Systemic gene transfer of binding immunoglobulin protein (BiP) prevents disease progression in murine collagen-induced arthritis

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Summary

Recombinant human binding immunoglobulin protein (BiP) has previously demonstrated anti-inflammatory properties in multiple models of inflammatory arthritis. We investigated whether these immunoregulatory properties could be exploited using gene therapy techniques. A single intraperitoneal injection of lentiviral vector containing the murine BiP (Lenti-mBiP) or green fluorescent protein (Lenti-GFP) transgene was administered in low- or high-dose studies during early arthritis. Disease activity was assessed by visual scoring, histology, serum cytokine and antibody production measured by cell enzyme-linked immunosorbent assay (ELISA) and ELISA, respectively. Lentiviral vector treatment caused significant induction of interferon (IFN)-γ responses regardless of the transgene; however, further specific effects were directly attributable to the BiP transgene. In both studies Lenti-mBiP suppressed clinical arthritis significantly. Histological examination showed that low-dose Lenti-mBiP suppressed inflammatory cell infiltration, cartilage destruction and significantly reduced pathogenic anti-type II collagen (CII) antibodies. Lenti-mBiP treatment caused significant up-regulation of soluble cytotoxic T lymphocyte antigen-4 (sCTLA-4) serum levels and down-regulation of interleukin (IL)-17A production in response to CII cell restimulation. *In-vitro* studies confirmed that Lenti-mBiP spleen cells could significantly suppress the release of IL-17A from CII primed responder cells following CII restimulation *in vitro*, and this suppression was associated with increased IL-10 production. Neutralization of CTLA-4 in further co-culture experiments demonstrated inverse regulation of IL-17A production. In conclusion, these data demonstrate proof of principle for the therapeutic potential of systemic lentiviral vector delivery of the BiP transgene leading to immunoregulation of arthritis by induction of soluble CTLA-4 and suppression of IL-17A production.

Keywords: BiP, collagen-induced arthritis, gene therapy, lentiviral vector

Introduction

Rheumatoid arthritis (RA) is a chronic, progressive autoimmune disease characterized primarily by a destructive inflammatory polyarthritis leading to joint failure, deformity and disability [1]. A growing armamentarium of biological therapies exist to treat ongoing disease and prevent RA progression; however, gene therapy has the potential to be superior to conventional pharmacological therapy for RA. *Cis* and *trans* acting regulatory elements may allow therapeutic gene expression to be placed under anatomical control or responsive to stress and inflammation, thus

minimizing unwanted immunosuppression following systemic delivery [2–4]. Using a variety of viral and non-viral vectors, gene therapy has already delivered promising results in disease models of inflammatory arthritis [5–7] and the rheumatology clinic [8].

Binding immunoglobulin protein (BiP/GRP78) is a highly conserved endoplasmic reticulum chaperone that displays potent extracellular anti-inflammatory and immunomodulatory activity in both mouse and man on release from stressed cells [9]. Recombinant human BiP (rhuBiP) binds to an unidentified receptor expressed predominantly on the surface of human peripheral blood

monocytes and induces an anti-inflammatory gene expression profile dominated by interleukin (IL)-10 secretion, attenuation of tumour necrosis factor (TNF)-α production and reduced CD86 and human leucocyte antigen D-related (HLA-DR) expression [10]. Human monocyte-derived dendritic cells treated with rhuBiP during their differentiation fail to fully mature and induce cytotoxic T lymphocyte antigen-4 (CTLA-4)$^+$ regulatory T cell development [11]. Moreover, rhuBiP has demonstrated therapeutic efficacy in a number of animal models of RA, including murine collagen-induced arthritis (CIA) [12], rat adjuvant arthritis [13] and the xenogeneic model of rheumatoid synovial membrane transplanted into severe combined immunodeficient mice [14]. Previous gene transfer experiments have shown BiP to have immunomodulatory and protective effects in a model of islet transplantation [15]. Accordingly, we have proposed that BiP be classed within a family of stress proteins called the resolution-associated molecular patterns, molecules that are released from stressed and necrotic cells that act as molecular counterpoints to damage-associated molecular patterns, feeding pro-resolution signals into immunological networks [16].

In this study we investigated the therapeutic potential of *in-vivo* lentiviral vector delivery of the murine BiP transgene (Lenti-mBiP) compared with the control green fluorescent protein (GFP) transgene in the mouse model of CIA. Lenti-mBiP-treated mice showed reduced pathology, disease markers and inflammatory mediators when compared with Lenti-GFP-treated animals. Possible mechanisms of BiP-mediated suppression of arthritis were also investigated.

Materials and methods

Preparation and production of protein and lentiviral vectors

Murine BiP (mBiP) cDNA (NCBI Accession number: BC050927) was acquired from the Mammalian Gene Collection (RZPD, Berlin, Germany). Quikchange site-directed mutagenesis (Stratagene, Amsterdam, the Netherlands) was used to exclude the 3′-Lys-Asp-Glu-Leu (KDEL) endoplasmic reticulum targeting sequence facilitating exaggerated BiP secretion.

