-like receptor-4 and up-regulation of CD14, followed by increased apoptosis, mediated via nuclear factor of activated T cells (NFATc)-2 signalling. LPS-primed BMDC are not only homotolerant to LPS but are heterotolerant to alternative pathogen-associated molecular pattern ligands, such as mycobacterial protein extract (*Mycobacterium tuberculosis*). Specifically, while *M. tuberculosis* protein extract induces secretion of IL-1β, TNF-α and IL-6 in unprimed BMDC, LPS-primed BMDC fail to secrete these cytokines in response to *M. tuberculosis*. We propose that LPS priming of BMDC, by exposure to high doses of LPS for 24 hr, stabilizes their tolerogenicity rather than promoting immunogenicity, and does so by multiple mechanisms, namely (i) generation of tolerogenic apoptotic BMDC through CD14:NFATc signalling; (ii) reduction of NF-κB and IRF3 signalling and downstream pro-inflammatory cytokine production; and (iii) blockade of inflammasome activation.

Keywords: dendritic cells; experimental autoimmune uveoretinitis; endotoxin tolerance; heterotolerance; uveitis.

Introduction

Uveitis is a major cause of blindness in humans.[1,2] In most cases the aetiology of uveitis is unknown, although bacterial and viral infections, as well as systemic diseases, are recognized causes and associations. Autoimmunity to retinal or other ocular antigens is considered to be mechanistic in non-infectious uveitis.[3] Treatment of non-infectious uveitis centres around non-specific therapy using immunosuppressive agents, or biologics such as anti-tumour necrosis factor-α (TNF-α) antibodies.[4,5] These therapies are associated with considerable toxicity and so much research effort has focused on the development of customized and targeted immunotherapies[5,6] including cell-based therapies.[7]

Dendritic cells (DC) are professional antigen-presenting cells specialized in uptake, processing and presentation of antigens to T cells. DC play an important role in the priming of the adaptive immune response due to their ability to directly stimulate naive T cells. However, one of their main functions appears to be a homeostatic one of maintaining tolerance to self-antigens.[8–12] Recently autologous tolerogenic DC (tolDC) modified *in vitro* with a nuclear factor-κB (NF-κB) inhibitor and loaded with citrullinated peptide antigens have been successfully used to treat patients with rheumatoid arthritis in a Phase 1 clinical trial.[13] Indeed, inoculation of tolDC into the site of inflammation is being trialled as a means to induce local tissue tolerance.[14] However, most work on tolDC has been in pre-clinical disease models, including autoimmune uveoretinitis.[15–17]

Traditionally, non-activated tolerogenic "immature" DC have high endocytic capacity and express low levels of "activation" markers (MHC II, CD40, CD80, CD83 and CD86) whereas immunogenic DC have reduced endocytic function, express high levels of "activation" markers and have strong T-cell priming ability.[18] Ligation of innate immunoreceptors such as Toll like receptors (TLR) by bacterial products such as lipopolysaccharide (LPS) leads to activation of DC and promotes immunity, specifically in the form of T-cell responses, if appropriate antigens are presented by the activated DC.[19,20] However, phenotypic characterization alone is insufficient to determine whether DC will induce tolerance or immunity.[21,22] In addition, it has been suggested that irrespective of the desired therapeutic outcome (induction of tolerance for prevention of autoimmune disease or immunity for treatment of cancer) DC activation is essential for the effectiveness of immunotherapy as activated DC preferentially migrate from the peripheral tissues into the draining lymph nodes where antigen-specific interaction with T cells occurs.[23–25]

Interestingly, there are considerable experimental data showing that pre-treatment of DC with LPS generates cells that promote tolerance rather than immunity[15,26–32] although the mechanism of tolerance induction in DC is not fully understood. Lipopolysaccharide-activated macrophages and DC become refractory to further stimulation with LPS,[33] a phenomenon known as endotoxin tolerance (ET), which has been attributed to various factors including: (i) the blockade of intracellular signalling events and subsequent gene re-programming; (ii) up-regulation of anti-inflammatory cytokines like interleukin-10 (IL-10) and transforming growth factor-β (TGF-β); and (iii) down-regulation of surface expression of the TLR4 receptor.[34] The majority of studies in myeloid cells on ET have been conducted using macrophages and have shown decreased phosphorylation levels of NF-κB as well as other signalling molecules such as p38 mitogen-activated protein kinase (p38 MAPK) and c-Jun N-terminal kinase (JNK) while displaying increased levels of phosphorylated extracellular signal-regulated kinase and IL-10 secretion.[35–39] In further studies it was shown that previous exposure to LPS led to impaired activation of TANK-binding kinase 1 (TBK1) and interferon regulatory factor 3 (IRF3) signalling through TIR-domain-containing adapter-inducing interferon-β (TRIF) pathway,[38,40] which has been attributed to the lipid A component of LPS.[41] LPS priming of DC has shown similar results for activation of myeloid differentiating factor 88 (MyD88) downstream signalling[35] but a decrease in activation of the TRIF pathway in endotoxin-tolerant DC (ET-DC) has not been reported to date. A major difference between ET-macrophages and ET-DC, however, has been in the induction of apoptosis: ET-macrophages, although down-regulated/modified in several of their pro-inflammatory signalling pathways, continue to survive in an alternatively activated state, whereas ET-DC progress to apoptosis after some days in culture (reviewed in ref. 30).

We have previously shown that LPS-primed bone-marrow-derived DC (BMDC), inoculated subcutaneously (s.c.) as a single injection, suppressed experimental autoimmune uveoretinitis (EAU) in the C57BL/6 mouse, induced using interphotoreceptor retinoid-binding protein (IRBP) peptide emulsified in complete Freund's adjuvant (CFA) containing *Mycobacterium tuberculosis*.[15,42] However, in our previous work the LPS extract contained small amounts of other TLR.[15,43,44] Here, we show that TLR4-specific ultrapure (up)LPS-primed phenotypically activated BMDC, rather than worsening the severity of EAU, significantly prevent the development of EAU. We show that upLPS-primed BMDC are endotoxin homotolerant (ET-BMDC) and further show that they are heterotolerant to *M. tuberculosis* protein extract in that they are (i) susceptible to apoptosis[45–47] (confirmed here) through a CD14/nuclear factor of activated T cells (NFATc)-associated mechanism, and (ii) fail to secrete IL-1β on

exposure to *M. tuberculosis* extract. As *M. tuberculosis* mediated C-type lectin receptor signalling via the Syk/CARD-9 complex,[48] a major route for inflammasome activation, has been shown to be an essential mediator of IRBP-CFA-induced EAU,[48,49] we propose that inhibition of IL-1β secretion is one mechanism whereby heterotolerant LPS-primed BMDC promote immunological tolerance. We also show that additional mechanisms are at play including induction of BMDC apoptosis as well as disruption of NF-κB and IRF3-mediated cytokine secretion. However, as signalling through the IL-1 receptor has recently been shown to be critical for the development of EAU, we suggest that *M. tuberculosis* antigen, LPS-activated, heterotolerant BMDC mediate their tolerogenicity primarily through suppression of IL-1β production.[50]

Materials and methods

Animals

Inbred 8- to 12-week-old C57BL/6J mice were provided by the Medical Research Facility at the University of Aberdeen. TLR4-deficient mice, originally generated by Dr Shizuo Akira (Osaka University, Osaka, Japan), were obtained from Professor Gordon Brown (University of Aberdeen, UK). The procedures adopted conformed to the regulations of the Animal License Act (UK) and to the Association for Research in Vision and Ophthalmology statement for The Use of Animals in Ophthalmic and Vision Research.

