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Binding immunoglobulin protein-treated peripheral blood monocyte-derived dendritic cells are refractory to maturation and induce regulatory T-cell development

Valerie M. Corrigall, Olivier Vittecoq and Gabriel S. Panayi

Department of Academic Rheumatology, King's College London School of Medicine at Guy's, King's and St Thomas' Hospitals, Guy's Hospital, London, UK

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Correspondence: Dr V. M. Corrigall, Department of Academic Rheumatology, King's College London Medical School at Guy's, King's and St. Thomas' Hospitals, 5th Floor Southwark Wing, Guy's Hospital, London SE1 9RT, UK.
Email: valerie.corrigall@kcl.ac.uk
Senior author: Gabriel S. Panayi,
Email: gabriel.panayi@kcl.ac.uk

Summary

Binding immunoglobulin protein (BiP) has been shown previously to have immunomodulatory functions. Herein we investigated whether BiP could affect the differentiation of monocytes into dendritic cells (DCs) and thence the development of regulatory T cells. Peripheral blood monocyte-derived DCs were matured with lipopolysaccharide in the presence or absence of BiP. DC development and T-cell changes were monitored by flow cytometry and regulatory T-cell function was measured by uptake of tritiated thymidine. More BiP-treated DCs ($DC_{(BiP)}$s) expressed amounts of intracellular indoleamine 2,3-dioxygenase (IDO) and cell surface leucocyte immunoglobulin-like receptor subfamily B member 1 (LILRB1), retained CD14 expression but down-regulated expression of human leucocyte antigen (HLA)-DR and CD86, and produced copious amounts of interleukin (IL)-10, when compared with control DCs. T cells co-cultured with $DC_{(BiP)}$s developed regulatory function with increased surface expression of $CD4^+$ $CD25^{hi}$ $CD27^{hi}$ but with no concomitant increase in forkhead box P3 (Foxp3). These T cells also showed significantly higher levels of intracellular cytotoxic T-lymphocyte antigen (CTLA)-4. The latter could be inhibited by the presence of the IDO inhibitor 1 methyl tryptophan. The addition of neutralizing anti-IL-10 antibody or the specific mitogen-activated protein kinase (MAPK) p38 inhibitor SB203580 reversed the inhibition of DC differentiation by BiP. In conclusion, BiP is an immunomodulator able to arrest inflammation through induction of tolerogenic DCs and subsequent generation of T regulatory cells.

Keywords: BiP; dendritic cells; idoleamine 2,3 dioxygenase; regulatory T cells

Introduction

The interactions between the innate and adaptive immune systems that determine the type of immune response that follows a particular antigenic assault are currently being dissected. Probably one of the most important players in the initiation of an appropriate immune response is the dendritic cell (DC). The maturation status of the DC and its local environment, with respect to the presence of inflammatory mediators, influence the subsequent cross-talk between DC and T cells and are crucial factors in the immune response directing T cell development towards T helper (Th) 1 cells,[1] associated with interleukin (IL)-12 production, Th17 cells, developed in the presence of transforming growth factor (TGF) β and IL-6,[2] or Th2 cells, associated with IL-4.[3] The production of IL-10 by DCs in the early stages of culture and the DC phenotype clearly also have an effect on the differentiation of regulatory T cells.[4] The nature of the initiating molecules that drive a DC to become pro- or anti-inflammatory, however, is still uncertain.

Stress proteins (SPs) or heat shock proteins (HSPs) are evolutionarily highly conserved proteins.[5] They have

Abbreviations: BiP, binding immunoglobulin protein; IDO, indoleamine 2,3 dioxygenase; LILRB1, leucocyte immunoglobulin-like receptor subfamily B member 1.

important intracellular functions as chaperones and protectors of cells from stress,[5] but more recently they have been recognized to have extracellular functions mediated by signalling through cell surface-expressed receptors. Thus they are able to influence monocyte differentiation through ligation of surface-expressed receptors, such as Toll-like receptor 2 (TLR2)[6] and TLR4,[6] CD14, CD40,[7] lectin-like oxidised low-density lipoprotein receptor-1[8] and CD91.[9,10] The majority of these SPs, such as HSP70, have been shown to stimulate monocytes to drive an overall pro-inflammatory response, reflecting the 'danger' hypothesis,[11] with accelerated maturation of DCs that produce interleukin (IL)-12, tumour necrosis factor (TNF)-α and IL-1β.[12,13] In direct contrast, we have shown that binding immunoglobulin protein (BiP), although a member of the HSP70 family, affects peripheral blood monocytes[14] via an as yet uncharacterized receptor, to reduce human leucocyte antigen (HLA)-DR and CD86 expression and induce copious production of IL-10.[14] A functional consequence of these changes is reduced proliferation of human peripheral blood mononuclear cells (PBMC) in response to the recall antigen tuberculin purified protein derivative (PPD).[14] In addition, BiP will drive differentiation of T cells towards a Th2 profile with anti-inflammatory properties. Thus we have expanded, from PBMC, BiP-specific CD8 T-cell clones that produce IL-10, IL-4 and IL-5 in various combinations with little or no interferon (IFN)-γ.[15] Similarly, *in vitro* investigation of the lymph node and spleen cells from BiP-treated mice showed secretion of IL-4[16] and IL-10 on re-stimulation with BiP. *In vivo*, a single intravenous injection of BiP given to DBA/1 or HLA-DR1 transgenic mice prevented or treated ongoing collagen-induced arthritis (CIA). Importantly, adoptive transfer of the splenocytes and lymph node cells from these BiP-treated animals will prevent or treat CIA. Interestingly, the protective effect of BiP is ablated in IL-4 knockout mice.[16] Thus there seems to be an analogy between mice and humans in terms of BiP being able to stimulate the release Th2 cytokines, especially IL-10 and IL-4.

Collectively, these results suggest that BiP is able to modify the immune response through interaction with monocytes and to produce T cells that may have anti-inflammatory and immunomodulatory properties. In particular, we have hypothesized that BiP may act after the initial inflammatory immune response to resolve inflammation and restore homeostasis.[17] In this study, we present evidence that *in vitro* BiP stimulation modulates human monocyte differentiation into mature DCs (mDCs) and that subsequent T-cell contact with BiP-treated mDCs ($mDC_{(BiP)}s$), either autologous or allogeneic, augments regulatory T-cell development. Overall, these data provide more direct experimental evidence of the effects of BiP on the inflammatory process.