Lentiviral vectors

For gene therapy application the pRRLSIN18 third-generation, self-inactivating, HIV-1 lentiviral vector was used, as described previously [17], and contained either an enhanced GFP reporter gene or a KDEL$^-$ murine BiP cDNA under control of the hCMV promoter. Vesicular stomatitis virus glycoprotein (VSV-G) pseudotyped recombinant lentiviral vector particles were produced by bulk transient transfection of human embryonic kidney (HEK) 293T cells,

as described previously [18,19]. Recombinant lentivirus was aliquoted and stored in OptiMEM (Invitogen, Paisley, UK) at −80°C. Titration of lentivirus was carried out by transduction of HEK293T cells in the presence of polybrene, as described previously [18,19]. The titres of vector preparations without fluorescent transgenes were determined by quantitative polymerase chain reaction (PCR) relative to a preparation of lentivirus of known titre. In these cases, 100 ng of total genomic DNA was amplified using primers and probes directed against the lentiviral vector long terminal repeats (LTR) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an internal reference standard [19]. Culture supernatants were harvested and tested for secreted mBiP by enzyme-linked immunosorbent assay (ELISA).

Validation of mBiP function

Peripheral blood mononuclear cells (PBMC) from healthy, consenting volunteers were separated by density gradient centrifugation using lymphoprep (Axis-Shield Diagnostics, Dundee, UK) and cultured in RPMI supplemented with 10% fetal calf serum (FCS); 1×10^6 cells were cultured with or without 20 µg/ml rhuBiP or mBiP. Supernatants were harvested after 24 h and IL-10 and TNF-α concentrations determined by ELISA. Human studies had the full approval of the Guy's Hospital Ethical Committee.

Collagen-induced arthritis model

CIA was induced in 8–12-week-old dilute brown non-Agouti/1 (DBA/1) mice (Harlan, Oxfordshire, UK) as described previously and clinical arthritis scored in a blinded fashion [12]. Animals were allocated randomly to treatment or control groups. In the treatment groups, mice were given a single intraperitoneal injection with either 1×10^7 (low-dose study) or $2 \cdot 5 \times 10^7$ (high-dose study) infectious lentiviral particles diluted in 100 µl phosphate-buffered saline (PBS) at arthritis onset. Arthritis onset was defined by a mean clinical score of 1 in the low-dose study (performed at Wichita State University) and a mean clinical score of 2·5 in the high-dose study (performed at King's College London). All animals were maintained in humane conditions, as dictated by local procedures and the US Animal Welfare Act 1968 and/or the King's College London Institutional Animal Care and Ethics Review Committee. All procedures were conducted in accordance with UK Home Office guidelines and UK Home Office project licence number PPL 70/6547.

Immunological analysis

Single-cell suspensions of spleen and inguinal lymph node cells were prepared as described previously [12]. For stimulation assays, cells ($2 \cdot 5 \times 10^6$ cells/well) were cultured in

48-well plates (Appleton Woods, Birmingham, UK) in a final volume of 1 ml RPMI supplemented with 10% heat-inactivated FCS and stimulated with 2 µg/ml concanavalin A (ConA) or 40 µg/ml collagen type II (CII). Flow cytometry was used to determine the percentage of CD3^{+}, CD4^{+}, CD8^{+}, B220^{+} and CD11b^{+} cells comprising each culture; no differences were observed between immune cell subpopulations and the cultures. In *ex-vivo* co-culture cytokine production experiments, 1.25×10^6 CIA responder splenocytes were co-cultured with 1.25×10^6 Lenti-BiP-, Lenti-GFP- or control-treated splenocytes and stimulated with 40 µg/ml CII. Cytokine secretion from cell cultures was determined at 72 h using the previously described carboxyethyl lysine ELISA (CelELISA) technique [20].

Primed animals for *in-vitro* co-culture experiments

DBA/1 mice were primed subcutaneously with either CII emulsified in complete Freund's adjuvant (CFA) (100 µg CII) or rhuBiP (100 µg rhuBiP) and left for 12 days. After 12 days, spleen and lymph nodes were dissected from euthanized mice and single-cell suspensions prepared. The cells were either cultured separately or at a 2:1 CII : BiP-primed cell ratio, respectively, and restimulated with CII (40 µg/ml) in the presence or absence of neutralizing antibody to CTLA-4 (1.5 µg/ml) (BD Pharmingen, Oxford, UK) or an isotype control (1.5 µg/ml) (eBioscience, Hatfield, UK). Cytokine secretion from cell cultures was determined at 72 h using the previously described CelELISA technique [20]. Some cells were also removed and prepared for the flow-cytometric analysis of intracellular staining for CTLA-4.

Histological analysis

Mouse paws were removed and stored in 10% neutral buffered formalin solution (Surgipath, Richmond, IL, USA). Decalcification, sectioning, staining and histological analysis was performed independently (Professor Paul Wooley's laboratory, Wichita State University, KS, USA), as described previously [21]. Sections were stained with haematoxylin and eosin to assess inflammation. Histological scores were confirmed by light microscopy using previously described parameters [21].

Serological analysis

Serum was collected at the beginning of CIA experiments and terminal exsanguination performed at the close of the CIA study. Total anti-CII immunoglobulin (Ig)G and anti-BiP IgG was determined by ELISA, as described previously [12,22].