Isolation and culture of BMDC

The BMDC were prepared and cultured as described previously, with modifications.[15] In brief, BM was flushed from tibias and femurs of C57BL/6J mice and after purification (depletion of T cells, B cells and MHC II$^+$ cells), was cultured at 6×10^5 cells/ml in bacteriological Petri dishes with complete RPMI-1640 containing 10 ng/ml recombinant granulocyte–macrophage colony-stimulating factor (GM-CSF; R&D Systems, Minneapolis, MN). Fresh medium was added on days 2 and 4. On day 6 cells were harvested and depleted of contaminating granulocytes. The remaining cells were plated at 1×10^6 cells/ml and after *in vitro* stimulation with LPS *Escherichia coli* 0111:B4 [standard purity grade LPS from Sigma (St Louis, MO), upLPS from Invivogen (San Diego, CA); 1 µg/ml] or *M. tuberculosis* extract [generated by sonication of non-viable *M. tuberculosis* H37Ra purchased from Difco (BD, Franklin Lakes, NJ); 15 µg/ml] used for adoptive transfer experiments or analysis by flow cytometry, Western blotting or confocal microscopy. For some experiments BMDC were pre-incubated with purified anti-CD14 antibody (15 min, 10 µg/ml; BD Biosciences, San Jose, CA).

Flow cytometry

The BMDC were incubated with purified anti-CD16/32 antibody followed by surface staining with antibodies against CD11c-allophycocyanin (APC), CD11b-peridinin chlorophyll protein (PerCP) Cy5.5, CD86-phycoerythrin (PE), MHC II I-A^b-FITC, CD40-BV421, F4/80-PE, Gr-1-APC-Cy7, CD115-PE-Cy7 (eBioscience, San Diego, CA), CD14-APC (BioLegend, San Diego, CA), TLR4-PE (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany), Annexin V-FITC and -7AAD. Antibodies were purchased from BD Biosciences unless otherwise stated. Multi-colour flow cytometry experiments were performed using LSR-II and LSR-Fortessa analysers (BD Biosciences). The FACS data files obtained were analysed with BD FACS DIVA and FLOWJO (Flowjo, Ashland, OR) software. Unstained sample and fluorescence minus one controls were used to set gates during analysis.

Measurement of cytokine production

To measure cytokine production by BMDC, cell culture supernatant was collected and analysed for the presence of IL-6, IL-10, IL-12, IL-1β and TNF-α using the Mouse Inflammatory Cytometric Bead Assay kit and FACS Array system (BD Biosciences). Interferon-β (IFN-β) was detected by ELISA (PBL Biomedical Laboratories, Piscataway, NJ).

Adoptive transfer experiments

On day 6 of culture, BMDC were stimulated with LPS (standard or upLPS) at 1 µg/ml, and control BMDC were incubated in medium alone. IRBP 1–20 peptide (GPTHLFQPSLVLDMAKVLLD; New England Peptide, Gardner, MA) was also loaded at 30 µg/ml to induce an antigen-specific response. After addition of LPS and peptide, the cells were cultured overnight before being harvested with Accutase (PAA Laboratories). A total of 10^6 BMDC in a volume of 100 µl PBS were injected s.c. into the nape of the neck of each mouse. For studying the effects of BMDC on EAU (see below), mice were immunized with IRBP 1–20 peptide 24 hr after BMDC transfer.

Disease induction and evaluation

EAU was induced by immunizing s.c. with 500 µg of IRBP 1–20 peptide emulsified in CFA supplemented with anadditional 2·5 mg/ml *M. tuberculosis* H37Ra (Difco) at the back of the hind legs. Pertussis toxin (1 µg; Health Protection Agency, Chorley, UK) was also administered intraperitoneally at the time of IRBP peptide immunization.

Twenty-eight days post-immunization eyes were examined and fundus images were taken from mice using an endoscopic imaging system (see next section). Mice were then killed by asphyxiation in CO_2, and eyes were harvested and immediately fixed and embedded for sectioning.

Imaging mouse fundus

Images of the retinal fundus were acquired using an endoscopic imaging system described previously.[51] Mice were anaesthetized with an intraperitoneal injection of a mixture of 20 mg/ml Ketaset (Fort Dodge Animal Health LDT, Overland Park, KS) and 1 mg/ml Domitor (Orion Pharma, Espoo, Finland) diluted in PBS. Pupils were dilated with one drop of 0·5% (weight/volume) tropicamide (Chauvin Pharmaceuticals, Kingston-upon-Thames, UK) and one drop of phenylephrene hydrochloride (Chauvin Pharmaceuticals). Viscotears liquid gel (Novartis, Basel, Switzerland) was used on the corneal surface during the imaging. Several images of the fundus from different directions were taken. Images were scored according to clinical scoring system described by Xu *et al.* with modifications.[51]

Histology

To correlate the clinical observations with the pathological changes, first we imaged the eyes, then mice were killed and their eyes were collected for histological examination. Eyes were fixed in 2·5% glutaraldehyde, embedded in resin and sectioned. Sections (~3 μm) of each globe were taken at several different levels. The sections were subsequently stained with haematoxylin & eosin. Severity of disease was scored on a scale of 0 (no disease) to 4 (maximum disease) in half-point increments, according to a semi-quantitative scoring system described previously.[52]

Western blotting

Whole-cell protein lysates from cultured BMDC were prepared using cell lysis buffer (50 mM Tris–HCl, 1 mM EDTA, 1 mM ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 10 mM sodium β-glycerol 1-phosphate, 1 mM dithiothreitol, 1 mM sodium orthovanadate, 0·27 M sucrose, 1% Triton X-100) supplemented with a complete protease inhibitor cocktail and PhosSTOP phosphatase inhibitor cocktail. Equivalent amounts of cell lysates (30 μg) were loaded and separated by SDS–PAGE followed by electrotransfer to nitrocellulose membranes (Invitrogen, Waltham, MA). The following anti-mouse antibodies were used for immunoblotting: phospho-TBK1/NAK (Ser172) (D52C2), TBK1 (108A429; Santa Cruz Biotechnology, Santa Cruz, CA), phospho-NF-κB

p65 (Ser536) (93H1), NF-κB p65 (C22B4), phospho-stress-activated protein kinase (SAPK)/Jun amino-terminal kinase (JNK) (Thr183/Tyr185) (98F2), SAPK/JNK, phospho-IRF3 (Ser396) (4D4G), IRF3 (D83B9), phospho-p38 MAPK (Thr180/Tyr182) (D3F9), p38 MAPK, IκB-α (112B2), phospho-MAP Kinase Activated Protein Kinase (MAPKAPK)-2/MAPK-activated protein kinase 2 (MK2) (Thr222) (9A7), MAPKAPK-2, caspase-1 (clone 4B4; Genentech, South San Francisco, CA), ASC/CARD5 (clone 8E4.1; Genentech), caspase 11 (clone 17D9; Novus Biologicals, Littleton, CO) and GAPDH (Abcam, Cambridge, UK). Antibodies were purchased from Cell Signaling Technology (Danvers, MA) unless otherwise stated. The membranes were imaged with the Odyssey Infrared Imaging System (Licor Biosciences, Lincoln, NE).