Materials and methods

Preparation of recombinant human BiP (rhuBiP)

6X histidine tagged recombinant human BiP was prepared as described previously.[18] Briefly, *Escherichia coli* expression strain BL21-(DE3) containing the recombinant pET30a-BiP plasmid was grown at 37° in Luria-Bertani (LB) medium containing kanamycin (50 mg/ml). Isopropyl-D-thiogalactopyranoside (1 mM) was added to the medium to induce expression of the recombinant protein. The culture was incubated for a further 4 hr at 37°. Cells were pelleted by centrifugation and stored at −70°. For purification of the recombinant bacterial proteins, the bacterial pellets were lysed in binding buffer [20 mM Na_2HPO_4, 500 mM NaCl, 5 mM imidazole, 1 mM phenylmethylsulphonyl fluoride (PMSF), 1 mg/ml lysozyme, 5 mg/ml DNAse and 0·1% Triton X-100, pH 7·4]. The lysate was cleared by centrifugation and passed over a binding buffer equilibrated chelating Hi-trap affinity column (Pharmacia, Amersham, UK). The non-specifically bound protein was washed from the column under stringent conditions using a series of three wash buffers. The primary washes were performed using 100 ml of binding buffer without and then with 5 μg/ml polymyxin B. This was followed by a high-stringency low-pH wash (20 mM Na_2HPO_4, 500 mM NaCl and 0·1% Triton X-100, pH 5·5) and an additional high-stringency wash using 100 ml of 20 mM Na_2HPO_4, 500 mM NaCl, 0·1% Triton X-100 and 50 mM imidazole, pH 7·4. The histidine-tagged recombinant proteins were eluted from the column by stripping with 50 mM ethylenediaminetetraacetic acid (EDTA). Eluted proteins were dialysed against phosphate-buffered saline (PBS) to remove EDTA and nickel contaminants. The protein purity, as assessed by polyacrylamide gel electrophoresis and silver staining, was greater than 95%. Associates of Cape Cod (Liverpool, UK) assessed endotoxin contamination at < 30 EU/mg protein.

Isolation of PBMC, T cells and monocytes (MOs)

Heparinized venous blood was obtained from healthy volunteers after informed consent and approval of the project by the Guy's and St Thomas' Hospital Ethical Committee had been given. PBMC were isolated by density centrifugation over Lymphoprep (Nycomed-Pharma, Amersham, UK). T cells and MOs were purified from PBMC by negative selection using the appropriate immunomagnetic kit (Dynal, Bromborough, UK).

Differentiation of MO-derived mDCs

Enhanced MO cultures (> 85% monocytes; $1{\cdot}5 \times 10^6$ cells/flask; Corning Costar, High Wycombe,

UK) were incubated in 5 ml of tissue culture medium (TCM) (RPMI-1640; Sigma, Poole, UK) supplemented with 10% heat-inactivated fetal calf serum (FCS) (Life Technologies, Paisley, UK). DCs were generated by culturing MOs with granulocyte–macrophage colony-stimulating factor (GM-CSF; 1000 U/ml; Novartis Research Institute, Vienna, Austria) and IL-4 (500 U/ml; R&D Systems, Oxford, UK) for 7 days, either alone or in the presence of rhuBiP (20 μg/ml). Cytokines and BiP were replenished every 2 days. DCs were matured by addition of lipopolysaccharide (LPS; 500 ng/ml) for the final 2 days of a 7-day culture.

IL-10 neutralization and mitogen-activated protein kinase (MAPK) inhibition

To neutralize IL-10 within the cultures, anti-IL-10 antibody (5 μg/ml; hybridoma clone 11676; ATCC, Rockville, MD) was added at day 0 to GM-CSF- and IL-4-treated cultures in the presence and absence of BiP (20 μg/ml). Two specific cell-permeable inhibitors of the MAPK intracellular signalling pathways were also used, SB203580 (SB; 10 μM; Calbiochem, Darmstadt, Germany), which inhibits p38 MAPK, and PD98059 (PD; 10 μM; Calbiochem), an inhibitor of MAPK extracellular signal regulated kinase (ERK). MOs were pretreated with the inhibitors for 2 hr before the addition of GM-CSF and IL-4 in the presence or absence of BiP.

T cell:DC co-culture and determination of regulatory T-cell activity

Purified T cells (> 95% T cells; 2×10^6 cells/ml/well) were cultured in the absence or presence of autologous DC populations in 24-well flat-bottomed plates at a DC:T-cell ratio of 1 : 1 for 4 days. T cells were isolated from the autologous co-cultures and incubated with anti-HLA-DR antibody (hybridoma clone L243; ATCC; 1/20 dilution of ammonium sulphate precipitated protein) and the DCs were removed with goat anti-mouse coated immunomagnetic beads (Dynal). T cells (10^6 cells/ml; 96-well plate) were then cultured with fresh autologous responder T cells and irradiated autologous PBMC (1 : 1 : 1 cell ratio) and cultured with or without anti-CD3 (hybridoma clone OKT3; ATCC; 1/2000 dilution of ammonium sulphate precipitated protein) for 3 days. The cells were pulsed with [^{3}H]thymidine (0·2 μCi/well) (Pharmacia Biotech, Amersham, UK) for the final 18 hr of culture.

Cytokine measurement

Supernatant samples were aliquoted and frozen at −70° until required. Production of the anti-inflammatory cytokine IL-10 was quantified by ELISA using paired antibodies and recombinant standards (PharMingen, Oxford, UK) according to the manufacturer's instructions.