Soluble CTLA-4 ELISA

ELISA plates (Nunc Maxisorp) were coated with anti-CTLA-4 capture antibody (2 µg/ml) (clone 9H10;

Pharmingen, Oxford, UK) in carbonate buffer (0.1 M, pH 9.6) overnight at 4°C. Plates were washed once with Hanks's balanced salt solution (HBSS; PAA Laboratories, Yeovil, UK), blocked with 3% PBS–BSA for 1 h at 37°C then washed twice with HBSS. Serum samples (1:4 dilution in HBSS) were added and incubated overnight at 37°C in a humidified atmosphere. Plates were washed twice with PBS-Tween 20, before the addition of biotin-conjugated anti-sCTLA-4 (1 µg/ml) (clone UC10-4B9; eBioscience). Following incubation for 2 h at room temperature plates were washed with PBS-Tween 20. Extravidin–alkaline phosphatase (Sigma, Poole, UK) was added for 90 min at room temperature. Finally plates were washed with PBS-Tween 20 before the addition of phosphatase substrate (Sigma, Poole, UK). The plates were developed in the dark for 24–120 h. A culture control supernatant from a ConA-stimulated mouse cell culture was arbitrarily given the concentration of 1000 relative units (RU) and diluted to give a standard curve. Optical density (OD) was read at 410 nm.

Statistical analysis

Data were collected and analysed using both Microsoft Excel and GraphPad Prism versions 4 and 5. Statistical analysis between treatment groups was carried out using Kruskal–Wallis non-parametric analysis of variance (ANOVA) (comparison of cohorts of mice treated *in vivo* with saline, Lenti-GFP or Lenti-BiP) and two-tailed unpaired Student's *t*-test (individual treatment groups in *ex-vivo* and *in-vitro* serological and immunological experiments). *P*-values of less than 0.05 were regarded as significant.

Results

Biological activity of mBiP gene product

Supernatants from cells transfected with the mBiP gene were collected during 96 h and showed clear accumulation of secreted mBiP, in comparison with untransfected cells and those transfected with the empty vector (Fig. 1a). Vector-derived mBiP demonstrated similar immunological characteristics as rhuBiP. Although rhuBiP induced significantly higher concentrations of IL-10 than mBiP at all serial dilutions, the pattern of cytokine induction, including the IL-10 : TNF-α ratios, were not significantly different between mBiP and rhuBiP or between any serial dilution of either proteins (Fig. 1b). mBiP and rhuBiP also stimulated equivalent concentrations of TNF-α and IL-10 from murine spleen and lymph node cells after 24 h incubation (Fig. 1c). Collectively, this was taken as evidence that vector produced secreted mBiP, with immunological properties similar to an established immunoregulatory protein (rhuBiP).
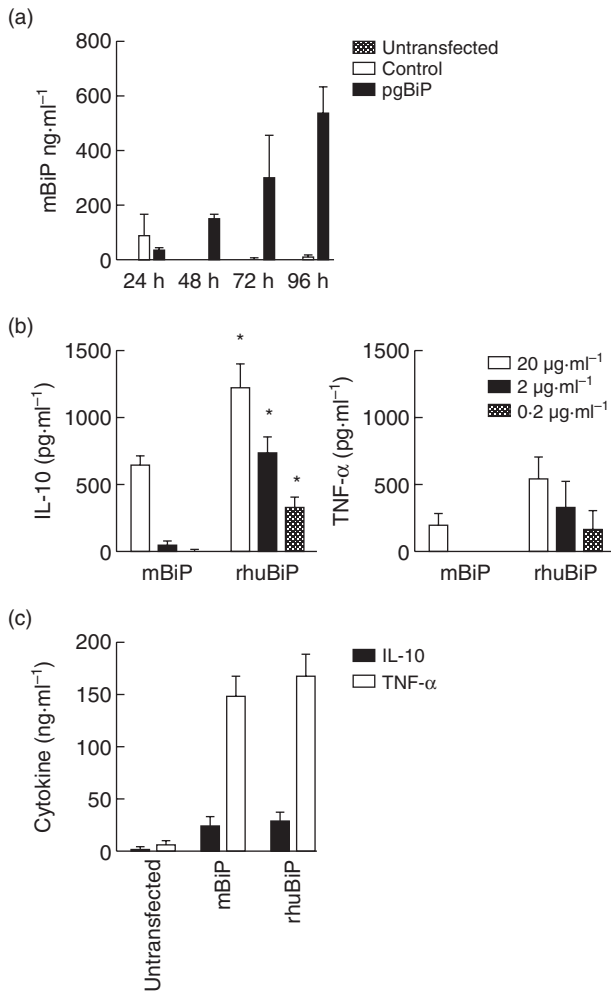
Fig. 1. The binding immunoglobulin protein (BiP) transgene induced biologically active BiP protein production: (a) 5×10^4 human embryonic kidney (HEK) 293T cells were transfected with empty vector or pgBiP. Serial BiP concentration in culture supernatants were measured at 24, 48, 72 and 96 h after transfection by enzyme-linked immunosorbent assay (ELISA). (b) Serial dilutions of mBiP or recombinant human (rhu)BiP were used to stimulate 1×10^6 cultured human peripheral blood mononuclear cells (PBMCs). The production of cytokines, interleukin (IL)-10 and tumour necrosis factor (TNF)-α, induced by the addition of mBiP or recombinant human (rhu) BiP protein, was measured at 24 h. (c) Mixed splenocytes/lymph nodes ($2\cdot5 \times 10^6$) from dilute brown non-Agouti/1 (DBA/1) mice were stimulated with 20 µg of rhuBiP or mBiP. Induced IL-10 and TNF-α were measured at 24 h. Significance was determined by Student's unpaired t-test. $*P < 0\cdot05$.