Confocal microscopy

For assessing the effect of LPS on nuclear translocation and/or activation of IRF3, NF-κB and NFATc2, BMDC were seeded (1×10^5/well) in polylysine or collagen-coated 16-well chamber slides (Thermo Scientific Nunc, Waltham, MA) and cultured overnight. The next day BMDC were stimulated with 1 μg/ml LPS for 1 hr (challenge) or 24 hr (priming). In addition, in some experiments 24 hr LPS-primed BMDC were further challenged with LPS for 1 hr. After stimulation, culture medium was removed and cells were fixed in 4% formaldehyde, permeabilized with 0·2% Triton X-100 and blocked with 10% donkey serum (Bio-sera, Kansas City, MO) in PBS/0·2% BSA for 1 hr. Cells were incubated for 1 hr at room temperature with rabbit monoclonal antibodies against total NF-κB p65 or IRF3 (Cell Signaling). Alternatively, cells were incubated overnight at 4° C with NFATc2 antibody (ImmunoGlobe, Himmelstadt, Germany). After primary antibody incubation, cells were washed with PBS/0·2% BSA and incubated with donkey anti-rabbit Alexa Fluor 488 secondary antibody (Invitrogen) and/or Phalloidin (Sigma). Following a wash step as above, cells were stained with DAPI. All samples were viewed using Zeiss 700 Laser Scanning Confocal Microscope (Carl Zeiss, Oberkochen, Germany). Acquired images were analysed using the ZEN 2009 LE software (Carl Zeiss). Nuclear translocation was quantified by counting the number of cells with nuclear localization of IRF3, NF-κB and NFATc2 and is expressed as percentage of the total number of DAPI-stained nuclei (cells touching the edge of the field were excluded).

Statistics

Data are presented as mean $\pm$ SEM; n is indicated in the figure legends. Statistical significance in *in vivo* EAU experiments was analysed using Kruskal–Wallis test and differences between groups of interest were measured using two-tailed Mann–Whitney U-test. Analysis of all

other data was done using non-parametric unpaired two-tailed *t*-test. *P*-values < 0·05 were considered significant (GRAPHPAD PRISM 5.04; GraphPad, San Diego, CA).

Results

Phenotypically activated LPS-primed BMDC reduce the severity of EAU in C57BL/6 mice

We have previously demonstrated that 24 hr LPS-primed BMDC inhibit EAU in C57BL/6 mice.[15] However, these data were difficult to interpret mechanistically due to the relative impurity of the LPS, which has subsequently been shown to contain both TLR4 and TLR2 ligands.[43,44] In the present study, IRBP 1–20 peptide-pulsed, upLPS-primed BMDC, which were on average 85% CD11c⁺ CD11b⁺ (Fig. 1, upper panel), expressed increased levels of CD86 (CD86-PE median: 134·3 ± 7·4 unstimulated BMDC versus 3427·0 ± 264·2 upLPS-treated BMDC; *P* < 0·0001), MHC II (MHC II-FITC median:

462·8 ± 21·6 unstimulated BMDC versus 758·5 ± 32·3 upLPS-treated BMDC; *P* = 0·0003) and CD40 (CD40-BV421 median: 305·0 ± 9·9 versus 2804·0 ± 39·45, respectively; *P* < 0·0001; Fig. 1 lower panel). In addition, assessment of CD115, F4/80 and Gr-1 expression on the cell surface revealed that GM-CSF cultured upLPS-primed BMDC used in this study contained a sub-population of macrophages (see Supplementary material, Table S1). Adoptive transfer of IRBP-pulsed LPS-primed BMDC significantly suppressed the signs of EAU both clinically and histologically (Fig. 2a,b). The data reported here indicate that upLPS-primed BMDC although phenotypically "activated" had a tolerizing effect that was specifically mediated through TLR4.

LPS priming of BMDC induces up-regulation of CD14, NFATc2 activation and progressive apoptosis

We wished to determine the molecular events occurring in LPS-primed BMDC and assess their potential role in
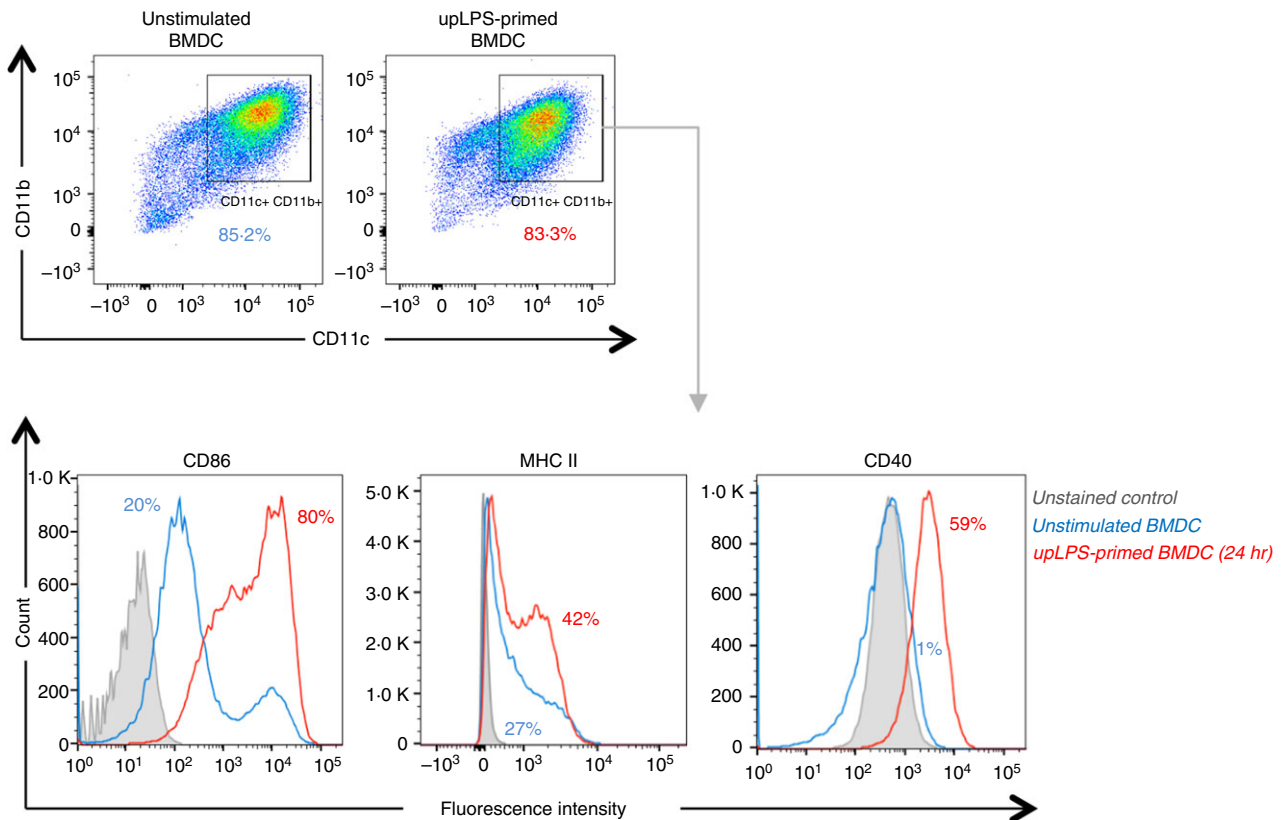