Immunofluorescent staining and flow cytometric analysis

Immunofluorescent staining was performed, as described previously,[19] the DC were harvested at day 7 and either stained or washed. Washed DC were set up in co-culture with autologous T cells and after 4 days co-culture these T cells were harvested and stained. Cell surface staining for DCs used anti-CD14, CD86, CD80, HLA-DR, CD11c, CD1a, CD83 and leucocyte immunoglobulin-like receptor subfamily B member 1 (LILRB1), and for regulatory T cells, CD3, CD4, CD25 and CD27 monoclonal antibodies all directly conjugated to fluorescein isothiocyanate (FITC), phycoerythrin (PE) or peridium chlorophyll (Per-CP) as required [all from Becton Dickinson/PharMingen (BD), Oxford, UK]. Intracellular staining for CD152/CTLA-4.PE (BD), forkhead box P3 (Foxp3) (eBiosciences, Insight Biotechnology, Wembley, UK) and IDO (Santa Cruz, Autogen Bioclear, Calne, UK) was carried out following fixation of cells with 1% paraformaldehyde for 5 min. Cells were then washed with immunofluorescence buffer [PBS/0·1% bovine serum albumin (BSA)/0·05% azide] containing 0·3% saponin. 0·3% saponin was present in all solutions thereafter. Isotype controls, mouse immunoglobulin G1 (IgG1) and IgG2 (Becton Dickinson, Oxford, UK) were used in parallel. Cells were analysed on FACScan/FACScalibur cytometers using CELLQUEST software (Becton Dickinson).

Statistical analysis

All experiments were performed at least three times. Data were compared using Student's *t*-test and are expressed as mean ± standard deviation in the text.

Results

In the experiments described below, BiP was only present in the initial cultures along with GM-CSF and IL-4 to drive the differentiation of PB monocytes into mature DCs.

BiP-treated mDCs ($mDC_{(BiP)}$s) showed phenotypic and functional differences from control mDCs

Changes in mDC phenotype were monitored over a 7-day differentiation period with maturation induced by LPS over the final 48 hr. Intracellular indoleamine 2,3-dioxygenase (IDO) was consistently detected in more cells in the $mDC_{(BiP)}$ cultures (8·5 ± 7 fold increase; range 2·0–20·0 fold) compared with control mDCs (mDCs,

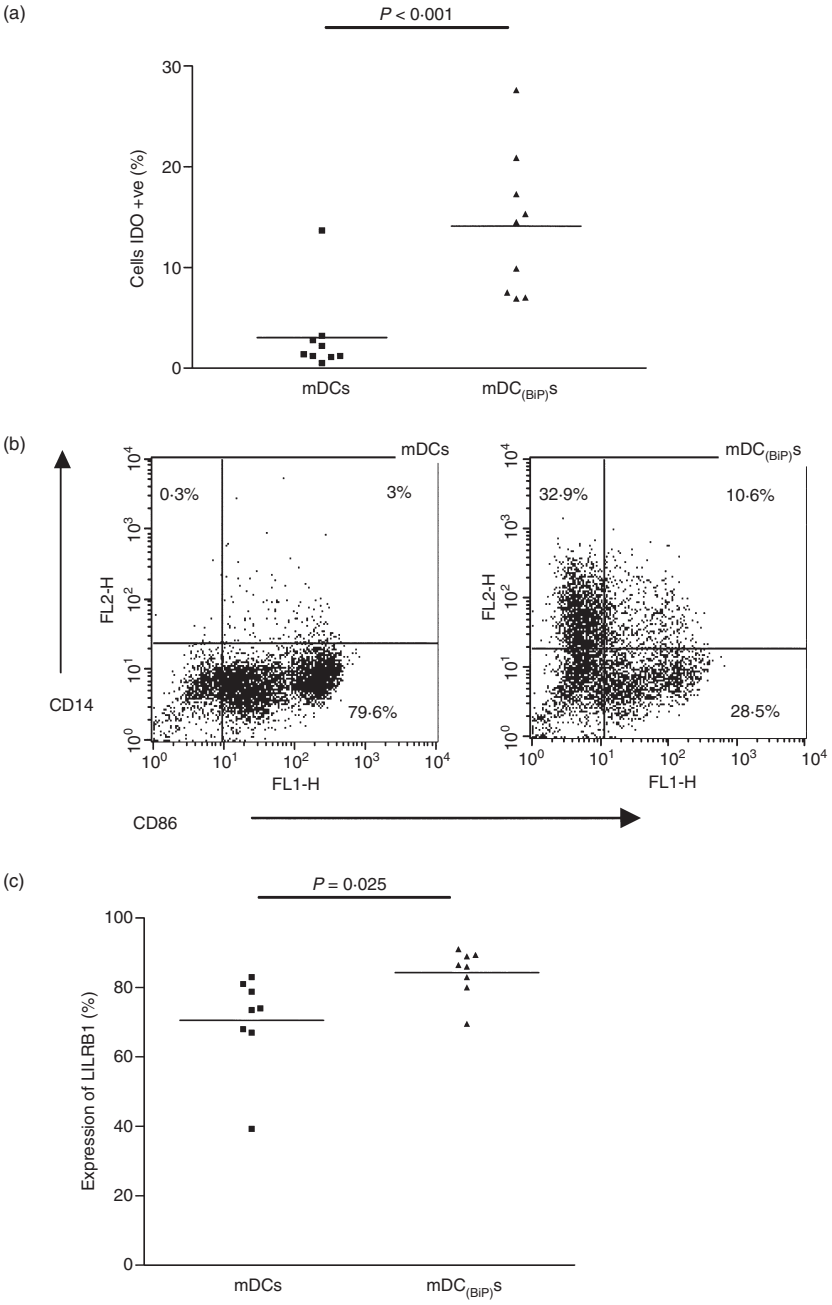

Figure 1. Binding immunoglobulin protein (BiP) alters expression of indolamine-2,3 dioxygenase (IDO), costimulatory molecules and regulatory molecules by dendritic cells (DCs). Peripheral blood monocytes were cultured with granulocyte–macrophage colony-stimulating factor (GM-CSF) and interleukin (IL)-4 in the presence or absence of BiP (20 μg/ml) for 7 days. Lipopolysaccharide (LPS; 500 ng/ml) was added for the final 48 hr to mature dendritic cells (mDCs). (a) Flow cytometry was used to detect intracellular expression of IDO after DC maturation in nine different experiments. (b) The expression of CD14 and CD86 on mDCs or $mDC_{(BiP)}$s in a representative experiment and (c) the expression of leucocyte immunoglobulin-like receptor subfamily B member 1 (LILRB1) by mDCs and $mDC_{(BiP)}$s in eight different experiments.