Systemic BiP gene therapy is therapeutic in CIA

Low-dose study. Treatment of diseased animals with Lenti-mBiP demonstrated significant therapeutic efficacy when compared with the untreated CIA control mice and those that received Lenti-GFP (Fig. 2a). Statistically significant suppression of arthritis scores was evident between the groups treated with vehicle and Lenti-mBiP on days 10 and

18. There was no significant difference between the Lenti-GFP and Lenti-mBiP groups. None the less, histological scores revealed clear differences in the severity of joint damage when comparing the paws from animals with median severity of clinical arthritis from each group (Fig. 2b). Joints from the CIA and Lenti-GFP control groups show pannus (P) invasion and cartilage destruction in contrast to those from Lenti-mBiP animals, where the joint space and cartilage remain relatively well preserved (Fig. 2b).

Furthermore, analysis of serum antibodies to CII confirmed that treatment with Lenti-mBiP significantly lowered the antibody titre compared with CIA control mice ($P < 0\cdot02$). Although there was a trend, there was no significant difference in antibody levels between the Lenti-GFP and the Lenti-mBiP groups (Fig. 2c).

Systemic lentiviral expression of a soluble transgene product in mice has previously generated serum concentrations in the pg/ml range [23]. In our study, mBiP was not detectable in the pretreatment state or in any of the treatment groups (data not shown). This most probably represents the relatively poor sensitivity of the available ELISA for mBiP, which can detect only to a minimum ng/ml level [23]. Additional evidence that the mBiP transgene was active was provided by the serum anti-BiP antibody levels, which were raised significantly in the Lenti-mBiP group compared with the CIA group ($P < 0\cdot05$) after individual normalization of pretreatment to post-treatment levels of anti-BiP antibodies (Fig. 2d). This contrasted with a small but insignificant increase in the anti-BiP antibody titre in the Lenti-GFP mice when compared with the CIA group. Anti-BiP antibodies titres did not correlate with final clinical disease activity scores, suggesting that these effects were mainly a consequence of *in-vivo* cell transduction and transgene expression from the lentiviral vector.