Figure 1. Lipopolysaccharide (LPS)-primed bone-marrow-derived dendritic cells (BMDC) are phenotypically "activated". T-cell, B-cell and MHCII⁺ cell-depleted bone marrow cells were cultured with granulocyte–macrophage colony-stimulating factor for 6 days. The cells were then further depleted of Gr-1-positive granulocytes and cultured for 24 hr with or without ultra pure LPS (upLPS). After 24 hr BMDC were harvested and analysed by flow cytometry. Cells were stained with fluorescent antibodies against CD11c, CD11b, CD86, MHCII and CD40. In the upper panel, the numbers correspond to percentage of CD11c CD11b double-positive cells in each gate. The histograms in the lower panel show surface expression of CD86, MHC II and CD40 by CD11c⁺ CD11b⁺ BMDC. The numbers on the histograms correspond to the percentage of indicated "activation" marker in unstimulated or upLPS-primed CD11c⁺ CD11b⁺ BMDC. Filled grey histograms represent unstained controls. Experiments were performed in four replicates per experimental condition, histograms are representative of three separate experiments with similar results.
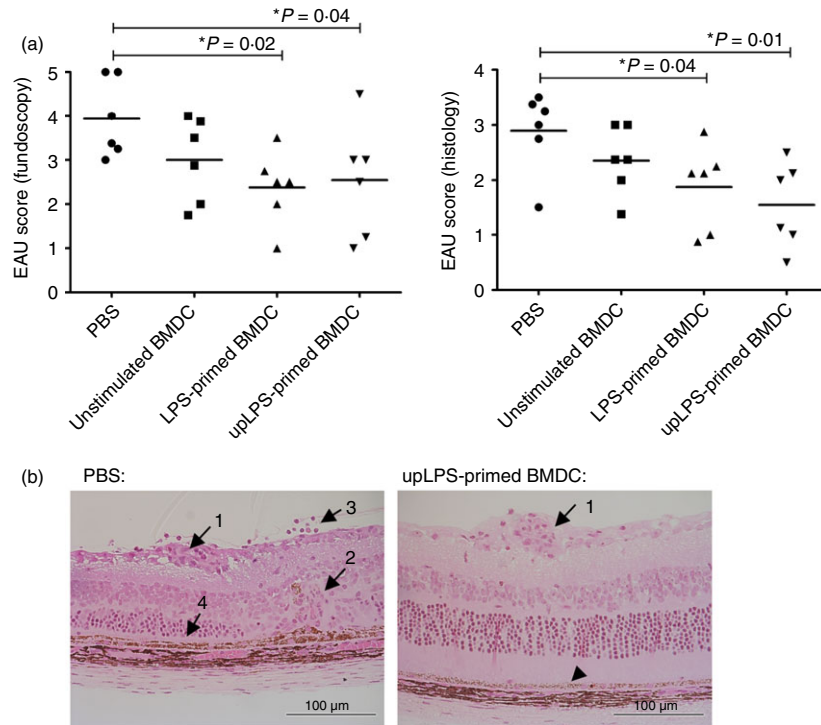
Figure 2. Lipopolysaccharide (LPS) -primed "activated" bone-marrow-derived dendritic cells (BMDC) suppress experimental autoimmune uveoretinitis (EAU). After 6 days of culture in the presence of granulocyte–macrophage colony-stimulating factor, purified BMDC were further depleted of Gr-1-positive granulocytes and stimulated for further 24 hr with interphotoreceptor retinoid-binding protein (IRBP) 1–20 peptide with or without addition of LPS [standard or ultrapure (upLPS)]. To test the tolerogenic properties of IRBP-specific LPS-primed BMDC, cells were inoculated subcutaneously into the neck of C57BL/6 mice 24 hr before EAU induction. Control mice were treated with PBS only. (a) The upLPS-primed BMDC significantly suppressed EAU as assessed by clinical fundoscopy and histology and were as effective as "standard" LPS-primed BMDC. One representative experiment is shown; two independent experiments were performed; each experiment had six independent replicates of each group. Each point represents mean EAU score from one mouse, data from each group are presented as mean ± SEM. Statistical analyses performed include Kruskal–Wallis test ($P = 0.065$ and $P = 0.029$ for fundoscopic and histological scores, respectively) followed by two-tailed Mann–Whitney *U*-test ($P = 0.04$ and $P = 0.01$ for upLPS-primed BMDC by fundoscopy and histology, respectively) to assess differences between groups of interest. (b) Representative histopathology of the eyes from C57BL/6 mice injected with PBS or upLPS-primed BMDC (magnification ×40, scale bar 100 μm). Black arrows indicate as follows: 1, vasculitis (inflamed blood vessel); 2, granuloma (group of inflammatory cells); 3, vitritis (inflammatory cells in the vitreous); 4, inflammation-induced loss of photoreceptor outer segments in PBS-treated group compared with mice treated with upLPS-primed BMDC (arrowhead).

promotion of immunological tolerance. In agreement with other studies[15,35,53] we observed a 3·2-fold down-regulation of TLR4 expression [PBS-treated BMDC mean fluorescence intensity (MFI): 168.7 ± 0.7; LPS-treated BMDC MFI: 53.0 ± 1.0; $P < 0.0001$] in LPS-primed BMDC and a corresponding 1·7-fold up-regulation of CD14 co-receptor expression (PBS-treated BMDC MFI: 15941.0 ± 385.6; LPS-treated BMDC MFI: 27823