3·0 ± 4·2% versus $mDC_{(BiP)}$s, 14·1 ± 7·1%; $P = 0·001$, $n = 9$) (Fig. 1a).

Compared with control mDCs, $mDC_{(BiP)}$s maintained CD14 expression, failed to up-regulate CD86 (Fig. 1b, Table 1) and failed to mature after the addition of LPS as determined by the up-regulation of CD83 (Table 1). BiP treatment of DCs also reduced the mean fluorescent intensity (MFI) of CD80 ($P = 0·07$; not significant) (Table 1) and HLA-DR ($P = 0·04$) (Table 1) expression, although the per cent expression remained high. In contrast, an increase in cell surface expression of LILRB1 (ILT2) was observed (mDCs, 72·5 ± 6·4% versus $mDC_{(BiP)}$s, 87·2 ± 3·5%; $P = 0·008$, $n = 4$) (Fig. 1c).

T cells co-cultured with $DC_{(BiP)}$s show phenotypic and functional characteristics consistent with the generation of T regulatory cells

Up-regulation of T regulatory cell markers

T-cell surface expression for $CD4^+$ $CD25^{hi}$ $CD27^{hi}$ (mDCs, 0·8 ± 0·2% versus $mDC_{(BiP)}$s, 2·5 ± 0·2%; $P = 0·016$, $n = 5$) (Fig. 2a) showed a small but significant

Table 1. Surface expression of phenotypic markers

	% cells			MFI		
	mDCs	$mDC_{(BiP)}s$	*P*	mDCs	$mDC_{(BiP)}s$	*P*
CD14	14 ± 19	48 ± 22	0·003	60 ± 19	366 ± 216	0·04
CD83	44 ± 18	10 ± 6	0·002	78 ± 29	49 ± 11	0·05
CD86	62 ± 13	27 ± 8	0·0008	343 ± 49	112 ± 14	0·04
CD80	94 ± 4	91 ± 6	NS	946 ± 166	395 ± 31	0·07 NS
HLA-DR	95 ± 1	95 ± 4	NS	2843 ± 168	1660 ± 167	0·044

Purified monocytes were cultured with granulocyte–macrophage colony-stimulating factor (GM-CSF) and interleukin (IL)-4 in the presence or absence of binding immunoglobulin protein (BiP) (20 μg/ml) for 7 days with lipopolysaccharide (LPS) (500 ng/ml) added for the final 2 days to give mature dendritic cells (mDCs).

Results are expressed as mean ± standard deviation of the per cent positive expression by DCs or mean fluorescent intensity (MFI) from seven different experiments.

HLA, human leucocyte antigen; $mDC_{(BiP)}$, BiP-treated mature dendritic cell; NS, not significant.

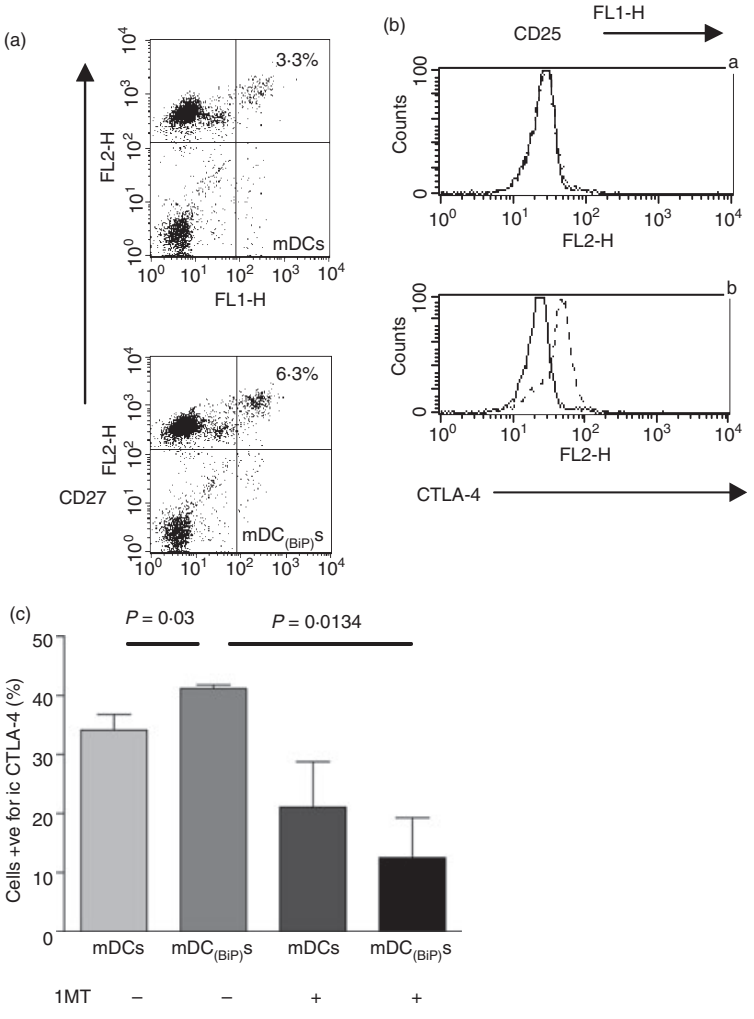

Figure 2. Co-culture of T cells with binding immunoglobulin protein (BiP)-treated dendritic cells (DCs) up-regulates regulatory markers. T cells were co-cultured with autologous mature DCs (mDCs) or BiP-treated mDCs ($mDC_{(BiP)}s$) for 4 days. Data from a representative experiment are shown in (a) a dot plot of $CD27^+$ $CD25^{hi}$ cells, gated for $CD4^+$ cells only, and (b) a histogram representing intracellular $CD4^+$ $CTLA\text{-}4^+$ cells (dashed line) compared with isotype control cells (solid line) after co-culture with mDCs (ba) or $mDC_{(BiP)}s$ (bb). (c) T cells were cultured with autologous mDCs or $mDC_{(BiP)}s$ in the absence or presence of the indolamine-2,3 dioxygenase (IDO) inhibitor 1 methyl tryptophan (1MT) (200 μM). After 4 days the number of intracellular cytotoxic (ic) T-lymphocyte antigen $(CTLA)\text{-}4^+$ $CD4^+$ cells in each co-culture was measured by intracellular immunofluorescence and flow cytometry. Data from three different experiments are shown as mean ± standard deviation.

up-regulation following contact with $mDC_{(BiP)}$s, although these cells showed no significant or consistent up-regulation of Foxp3 (data not shown).