Overall, this low-dose study demonstrated that the Lenti-mBiP transgene was active and had the potential to modulate clinical and immunological parameters of CIA. However, it was not possible to exclude a generic effect of the lentiviral vector on CIA development, as there were no significant differences in arthritis between the two lentiviral groups. Indeed, preliminary evidence suggested that treatment of CIA with a lentivirus, regardless of the expressed transgene, led to an exaggerated interferon (IFN)-γ response to mitogenic stimulation by ConA (Fig. 2e,f) (CIA: 2250 ± 553 pg/ml *versus* Lenti-GFP: 5704 ± 536 pg/ml *versus* Lenti-mBiP: 4225 ± 975; $n = 5$ in each group; $P < 0\cdot05$ Lenti-GFP *versus* CIA) and a suppression of IL-10 responses to CII restimulation (CIA: $780\cdot4 \pm 54$ pg/ml *versus* Lenti-GFP: 273 ± 171 pg/ml *versus* Lenti-mBiP 416 ± 263, $n = 5$ in each group; $P < 0\cdot05$ Lenti-GFP *versus* CIA and Lenti-BiP *versus* CIA). Therefore, we sought evidence that the mBiP transgene had a specific therapeutic effect following treatment.
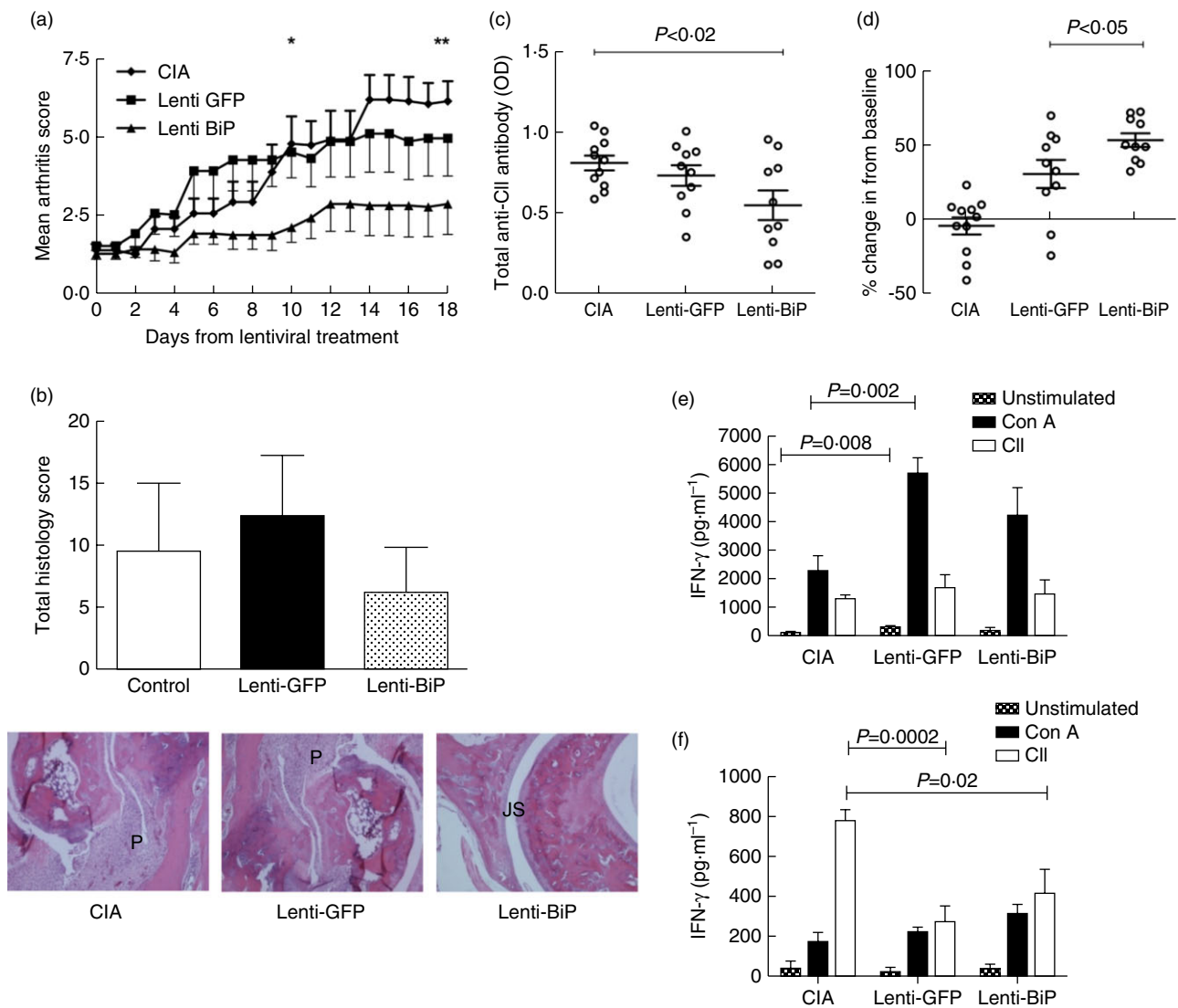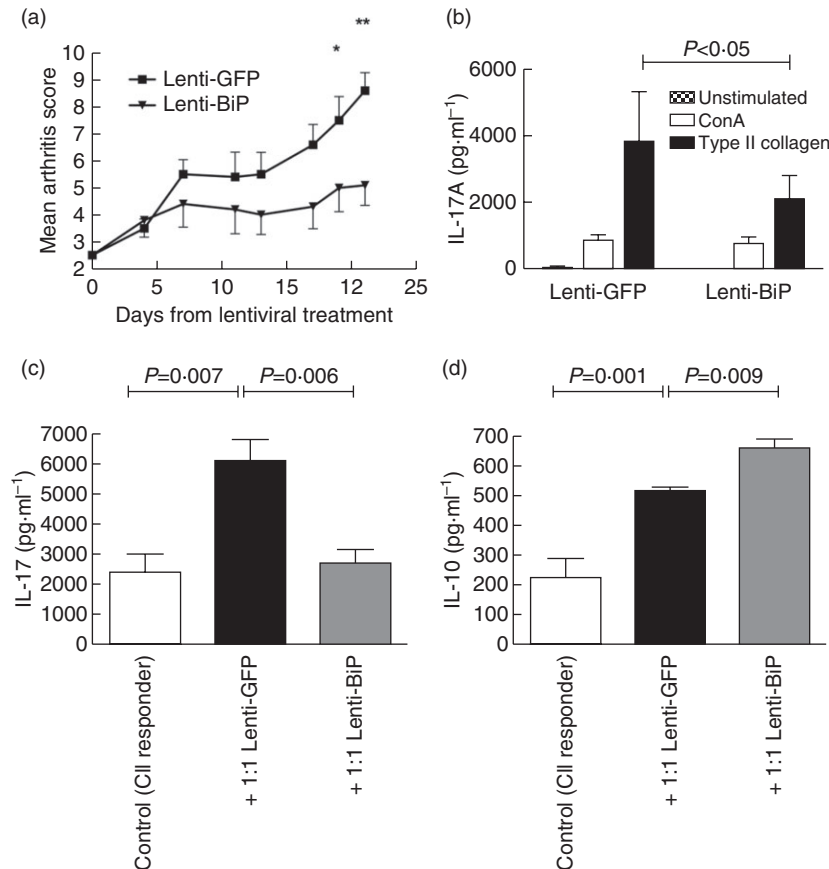
Fig. 2. Low-dose lentiviral vector containing the murine binding immunoglobulin protein (Lenti-mBiP) treatment improves histological and serological parameters of collagen-induced arthritis. Dilute brown non-Agouti/1 (DBA/1) mice ($n = 10$ or 11/group) were either untreated or treated with 1×10^7 infectious viral vector particles of either Lenti-green fluorescent protein (GFP) or Lenti-mBiP administered intraperitoneally at disease onset. (a) Clinical arthritis scores were assessed regularly up to day 18 post-treatment. Data represents the mean and standard error of the mean (*$P < 0.03$ Lenti-GFP *versus* Lenti-BiP; **$P < 0.01$ CIA *versus* Lenti-BiP; significance was determined using Kruskal–Wallis one-way analysis of variance) (b) Haematoxylin and eosin (H&E) staining (magnification ×100) of representative animals with median pathology in the hind paws from the different treatment groups. P = pannus, JS = normal joint space. A histogram shows the mean and standard deviation of the histological scores for each group of five animals. (c) At termination the serum level of total anti collagen type II (CII) immunoglobulin (Ig)G antibodies was measured by enzyme-linked immunosorbent assay (ELISA) and (d) the percentage increase in anti-BiP IgG levels from the individual baseline level at the start of the study was recorded. Mixed lymph node and splenocytes (2.5×10^6) were cultured and restimulated with 40 µg/ml CII or 2 µg/ml concanavalin A (ConA). Interferon (IFN)-γ (e) and interleukin (IL)-10 (f) concentrations in culture supernatants were determined at 72 h by carboxyethyl lysine ELISA (CelELISA) ($n = 5$/group). Statistical significance was analysed using GraphPad software and significance was determined using unpaired two-tailed Student's *t*-test. Significance was taken as <0.05 and the values are as shown.