Figure 3. Lipopolysaccharide (LPS) down-regulates Toll-like receptor 4 (TLR4) expression, induces secretion of pro- and anti-inflammatory cytokines and promotes apoptosis of bone-marrow-derived dendritic cells (BMDC). (a) Histograms show the surface expression of TLR4 and CD14 after priming with LPS for 24 hr (red) compared with unstimulated BMDC (blue). Cells were stained with antibodies against TLR4 and CD14. Experiments were performed in triplicate per experimental condition, histograms shown are representative of three separate experiments with similar results. (b) BMDC were pre-treated with anti-CD14 antibody followed by priming with LPS for 24 hr. BMDC culture supernatant was collected and analysed for presence of various cytokines. Data shown are mean of three separate experiments ± SEM. Significance was calculated between the LPS-primed group and CD14/LPS-treated group (*t* test). (c) BMDC were seeded in chamber slides and primed with LPS for 24 hr, cells in the control group were unstimulated. After 24 hr BMDC were fixed and stained with anti-NFATc2 antibody and DAPI as described in the Materials and methods section. Images were taken using an LSM700 microscope under magnification ×40, scale bar 50 μm. (d) BMDC were seeded in 24-well plates in 1 ml of cRPMI and stimulated with LPS for 24, 48 or 72 hr. After 24 hr, 0·5 ml of fresh cRPMI was added to each well. Cells were harvested at each time-point and stained with CD11c-allophycocyanin, Annexin V-FITC and -7AAD. Gating strategy: early apoptotic cells (Annexin V[+], 7AAD[−]), late apoptotic cells (Annexin V[+], 7AAD[+]), necrotic cells (Annexin V[−], 7AAD[+]). Data presented on the graphs are mean of three separate experiments ± SEM (*t*-test).
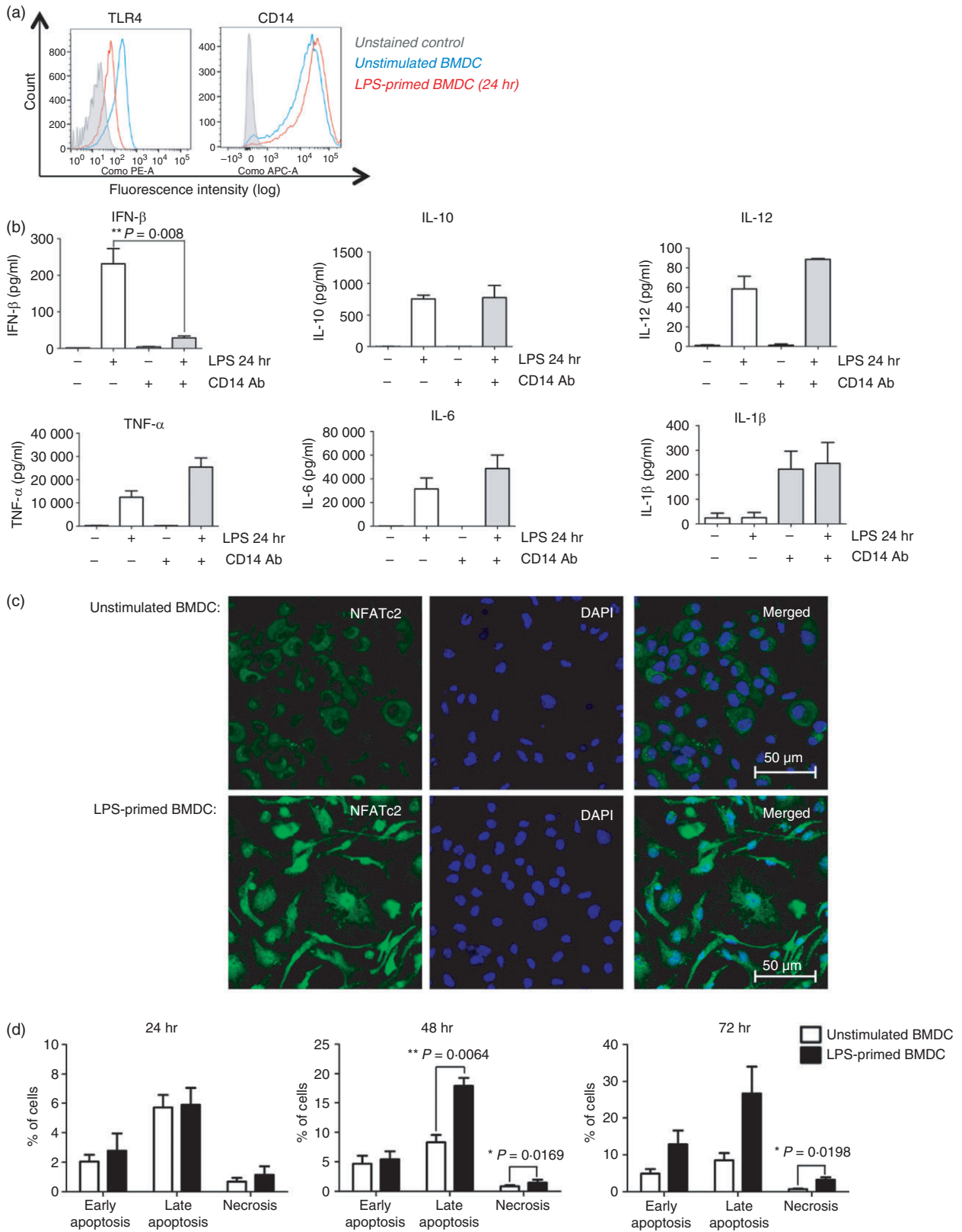
± 475·8; $P < 0.0001$; Fig. 3a). Lipopolysaccharide-primed BMDC were induced to secrete several cytokines including IFN-β, IL-10, TNF-α and IL-6 (Fig 3b). Small, probably non-physiologically significant, amounts of IL-12 were also produced. Of these, only IFN-β secretion was dependent on CD14 (Fig 3b). Interestingly, LPS-

primed BMDC did not produce significant amounts of IL-1β, although blockade of CD14 induced TLR4-independent IL-1β production (Fig. 3b). As previously reported by Zanoni *et al.*,[47] expression of CD14 by BMDC was associated with increased expression and nuclear translocation of NFATc2 (Fig. 3c; LPS priming increased nuclear localization of NFATc2 35-fold compared with unstimulated BMDC, $P < 0.0001$) as well as with increased levels of both late apoptosis and necrosis at 48 hr and necrosis at 72 hr after LPS exposure (Fig. 3d). Endogenous levels of apoptosis-associated proteins, Bim and Bcl2, were up-regulated by 24 hr LPS priming but no marked increase in either was observed after 1 hr duration re-challenge (data not shown).

LPS priming of BMDC induces endotoxin tolerance and disables activation of MyD88 and TRIF signalling pathways

Although induction of apoptosis in LPS-primed BMDC offers a potential explanation for their enhanced tolerogenicity,[45,54] tolDC are also known to be generated by inhibition of core transcription factor pathways such as NF-κB.[55] The LPS-primed macrophages and DC fail to be activated by a second exposure to LPS, a phenomenon known as ET.[56] In this study, we confirmed that activation of both the MyD88 and TRIF signalling pathways in LPS-primed BMDC is decreased or does not occur on second exposure to LPS (LPS–LPS homotolerance; Fig. 4a,b). BMDC challenged with LPS for 1 hr showed increased levels of phosphorylated NF-κB and concomitant loss of nuclear factor of κ light polypeptide gene enhancer in B cells inhibitor α (IκB-α) (Fig. 4a). In contrast, 24 hr LPS-primed BMDC challenged with LPS for the second time failed to induce NF-κB nuclear translocation (Fig. 4b; 19-fold reduction in the nuclear localization, $P < 0.0001$). Similar reduction in the response of LPS-primed BMDC upon LPS re-challenge was observed for p38 MAPK and MK2 as well as TBK1, IRF3 and JNK (Fig. 4a,b; LPS priming reduced nuclear localization of IRF3 upon re-challenge threefold; $P = 0.0066$). Concurrent with the reduced signalling in LPS-primed BMDC through MyD88 and TRIF signalling pathways on re-challenge with LPS, cytokine production including TNF-α, IL-6 and even the small amounts of IL-12 were markedly reduced whereas the amount of secreted IL-10 increased (Fig. 5). In addition, the ability of BMDC to secrete TGF-β was assessed; however, the amounts produced were below the limit of detection (data not shown). Importantly, the typical strong induction of IFN-β[57] by LPS was completely abrogated on re-challenge with LPS (Fig. 5).