Regulation of intracellular cytotoxic T-lymphocyte antigen (CTLA)-4 by IDO-containing DCs

In addition to Foxp3, up-regulated CTLA-4 expression has been cited as a prerequisite for functional regulatory T cells.[20] T cells that had been in contact with $mDC_{(BiP)}$s showed significant up-regulation of intracellular CTLA-4 compared with T cells cultured with mDCs (Fig. 2b). To investigate whether the increased expression of IDO in $mDC_{(BiP)}$s was directly responsible for increased CTLA-4 expression by co-cultured T cells, the IDO inhibitor 1 methyl tryptophan (1MT) was used. A significant reduction in the percentage of $CTLA^+$ T cells ($P = 0{\cdot}013$, $n = 3$) (Fig. 2c) was observed when 1MT was added to T cell/$mDC_{(BiP)}$ co-cultures.

T cells show regulatory activity post co-culture with $DC_{(BiP)}$s

The regulatory T-cell assay used responder T cells, pre-cultured T cells (from autologous mDC and $mDC_{(BiP)}$ co-cultures) and irradiated PBMC set up at a 1 : 1 : 1 ratio, with and without anti-CD3 antibody as a stimulus. Proliferation of autologous responder T cells in response to anti-CD3 antibody was inhibited by T cells co-cultured with $mDC_{(BiP)}$s ($63{\cdot}8 \pm 13{\cdot}7\%$ inhibition; $n = 5$). A representative experiment is shown (Fig. 3b).

Allogeneic response and cytokine production by BiP-treated early DC cultures

Study of monocytes 3 days after culture with GM-CSF and IL-4 in the presence or absence of BiP showed significant differences in the function and cytokine production of the pre-DC cultures. Early DCs cultured in the presence of BiP significantly inhibited an allogeneic T-cell response (Fig. 4a), unlike the control early DCs. The addition of a neutralizing anti-IL-10 antibody (Fig. 4a) or the specific MAPK p38 inhibitor SB203580 (Fig. 4b) to the BiP-treated cultures reversed this inhibitory activity, although the MAPK ERK inhibitor PD98059 had no significant effect (Fig. 4b). The induction of non-inflammatory DCs from monocytes was dependent on the timing of the addition of BiP, as evidenced by the fact that monocytes treated with BiP 24 hr after the initial dose of GM-CSF and IL-4 did not inhibit the allogeneic T-cell response to pre-DCs (data not shown).

On analysis of the culture supernatants, pre-$DC_{(BiP)}$s produced significantly more IL-10 than the control pre-DC cultures (Fig. 4c). IL-10 production was not detected in the presence of neutralizing anti-IL-10 or SB203580. However, PD98059 did not inhibit IL-10 production and consequently suppression of the T-cell proliferative response was observed. Significantly higher production of IL-10 by $mDC_{(BiP)}$s was also observed after 7 days of culture (mDCs, $132{\cdot}4 \pm 84{\cdot}1$ versus $mDC_{(BiP)}$s, $970{\cdot}1 \pm 684$ pg/ml; $P = 0{\cdot}0081$, $n = 8$) (Fig. 4d), although addition of BiP to differentiating monocytes at day 3/4 ($mDC_{(lateBiP)}$s) was not sufficient to up-regulate IL-10 to

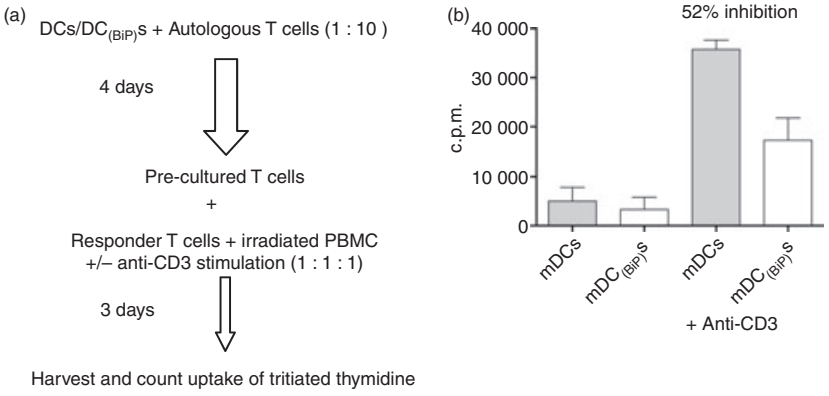

Figure 3. Co-culture with binding immunoglobulin protein (BiP)-treated dendritic cells (DCs) produces T cells with regulatory function. (a) A diagram of the protocol for the experiment is shown: after 4 days of pre-incubation with autologous mature DCs (mDCs) or BiP-treated mDCs ($mDC_{(BiP)}$s), T cells were separated, washed and set up in autologous cultures with irradiated peripheral blood mononuclear cells (PBMC) (3000 rads) and responder T cells (1 : 1 : 1 ratio) (10^5 cells/well) in the presence or absence of anti-CD3 antibody (1/2000 dilution). (b) Data showing the proliferative response in a representative experiment carried out as described in (a). The data were recorded as counts per minute of tritiated thymidine added to the cultures for the final 18 hr. Cultures were set up in triplicate and data are shown as mean ± standard deviation. Four experiments were carried out.