High-dose study. Animals were dosed when mild arthritis had developed (clinical score = 2.5). Treatment with Lenti-mBiP delayed the clinical progression of CIA from day 5 onwards compared to animals receiving the Lenti-GFP vector. Disease scores were reduced significantly compared

to animals receiving the Lenti-GFP control after day 19 of the study (day 19, $P = 0.03$, day 21, $P = 0.002$) (Fig. 3a).

Systemic lentiviral gene therapy with mBiP is associated with reduced IL-17A responses to type II collagen. IL-17A production was reduced significantly on CII restimulation of

Fig. 3. High-dose lentiviral vector containing the murine binding immunoglobulin protein (Lenti-mBiP) treatment ameliorates collagen-induced arthritis (CIA) and suppresses collagen-specific interleukin (IL)-17 responses: dilute brown non-Agouti/1 (DBA/1) mice ($n = 10$/group) were treated with $2 \cdot 5 \times 10^7$ infectious viral particles of either Lenti-green fluorescent protein (GFP) (Lenti-GFP) or Lenti-mBiP administered intraperitoneally at onset of mild arthritis (clinical score 2). (a) Clinical arthritis scores were assessed regularly for a further 21 days after treatment. The mean ± standard error of the mean is shown. *$P < 0 \cdot 05$ Lenti-GFP *versus* Lenti-BiP; **$P < 0 \cdot 005$ Lenti-GFP *versus* Lenti-BiP (significance was determined using Kruskal–Wallis one-way analysis of variance). (b) Spleens and lymph nodes were dissected at termination and $2 \cdot 5 \times 10^6$ cells were either unstimulated or stimulated with concanavalin A (ConA) (2 µg/ml) or type II collagen (CII)(40 µg/ml) as shown. Single-cell cultures of mixed splenocytes and lymph node cells from either control collagen-induced arthritis (CIA), Lenti-GFP or Lenti-mBiP individual mice were used in co-culture with CII primed cells ($1 \cdot 25 \cdot 10^6$). Cells were stimulated for 72 h with CII (40 µg/ml). (c) IL-17A and (d) IL-10 secretion was measured by carboxyethyl lysine enzyme-linked immunosorbent assay (CelELISA). The mean ± standard error of the mean is shown. ($n = 4$/group). Statistical significance was analysed using GraphPad software and significance was determined using the unpaired two-tailed Student's *t*-test. Significance was taken as <$0 \cdot 05$ and the values are as shown.

spleen and lymph node cells taken from animals treated with Lenti-BiP compared with those from Lenti-GFP-treated animals (Fig. 3b).

Further analysis showed the ability of Lenti-mBiP cells to regulate the production of IL-17A (Fig. 3b,c). On restimulation with CII, Lenti-mBiP cells produced significantly less IL-17A ($P = 0 \cdot 006$), and in co-culture with CII primed responder cells production of IL-17A remained low (Fig. 3c). Furthermore, co-culturing Lenti-BiP-treated cells with CII primed responder cells was associated with increased IL-10 secretion (Fig. 3d). These effects appear unique and attributable to conditioning by the mBiP transgene. In both experiments, the co-culture of cells exposed to lentiviral vectors *in vivo* with CII-primed

responder cells led to significant increases in the levels of both IL-10 and IL-17 produced *ex vivo*. This strongly suggests, as before, that exposure to the lentivirus itself alters the immunological parameters of the collagen-induced arthritis model and of lymphocytes derived from treated animals.