LPS-primed BMDC are heterotolerant to mycobacterial protein

Since adoptive transfer of upLPS-primed BMDC suppressed the development of EAU (Fig. 2a,b), and such DC are clearly refractory to a second challenge with LPS *in vitro* (Figs 4a,b, and 5), we hypothesized that their enhanced tolerogenicity might be due to the BMDC being refractory to a second challenge *in vivo*, i.e. they might display "heterotolerance", as in the EAU model the "second challenge" would be mediated by CFA containing heat-killed *M. tuberculosis* H37Ra. *Mycobacterium tuberculosis* activates the innate immune system through multiple pattern recognition receptors, including TLR4, TLR2, Dectin-1, mannose receptors and others.[58] We therefore tested whether LPS-primed BMDC were heterotolerant to *M. tuberculosis* by challenging the cells *in vitro* with a protein extract of *M. tuberculosis* with or without previous exposure to LPS.

We first examined the response of BMDC to a single exposure to *M. tuberculosis*. In contrast to LPS, *M. tuberculosis* had a minimal modifying effect, either as a single challenge or after LPS-priming, on the activation level of NF-κB and p38 MAPK and failed altogether to activate TBK1, IRF3 and JNK in BMDC (Fig. 4a). Accordingly *M. tuberculosis*-challenged BMDC did not produce IL-12 or IFN-β while the level of secreted IL-6 and TNF-α was also low compared with LPS-challenged BMDC (Fig. 5).

We also explored inflammasome activation and IL-1β production by BMDC. Interleukin-1β is a pro-inflammatory cytokine that plays an important role during IRBP-CFA-induced EAU.[59] Production of active IL-1β occurs by way of inflammasome activation through both canonical and non-canonical pathways (reviewed in refs 60–62). Activation of caspase 1 and apoptosis-associated speck-like protein containing CARD (ASC) is required for

Figure 4. Lipopolysaccharide (LPS) -primed bone-marrow-derived dendritic cells (BMDC) are refractory to a second challenge with LPS. BMDC were generated from wild-type (WT) or Toll-like receptor 4 knockout (TLR4 KO) mice according to the protocol described in Materials and methods. On day 6 of culture, BMDC were treated with LPS [standard or ultrapure (upLPS)] for 24 hr (LPS priming); a control group was incubated with complete medium alone. After 24 hr, BMDC were re-challenged for 1, 24, 48 or 72 hr with LPS or *Mycobacterium tuberculosis* (Mtb). Following the second stimulation BMDC were lysed and proteins were isolated, or in the case of cells cultured in the chamber slides, cells were fixed and stained for confocal microscopy as described in Materials and methods. (a) Influence of LPS priming on the responsiveness of BMDC and activation of TIR-domain-containing adapter-inducing interferon-β (TRIF) and myeloid differentiating factor 88 (MyD88) -dependent pathways. Representative Western blots shown ($n = 3$). GAPDH used as loading control. (b) Effects of LPS desensitization on the nuclear translocation of nuclear factor-κB (NF-κB) and interferon regulatory factor 3 (IRF3). Scale bar 50 μm.

(a)

(b)

canonical IL-1β production, but caspase 11 mediates non-canonical inflammasome activation.[61] As shown in Fig. 4(a) the phosphorylation level of caspase 11 was increased 24 hr after exposure to LPS (or *M. tuberculosis*), but was abrogated upon *M. tuberculosis* re-challenge. No differences were noted in the levels of caspase 1 and ASC
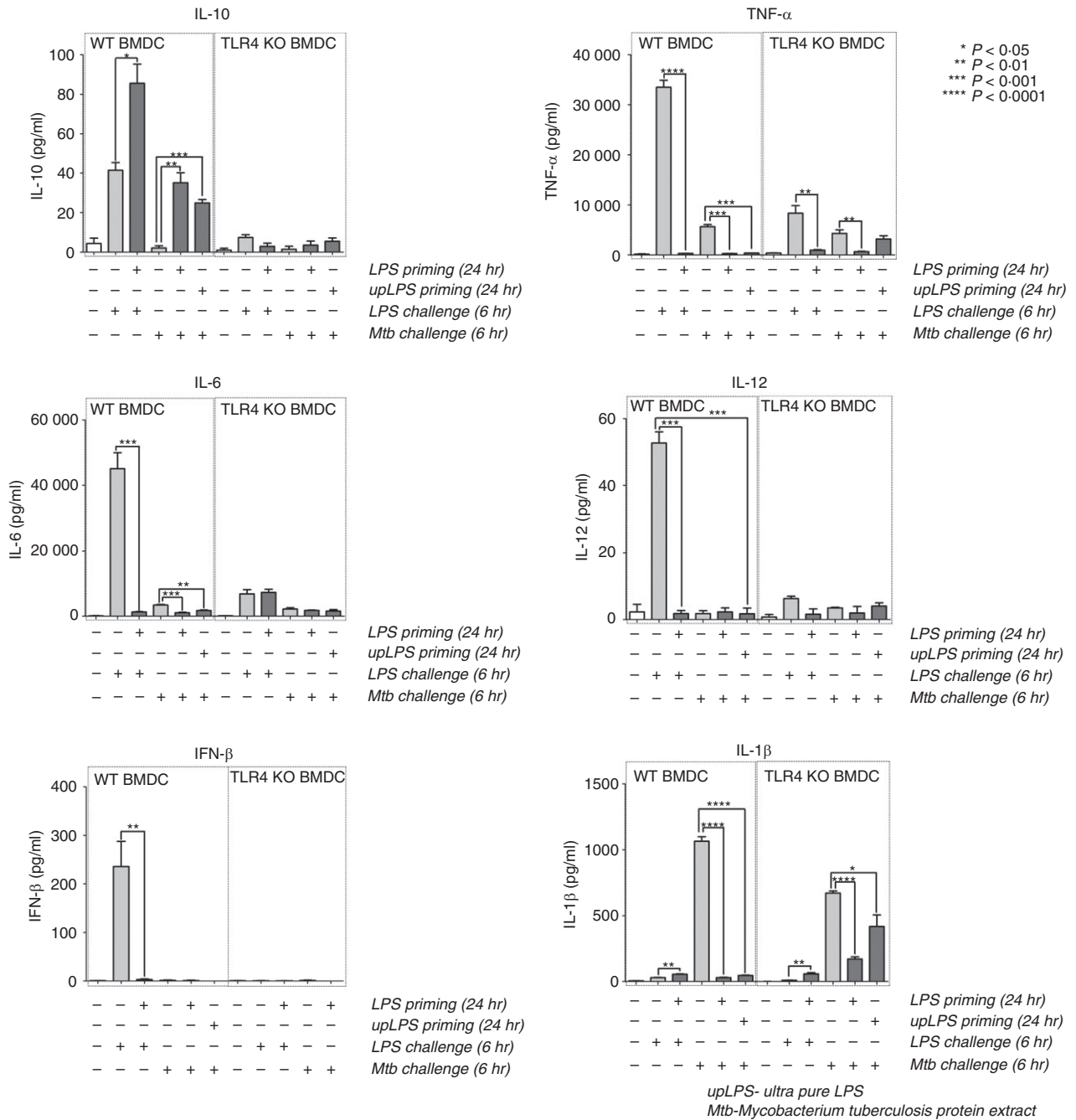
Figure 5. Analysis of cytokine secretion from lipopolysaccharide (LPS) -primed bone-marrow-derived dendritic cells (BMDC) upon re-stimulation. On day 6 of culture, BMDC from wild-type (WT) or Toll-like receptor 4 knockout (TLR4 KO) mice were primed with LPS [standard or ultrapure (upLPS)] for 24 hr, a control group was incubated with complete medium alone. After 24 hr, culture medium from both groups was gently removed and fresh medium was added. Cells were re-stimulated for 6 hr with LPS or *Mycobacterium tuberculosis* (Mtb). Following a second stimulation, culture supernatant was collected and levels of secreted cytokines including interleukin-10 (IL-10), tumour necrosis factor-α (TNF-α), IL-6, IL-12, interferon-β (IFN-β) and IL-1β were analysed using ELISA or cytometric bead assay. Data shown are mean of three separate experiments ± SEM. Significance was calculated using *t*-test.