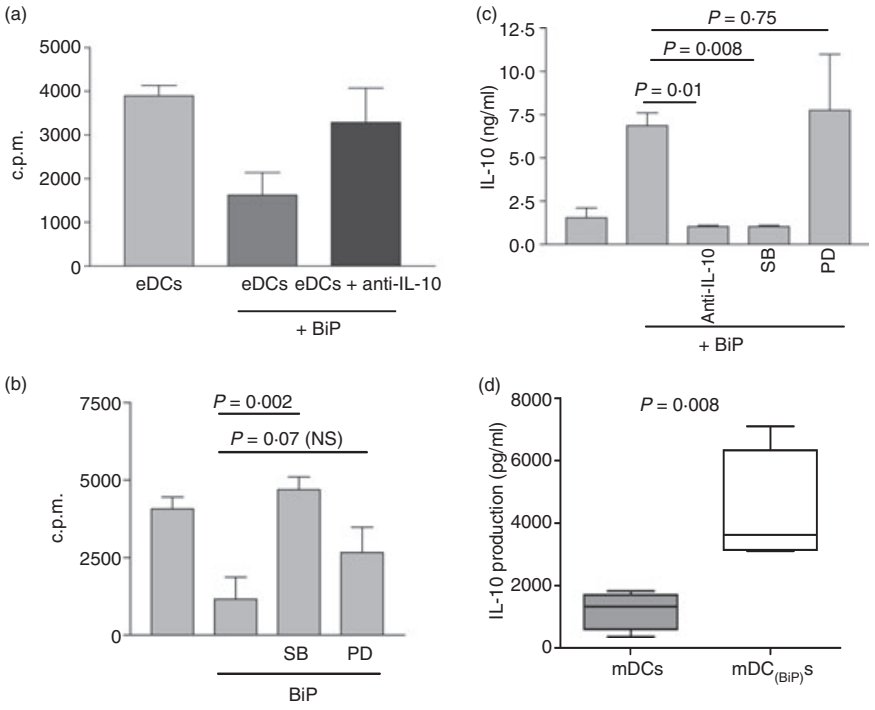

Figure 4. The effect of interleukin (IL)-10 and mitogen-activated protein kinase (MAPK) inhibitors on dendritic cell (DC) maturation. Allogeneic proliferative responses by peripheral blood mononuclear cells (PBMC) in culture with irradiated early DCs (eDCs) (3000 rads), after only 3 days in culture with granulocyte–macrophage colony-stimulating factor (GM-CSF) and IL-4 in the absence or presence of binding immunoglobulin protein (BiP) (20 μg/ml) at a ratio of 10 : 1 PBMC : early DCs (10^5 : 10^4 cells/well), were determined. The results are shown as allogeneic T-cell proliferation in response to eDCs or BiP-treated eDCs ($eDC_{(BiP)}s$) (a) with the addition of neutralizing anti-IL-10 (1/100 dilution) antibody or (b) following pretreatment (2 hr) of monocytes with the p38 inhibitor SB203580 (SB) (10 μM) or the extracellular signal regulated kinase 1/2 (ERK1/2) inhibitor PD98059 (PD) (10 μM), prior to the addition of GM-CSF, IL-4 and BiP to the cultures. Proliferation was measured by uptake of tritiated thymidine and recorded as counts per minute (c.p.m.). Enzyme-linked immunosorbent assays (ELISAs) were used to detect IL-10 in (c) the culture supernatants of the experiments shown in (a) and (b), and (d) the culture medium from mature DCs (mDCs) or BiP-treated mDCs ($mDC_{(BiP)}s$) differentiated for 7 days with GM-CSF and IL-4 with lipopolysaccharide (LPS; 500 ng/ml) added for the final 2 days.

the same level (mDCs, 218 ± 106 pg/ml versus $mDC_{(BiP)}s$, 965 ± 74 pg/ml versus $mDC_{(lateBiP)}s$, 330 ± 311; $n = 3$).

Discussion

In this paper, we have demonstrated the ability of extracellular BiP to inhibit full mDC differentiation and down-regulate inflammatory immune responses through induction of IDO^+ DCs, which drive the up-regulation of CTLA-4 in T cells with regulatory function. We have shown that BiP-treated DC cultures fail to mature and show down-regulation of CD86 and up-regulation of IDO even in the presence of LPS. Thus, BiP induced the phenotype of an anti-inflammatory DC which was stable despite being exposed to LPS, an inflammatory stimulant.

Following co-culture of T cells with $DC_{(BiP)}s$, there was a small but significant increase in the percentage of $CD4^+$ $CD25^{hi}$ $CD27^{hi}$ cells, a recognized subset of regulatory T cells.[21] CD27 has been identified as being a surface marker most closely associated with regulatory function for expanded regulatory T cells,[21] but we are currently also investigating whether other regulatory T-cell populations are induced. As the exact phenotype of the inducible CD4 regulatory T cell is still being characterized, we would just remark that the most notable evidence for an expansion of a cell that might be regarded as a regulatory T cell is the increase of intracellular CTLA-4 in $CD4^+$ cells following co-culture with $DC_{(BiP)}s$. These cells appeared to be regulatory in function but did not up-regulate Foxp3. However, Zheng *et al.*[22] have recently described regulatory $CTLA\text{-}4^+$ T cells, derived from $CD4^+$ $CD25^-$ cells, that lack Foxp3 expression and concluded that the up-regulation of CTLA-4 and regulatory function may be facilitated by, but is not dependent on, Foxp3 expression. We found that T-cell proliferation in response to anti-CD3 antibody was inhibited by T cells pre-incubated with $DC_{(BiP)}s$, in the complete absence of additional exogenous BiP. Therefore, using human cells *in vitro*, we have confirmed our observation in mice[16] that lymph node and spleen cells given BiP parenterally will suppress collagen-induced arthritis in recipient mice without the need for further BiP administration.[16]

It is suggested that the development of regulatory T cells within this system is driven by BiP-induced changes to the DC surface phenotype acting to reduce antigen presentation efficacy in conjunction with increased intracellular IDO. High levels of IDO are known to enhance the tolerizing ability of DCs,[23] although the exact mechanism by which this is brought about is still being debated. At least two mechanisms of action are possible; one involves a reduction in local levels of the essential amino acid tryptophan[23] and another involves inhibitory agents, such as kynurenines (downstream products of tryptophan catabolism), which can induce apoptosis.[23] Interpretation of the data can be further complicated because detection of intracellular IDO does not necessarily relate to activity. Enzymatic activity can be investigated functionally by using the IDO inhibitor 1MT.[24] In the context of this work, we have shown for the first time that $mDC_{(BiP)}$s consistently showed greater IDO expression. Furthermore, we have shown that up-regulation of intracellular CTLA-4 in either allogeneic or autologous T cells following contact with $mDC_{(BiP)}$s may be inhibited by the addition of 1MT. These 1MT-treated cells then show significantly improved allogeneic proliferative responses. Munn and Mellor[23] have already shown that CTLA-4 immunoglobulin can induce IDO in DCs *in vitro*, and work done in macaques shows that CTLA-4 blockade down-regulates IDO and TGF-β.[25] We have now shown that BiP up-regulates IDO in $DC_{(BiP)}$s and that direct contact between these $DC_{(BiP)}$s and T cells induces T-cell CTLA-4 expression, completing this paracrine control loop.