Human *in-vitro* studies have shown that BiP can induce CTLA-4+ regulatory T cells [11]. We therefore investigated whether soluble CTLA-4 (sCTLA-4) was detectable in mouse serum at the termination of the study. Strikingly, the Lenti-mBiP group showed significantly higher levels of sCTLA-4 compared with the Lenti-GFP group ($P = 0 \cdot 015$) (Fig. 4a).

Complementary experiments were undertaken to investigate whether the cytokine changes observed following

Fig. 4. Neutralization of cytotoxic T lymphocyte antigen-4 (CTLA-4) and interleukin (IL)-10 affect interferon (IFN)-γ and IL-17 production, respectively. (a) The total soluble CTLA-4 serum concentration was determined for each mouse at termination of the high-dose study by ELISA (n = 6–9/group). Single-cell cultures of mixed splenocytes and lymph node cells from individual mice were used throughout the following experiments. Cells isolated from binding immunoglobulin protein (BiP)-primed animals, given a subcutaneous injection of BiP (200 μg/ml) and culled after 12 days, were stained for intracellular cytokine expression of (b) *ex-vivo* CTLA-4 on CD3^{+} T cells from spleens and lymph nodes. (c) Collagen type II (CII) primed cells were co-cultured with cells from BiP primed animals in the absence and presence of neutralizing anti-CTLA-4 (1·5 μg/ml), anti-IL-10 (1·5 μg/ml) and isotype control (1·5 μg/ml) and stimulated for 72 h with CII (40 μg/ml). Cytokine production was measured by carboxyethyl lysine enzyme-linked immunosorbent assay (CelELISA) (n = 5/group). The mean ± standard error of the mean is shown. Statistical significance was analysed using GraphPad software and significance was determined using the unpaired two-tailed Student's *t*-test. Significance was taken as <0·05 and the values are as shown.
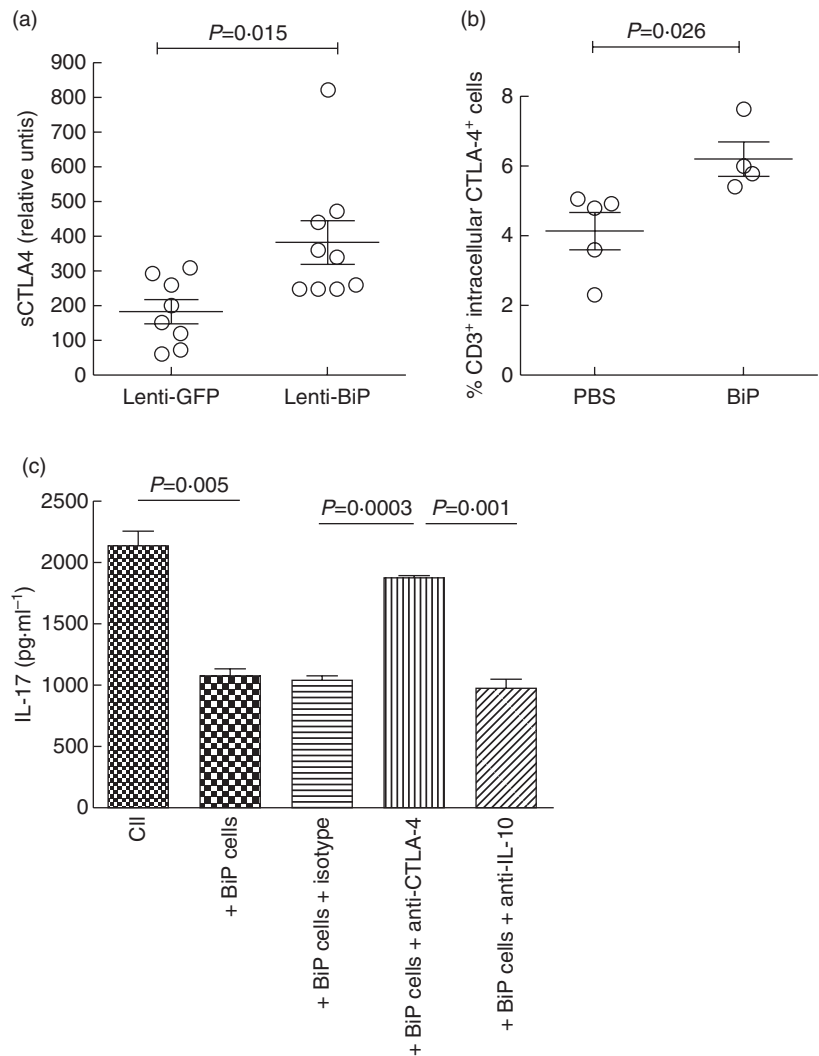
Lenti-mBiP treatment could be recapitulated by direct priming of animals with rhuBiP *in vivo*. BiP-primed mice showed an elevated frequency of CD3^{+} T cells expressing intracellular CTLA-4 when compared with control animals (Fig. 4b).