among differentially activated BMDC. Interestingly, LPS singly or on re-challenge failed to induce IL-1β secretion whereas challenge with *M. tuberculosis* induced prominent IL-1β secretion, which was markedly suppressed in LPS-primed BMDC (Fig. 5). This effect was only partially lost

in LPS-primed BMDC from TLR4 knockout mice, indicating that the *M. tuberculosis* ligands in this extract were acting by additional pathways, such as TLR2 and Dectin-1.[48] These data for the first time show that LPS (endotoxin) "heterotolerance" or "crosstolerance" to
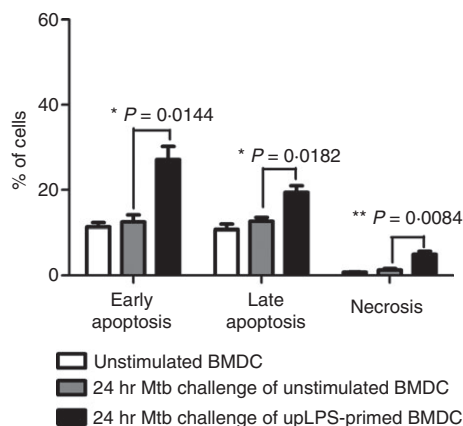
Figure 6. Effect of lipopolysaccharide (LPS) -priming on survival of bone-marrow-derived dendritic cells (BMDC) upon *in vitro* challenge with *Mycobacterium tuberculosis* (Mtb). BMDC were seeded in 24-well plates in 1 ml of cRPMI and primed with ultrapure LPS (upLPS) for 24 hr, controls were incubated in cRPMI alone. After 24 hr, medium from each well was removed and replaced with fresh medium, and the cells were either unstimulated or challenged with Mtb protein extract for a further 24 hr. The cells were then harvested and stained with anti-CD11c antibody-APC, Annexin V-FITC and 7AAD. Gating strategy: early apoptotic cells (Annexin V^+, $7AAD^-$), late apoptotic cells (Annexin V^+, $7AAD^+$), necrotic cells (Annexin V^-, $7AAD^+$).; $n = 3$, mean ± SEM (*t*-test).

M. tuberculosis ligands, i.e. where cells pre-exposed to LPS are refractory to a subsequent challenge with *M. tuberculosis* ligands, was inducible in BMDC. Heterotolerance has been previously shown *in vitro* using LPS and the TLR2 agonist Pam3Cys in the context of TNF-α signalling for both macrophages and DC.[63,64] However, here we provide the first evidence, using TLR4 knockout BMDC, that LPS hetero-/cross-tolerance occurs through LPS/TLR4 signalling and inflammasome signalling mediated via abrogation of non-canonical caspase-11 activation (Fig 4a) and IL-1β secretion (Fig 5). Interestingly, an opposite effect was observed with regard to IL-10 secretion: LPS-primed BMDC challenged with LPS or *M. tuberculosis* exhibited an increased IL-10 response which was also TLR4 specific (Fig. 5).

Earlier we showed (Fig. 3d) that LPS stimulation promotes apoptosis of BMDC. Here, we further explored the effect of LPS-priming on survival of BMDC upon *in vitro* challenge with *M. tuberculosis* (Fig. 6). Interestingly, BMDC primed with upLPS for 24 hr were significantly more susceptible to apoptosis/necrosis on encounter with *M. tuberculosis* compared with unstimulated BMDC (2·2-fold increase in early apoptosis; 1,5-fold increase in late apoptosis; 4-fold increase in necrosis).

Discussion

Recently, there has been considerable interest in developing specific cell-based immunotherapies using DC. Here

the mechanisms underlying the tolerogenic properties of BMDC in the suppression of EAU, the mouse model of human sight-threatening autoimmune uveitis, were examined. DC have the homeostatic function of promoting immunological tolerance to self-antigens thereby preventing the development of autoimmune disorders.[65] Both pre-clinical and clinical studies have shown that adoptively transferred DC have potent immunosuppressive properties.[14,66–68] However, there is a risk that DC placed in the pro-inflammatory environment of the host with active autoimmune disease would convert to activated self-antigen-presenting DC, promoting immunity rather than tolerance, thereby worsening the disease. Therefore, much effort has been directed to develop protocols that stabilize the tolerogenic properties of DC, which would ultimately ensure safety and effectiveness of DC-based vaccines.

The data in this report confirm previous studies[15,42] showing efficacy of LPS-primed BMDC in preventing the development of EAU, an effect mediated by LPS engagement of TLR4 on the BMDC. Similar results have been found in other models and also in parallel clinical studies using autologous DC.[69,70]

It is important to note that the population of GM-CSF-cultured BMDC used in this study is DC-enriched (GM-DC) and contains a sub-population of macrophages (see Supplementary material, Table S1). The depletion procedure used to purify BMDC was designed to maximize the proportion of DC progenitors in the initial plating out of the cells but, as has recently been described, some progenitors differentiate into macrophages (GM-Mφ).[71] However, GM-DC are migratory whereas GM-Mφ are sessile[71] and administration of BMDC by s.c. inoculation, as described here, preferentially permits BMDC homing to the draining lymph node.[15,72] We therefore attribute the tolerogenic effects to the migratory GM-DC in the mixed population.