Several candidates have been proposed as deactivation agents for human DCs. These include IL-10,[26] TGF-β,[26] vitamin D_3[27] and vasoactive intestinal peptide (VIP).[28] There is a general consensus that to generate anti-inflammatory DCs there must be down-regulation of the costimulatory molecules CD86 and/or CD80, often in conjunction with up-regulation of IL-10 and, as has been suggested more recently, IDO, although IDO levels in these cultures have not yet been reported. More subtle differences, however, may point to mechanistic diversity. For instance, BiP caused a marked decrease in CD86 expression while minimally affecting HLA-DR and CD80 positivity, while addition of recombinant IL-10 alone affects both HLA-DR and CD86 expression.[29] VIP also induces high concentrations of IL-10 during DC maturation with LPS, but causes only a slight loss of CD86 and CD80 expression and does not affect HLA-DR expression.[30] So, although BiP and VIP stimulate the persistent production of IL-10, there are differences in the final phenotype of $mDC_{(BiP)}$s or VIP-treated mDCs when compared with IL-10-treated DCs. Conversely, vitamin D_3-treated DCs, which produce little IL-10, induce high CD86 and low HLA-DR expression[27] but maintain features shared with $mDC_{(BiP)}$s, namely, high CD14 and low CD83 expression. Collectively, these findings suggest that these DCs have failed to fully mature in the presence of LPS. Alongside these changes, $mDC_{(BiP)}$s display other phenotypic changes such as a significantly higher expression of the inhibitory molecule LILRB1 associated with induction of tolerance.[31] Ligation of LILRB1 on the DC surface has been shown to reduce the ability of these cells to stimulate a proliferative T-cell response.[32] Phosphorylation of the ITIM motif in the cytoplasmic tail of LILRB1 is enhanced if there is co-ligation of HLA-DR. Thus, BiP treatment should promote inhibition and/or tolerance following up-regulation of LILRB1 in the presence of HLA-DR. In conjunction with the co-expression of CD40, there may be further enhancement of the inhibitory potential of these cells.

To investigate whether IL-10 was the dominant cause of suppression in our system, we decided to concentrate on its effect in the early stages of DC differentiation. BiP-treated pre-DCs were unable to stimulate a mixed lymphocyte reaction. Neutralization of IL-10 using either a blocking anti-IL-10 antibody or the specific inhibitor of the MAPK p38 pathway SB203580, on which IL-10 signalling is dependent, resulted in complete restoration of allogeneic T-cell proliferation supported by pre-$DC_{(BiP)}$s, indicating that IL-10 had a major influence in these cultures. In contrast, inhibition of the MAPK ERK1/2 pathway failed to restore the allogeneic T-cell reaction. The temporal sequence of events may be important in these experiments, as addition of IL-10 still affected DC differentiation after 6 days in culture.[31] However, we have been unable to show any effect of BiP on DC differentiation when it was added 24 hr or more after the initial addition of GM-CSF and IL-4. Thus, IL-10 remains a modifying factor in the first 6 days of culture whereas BiP needs to be present during the first 24 hr in order to change the DC phenotype. It may be concluded that BiP has effects on DC development that are separate from and/or are additional to those of the IL-10 released from these cells under the influence of BiP.

In conclusion, we have demonstrated that BiP modifies the maturation of DCs to produce a stable anti-inflammatory phenotype in such a way that direct contact between $DC_{(BiP)}$s and T cells drives T-cell development towards a regulatory cell profile. The above body of work supports our hypothesis that BiP is an immunomodulatory molecule that aids resolution of inflammation. It may thus be of potential therapeutic benefit in human inflammatory diseases such as rheumatoid arthritis (RA).

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Disclosures

VMC and GSP hold the patent for the use of BiP as an immunotherapy in rheumatoid arthritis. GSP is a director of the non-profit-making company Immune Regulation Ltd. OV has no disclosure to make.

References

1 Maldonado-Lopez R, Moser M. Dendritic cell subsets and the regulation of Th1/Th2 responses. *Semin Immunol* 2001; **13**:275–82.

2 Weaver CT, Hatton RD, Mangan PR, Harrington LE. IL-17 family cytokines and the expanding diversity of effector T cell lineages. *Annu Rev Immunol* 2007; **25**:821–52.

3 Romagnani S. Regulation of the T cell response. *Clin Exp Allergy* 2006; **36**:1357–66.

4 Gonzalez-Rey E, Chorny A, Fernandez-Martin A, Ganea D, Delgado M. Vasoactive intestinal peptide generates human tolerogenic dendritic cells that induce CD4 and CD8 regulatory T cells. *Blood* 2006; **107**:3632–8.

5 Gething MJ. Role and regulation of the ER chaperone BiP. *Semin Cell Dev Biol* 1999; **10**:465–72.

6 Asea A, Rehli M, Kabingu E, Boch JA, Bare O, Auron PE, Stevenson MA, Calderwood SK. Novel signal transduction pathway utilized by extracellular HSP70: role of toll-like receptor (TLR) 2 and TLR4. *J Biol Chem* 2002; **277**:15028–34.

7 Wang Y, Kelly CG, Karttunen JT *et al.* CD40 is a cellular receptor mediating mycobacterial heat shock protein 70 stimulation of CC-chemokines. *Immunity* 2001; **15**:971–83.