There are several possible mechanisms whereby upLPS-priming of BMDC mediates their tolerogenic effect in preventing EAU and other experimental autoimmune models. Our previous data[15,42] showing that s.c. administration of LPS-primed BMDC induces expansion of regulatory T cells at the skin-draining lymph nodes, suggest that $CD4^+$ regulatory T cells are likely to contribute toward the observed tolerogenic effect. Induction of regulatory T cells by LPS-primed ET-DC is probably a consequence of modified cell signalling induced in the DC by high-dose LPS, which includes reduced signalling through NF-κB (see also Fig. 4a,b), and specifically the NF-κB subunit RelB, which when combined with p50 is known to regulate DC activation and maturation.[73,74] Most recently RelB inhibitor-treated autologous DC have shown efficacy in a Phase 1 clinical trial of patients with rheumatoid arthritis.[13] Interestingly, although most of the studies investigating ET have been *in vitro* studies in

which macrophages,[39,75] and less so DC,[35] are refractory to a second challenge with LPS ("homotolerance") a few studies have shown that macrophages pre-exposed to one TLR-agonist become unresponsive to challenge with another TLR-agonist ("heterotolerance"). For instance, LPS-primed macrophages fail to respond to a second challenge with extracts from other Gram-negative bacteria.[37,64,76] One study has also shown that LPS-primed BMDC are heterotolerant to Pam3Cys, an effect controlled by IL-1 receptor-associated kinase M, which is an intracellular negative regulator of TLR signalling.[63] However, the present study is the first to report heterotolerance of LPS-primed BMDC to mycobacterial proteins. As ET is a feature of several conditions involving multiple organisms (e.g. those involved in sepsis or hepatic ischaemia) the above data may have therapeutic implications.

Inhibition of NF-κB downstream signalling may not be the only mechanism for tolerogenicity of LPS-primed BMDC. Zanoni *et al.*[30,47,77] have shown that LPS-primed BMDC are programmed for apoptosis, unlike LPS-primed macrophages which, although refractory to a second challenge of LPS, survive in culture for prolonged periods. Here we show that up to 25% of BMDC primed with LPS for 48 hr show signs of apoptosis after 8 days in culture (Fig. 3d). In addition, we confirm the link between LPS-induced BMDC apoptosis with increased expression of CD14 and the induction of NFATc2 (Fig. 3).[47] Apoptotic cells are known to promote immune tolerance when administered *in vivo*. In particular, the clearance of apoptotic cells has been identified as an event that is directly responsible for tolerance induction. Further, it has been demonstrated that administration of apoptotic BMDC suppresses immune responses and inhibits experimental diseases such as LPS-induced airway inflammation.[54] In contrast, necrotic cells are thought to play the opposite role and trigger inflammatory rather than regulatory responses. In our recent report[72] we showed that only a small percentage of the total inoculated tolerogenic BMDC traffics to the draining lymph nodes, suggesting that the majority of cells die at the site of administration. We believe that the latter is most likely to be responsible for the observed, although non-significant, dampening effect of unstimulated BMDC on EAU severity. Anderson *et al.*[23] have previously demonstrated that DC activation is required for their migratory capacity and as a result it is vital for effectiveness of DC-based vaccines both with tolDC and immunogenic DC. Accordingly we have confirmed that LPS-primed BMDC are capable of significantly reducing the severity of EAU (Fig. 2). In addition, we have previously shown that upon s.c. administration, LPS-primed BMDC selectively traffic and progressively accumulate in the draining lymph nodes (up to 6·5% of inoculated BMDC could be detected on day 6 post injection).[15] We believe that the enhanced migratory abilities of LPS-primed BMDC together with, but not limited to,

their increased susceptibility to apoptosis (Fig. 6) are directly correlated with their greater effectiveness, compared with non-activated BMDC.

Interestingly, human tolDC, generated with dexamethasone and the active form of vitamin D3, were shown not only to maintain their tolerogenic function upon activation with maturation stimuli (LPS) but were superior in terms of their migratory activity toward CCL19 (confirmed in our report[72]) and had enhanced antigen-presenting ability.[23] Citrullinated-peptide-pulsed tolDC have also been reported to be safe and effective in a Phase 1 clinical study in patients with rheumatoid arthritis.[13] Furthermore, tolDC generated according to the protocol described above (for safety purposes LPS has been substituted with the synthetic TLR4 ligand namely monophosphoryl lipid A) were recently tested in a Phase 1 clinical trial in patients with rheumatoid arthritis or inflammatory arthritis.[14] The outcome of this small Phase 1 clinical trial suggests that DC therapy is safe and potentially effective in treatment of rheumatoid arthritis. Furthermore, local induction of tolerance may be possible as autologous DC inoculated directly into the joint in rheumatoid arthritis patients appear to be safe and show early signs of effectivity.

Alternatively, tolerogenicity of LPS-primed BMDC *in vivo* in the EAU model may be a manifestation of heterotolerance in which the second challenge is provided by agents required for disease induction. In the model of IRBP-CFA-induced EAU, heat-killed mycobacteria in the CFA are the most likely candidate to provide this challenge. Here we have shown that LPS-primed BMDC are heterotolerant to *M. tuberculosis* protein extract. Unlike LPS, *M. tuberculosis* protein does not activate signalling by way of the TRIF pathway (TBK1/IRF3), so there appears to be no induction of type 1 IFN (Figs 4a and 5). However, a single exposure of BMDC to *M. tuberculosis* induces a high level of IL-1β even in the absence of TLR4, which is almost completely abrogated in LPS-primed BMDC (Fig. 5). Interleukin-1β is known to activate DC, including through an autocrine loop, towards a mature phenotype and functionality for antigen presentation. *Mycobacterium tuberculosis* activates DC through several ligands including TLR2 and Dectin-1, and less so TLR4 with downstream activation of the inflammasome and secretion of IL-1β. Furthermore, Dectin-1, Mincle and signalling through the Syk/Card 9 complex are required for induction of EAU by way of IRBP:CFA.[48,49] As signalling by the IL-1 receptor has recently been shown to be critical for the induction of EAU,[50] it is a distinct possibility that LPS-primed BMDC prevent IRBP:CFA-induced EAU by blocking BMDC inflammasome activation and IL-1β production.

In summary, LPS-priming of BMDC using the protocol described here clearly has an enhancing and stabilizing effect in preventing IRBP:CFA-induced EAU. At least

three potential mechanisms have been described which could mediate this effect including (i) induction of apoptosis in BMDC through CD14:NFATc2 signalling; (ii) reduction of NF-κB and IRF3 signalling and downstream pro-inflammatory cytokine production; and (iii) blockade of *M. tuberculosis*-induced inflammasome activation.

Initial clinical results in rheumatoid arthritis have shown that targeting NF-κB/RelB alone may be effective in controlling disease. However, therapeutic strategies which would allow targeting of several pathways towards immune tolerance, as identified here, might optimize DC-based cell therapy for many of these conditions.

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Authorship

IPK conceived, designed and performed experiments, analysed the data and wrote the manuscript; EM performed experiments; CMG suggested experiments, interpreted data and reviewed the manuscript; CM performed additional experiments and analysed the data; JVF suggested experiments, interpreted the data and wrote the manuscript.

Disclosures

The authors declare no financial or commercial conflict of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Ultrapure lipopolysaccharide (upLPS) - primed bone-marrow-derived dendritic cells (BMDC) generated by culture of purified bone marrow cells in the presence of granulocyte–macrophage colony-stimulating factor contain low numbers of macrophage/monocyte signature markers. The table shows the percentage of cells expressing CD115, F4/80 or Gr-1 (gated on total cell population). Data from one of the two experiments performed in four replicates per experimental condition are shown.