8 Delneste Y, Magistrelli G, Gauchat J *et al.* Involvement of LOX-1 in dendritic cell-ediated antigen cross-presentation. *Immunity* 2002; **17**:353–62.

9 Binder RJ, Han DK, Srivastava PK. CD91: a receptor for heat shock protein gp96. *Nat Immunol* 2000; **1**:151–5.

10 Binder RJ, Harris ML, Menoret A, Srivastava PK. Saturation, competition, and specificity in interaction of heat shock proteins (hsp) gp96, hsp90, and hsp70 with CD11b+ cells. *J Immunol* 2000; **165**:2582–7.

11 Matzinger P. The danger model: a renewed sense of self. *Science* 2002; **296**:301–5.

12 Asea A, Kraeft SK, Kurt-Jones EA, Stevenson MA, Chen LB, Finberg RW, Koo GC, Calderwood SK. HSP70 stimulates cytokine production through a CD14-dependant pathway, demonstrating its dual role as a chaperone and cytokine. *Nat Med* 2000; **6**:435–42.

13 Habich C, Baumgart K, Kolb H, Burkart V. The receptor for heat shock protein 60 on macrophages is saturable, specific, and distinct from receptors for other heat shock proteins. *J Immunol* 2002; **168**:569–76.

14 Corrigall VM, Bodman-Smith MD, Brunst M, Cornell H, Panayi GS. The stress protein, BiP, stimulates human peripheral blood mononuclear cells to express an anti-inflammatory cytokine profile and to inhibit antigen presenting cell function: relevance to the treatment of inflammatory arthritis. *Arthritis Rheum* 2004; **50**:1167–71.

15 Bodman-Smith MD, Corrigall VM, Kemeny DM, Panayi GS. BiP, a putative autoantigen in rheumatoid arthritis, stimulates IL-10-producing CD8-positive T cells from normal individuals. *Rheumatology (Oxford)* 2003; **42**:637–44.

16 Brownlie RJ, Myers LK, Wooley PH, Corrigall VM, Bodman-Smith MD, Panayi GS, Thompson SJ. Treatment of murine collagen-induced arthritis by the stress protein BiP via interleukin-4-producing regulatory T cells: a novel function for an ancient protein. *Arthritis Rheum* 2006; **54**:854–63.

17 Panayi GS, Corrigall VM, Henderson B. Stress cytokines: pivotal proteins in immune regulatory networks; Opinion. *Curr Opin Immunol* 2004; **16**:531–4.

18 Corrigall VM, Bodman-Smith MD, Fife MS *et al.* The human endoplasmic reticulum molecular chaperone BiP is an autoantigen for rheumatoid arthritis and prevents the induction of experimental arthritis. *J Immunol* 2001; **166**:1492–8.

19 Iannone F, Corrigall VM, Kingsley GH, Panayi GS. Evidence for the continuous recruitment and activation of T cells into the joints of patients with rheumatoid arthritis. *Eur J Immunol* 1994; **24**:2706–13.

20 Dieckmann D, Plottner H, Berchtold S, Berger T, Schuler G. Ex vivo isolation and characterization of CD4(+)CD25(+) T cells with regulatory properties from human blood. *J Exp Med.* 2001; **193**:1303–10.

21 Duggleby RC, Shaw TN, Jarvis LB, Kaur G, Gaston JS. CD27 expression discriminates between regulatory and non-regulatory cells after expansion of human peripheral blood CD4+ CD25+ cells. *Immunology* 2007; **121**:129–39.

22 Zheng Y, Manzotti CN, Burke F, Dussably L, Qureshi O, Walker LSK, Sansom DM. Acquisition of suppressive function by activated human CD4+CD25− T cells is associated with the expression of CTLA-4 not FoxP3. *J Immunol* 2008; **181**:1683–91.

23 Mellor AL, Munn DH. IDO expression by dendritic cells: tolerance and tryptophan catabolism. *Nat Rev Immunol* 2004; **4**:762–74.

24 Hwang SL, Chung NL, Chan JK, Lin CL. Indoleamine 2, 3-dioxygenase (IDO) is essential for dendritic cell activation and chemotactic responsiveness to chemokines. *Cell Res* 2005; **15**:167–75.

25 Hryniewicz A, Boasso A, Edghill-Smith Y *et al.* CTLA-4 blockade decreases TGF-beta, IDO, and viral RNA expression in tissues of SIVmac251-infected macaques. *Blood* 2006; **108**:3834–42.

26 Rutella S, Danese S, Leone G. Tolerogenic dendritic cells: cytokine modulation comes of age. *Blood* 2006; **108**:1435–40.

27 Canning MO, Grotenhuis K, de Wit H, Ruwhof C, Drexhage HA. 1-alpha,25-Dihydroxyvitamin D3 (1,25(OH)(2)D(3)) hampers the maturation of fully active immature dendritic cells from monocytes. *Eur J Endocrinol* 2001; **145**:351–7.

28 Delgado M, Gonzalez-Rey E, Ganea D. Vasoactive intestinal peptide: the dendritic cell –> regulatory T cell axis. *Ann N Y Acad Sci* 2006; **1070**:233–8.

29 Figueroa-Vega N, Galindo-Rodriguez G, Bajana S, Portales-Perez D, Abud-Mendoza C, Sanchez-Torres C, González-Amaro R. Phenotypic analysis of IL-10-treated, monocyte-derived dendritic cells in patients with systemic lupus erythematosus. *Scand J Immunol* 2006; **64**:668–76.

30 Delgado M, Gonzalez-Rey E, Ganea D. The neuropeptide vasoactive intestinal peptide generates tolerogenic dendritic cells. *J Immunol* 2005; **175**:7311–24.

31 Velten FW, Duperrier K, Bohlender J, Metharom P, Goerdt S. A gene signature of inhibitory MHC receptors identifies a BDCA3(+) subset of IL-10-induced dendritic cells with reduced allostimulatory capacity in vitro. *Eur J Immunol* 2004; **34**:2800–11.

32 Young NT, Waller EC, Patel R, Roghanian A, Austyn JM, Trowsdale J. The inhibitory receptor LILRB1 modulates the differentiation and regulatory potential of human dendritic cells. *Blood* 2008; **6**:3090–6.