Role of soluble CTLA-4 and IL-10 in BiP primed cell function. To determine the importance of sCTLA-4 and/or IL-10 to the system, mixed spleen and lymph node cells from animals primed with either CII or rhuBiP were co-cultured at a 2:1 ratio, respectively, and stimulated with CII in the absence and presence of neutralizing antibodies for CTLA-4 and IL-10. The addition of BiP-primed cells in co-culture with CII-primed cells significantly reduced production of IL-17A. The neutralization of CTLA-4 in these cultures increased the production of IL-17A to that of the CII primed cells alone (Fig. 4c). IL-17A inhibition was entirely consequent on CTLA-4 produced by the BiP-primed cells, as no effect on IL-17A production was observed following neutralization of CTLA-4 in cultures containing CII primed cells only (data not shown).

Discussion

This study demonstrates the therapeutic potential of systemic lentiviral delivery of an mBiP transgene in murine CIA. Striking similarities were observed between the properties of mBiP and rhuBiP in standardized and well-defined assays of BiP's immunological activity [24]. Subtle differences were observed in the total concentration of IL-10 generated from human PBMC following rhuBiP and mBiP stimulation. These differences were limited to IL-10, and no significant differences were observed in the ratio of IL-10 to TNF-α. No significant differences were observed when rhuBiP or mBiP were used to stimulate murine cells. This is not surprising, as mBiP and rhuBiP share 99·4% homology at an amino acid level and suggest that the immunological activity of BiP may be conserved across phyla. Those differences observed may be due, in part, to the eukaryotic or prokaryotc cell source of the protein and the consequent differences in folding and glycosylation.

Important differences between the actions of intracellular and extracellular BiP should be considered with respect to

the removal of the ER localizing KDEL sequence. The previously described immunological properties of extracellular BiP are consistent with extracellular BiP acting on a cell surface receptor [10,11]. These properties are quite distinct from those displayed by intracellular, endoplasmic reticulum localized BiP, which regulates the unfolded protein response and induces cytoprotective and anti-apoptotic signals when cells are challenged by endoplasmic reticulum stress [25]. Thus, intracellular BiP may augment chronic inflammation through anti-apoptotic protection of inflammatory cells, such as the rheumatoid fibroblast-like synoviocyte [26] yet, as an extracellular protein, regulate inflammation on secretion from stressed cells [16]. A more complete dissection of the relative contributions of intracellular and extracellular BiP to the pathogenesis and resolution of chronic inflammation will require greater understanding of cells transduced by lentiviral vectors, the receptor or receptors to which BiP binds on the cell surface and the downstream signalling pathways employed. Nevertheless, we have demonstrated that removal of the KDEL facilitates BiP secretion from transfected cells *in vitro*; the raised concentrations of anti-BiP antibodies in Lenti-mBiP-treated animals provides indirect evidence of vector-derived BiP secretion *in vivo*. However, any increase in serum BiP below the detection range of the BiP ELISA (ng/ml) proved that lentiviral vectors rarely induce serum concentrations of secreted proteins in excess of pg/ml quantities [23]. Previous studies have suggested that trafficking immune cells are transduced by intraperitoneal delivery of VSV-G pseudotyped lentivirus [23]. With respect to this study, we propose that vector-derived secreted BiP conditions transduced leucocytes towards an anti-inflammatory phenotype.

Extracellular rhuBiP has both prophylactic and therapeutic action in CIA [13]. In two independent experiments, Lenti-mBiP treatment was associated with amelioration of clinical, serological and immunological manifestations of disease, consistent with our previous work [13]. The subtle differences in the severity of overall arthritis in each experiment are due probably to the well-reported interexperiment variability within the CIA model [27]. A contributory factor to the lowered anti-CII levels observed in Lenti-mBiP-treated mice might be the relative lack of IL-17A in the Lenti-mBiP-treated mice [28]. The close association between IL-17 and anti-CII antibody production was apparent when CIA was induced in IL-17-deficient mice [29], resulting in reduced production of anti-CII antibodies and milder disease. IL-17 [30], especially IL-17A [31], but also IL-17B and IL-17C [32], are important pathogenic proinflammatory mediators in arthritis [33]. Spleen and lymph node cells derived from the Lenti-mBiP-treated mice inhibited IL-17A production significantly when compared with cells from Lenti-GFP-treated animals. Concomitantly, these Lenti-mBiP treated cells up-regulated IL-10 in co-culture with CII primed cells. These data support the

counter-regulation of IL-10 and T helper type 17 (Th17)/IL-17A, as reported in both CIA [6] and RA [34]. Of note is the previously reported observation that rhuBiP-primed cells ameliorate CIA following adoptive transfer, suggesting that BiP-primed cells have intrinsic regulatory properties [12].

The negative co-stimulatory signal of CTLA-4 has already been successfully exploited experimentally, using local and systemic gene therapies in CIA [35,36], and clinically in RA [37]. Both rhuBiP and Lenti-mBiP have shown potential to induce CTLA-4 production. Further proof of the regulatory importance of this was provided by the *in-vitro* neutralization of CTLA-4, which reversed the inhibitory effect of BiP on IL-17A production. This counter-regulation between IL-17A and sCTLA4 has been reported previously by Ying *et al.* [27] in BALB/c mice, where the blocking of the CTLA-4–CD80 interaction potentiated Th17 differentiation. In the current study the induction of CTLA-4 is dependent upon the mBiP transgene, rather than the lentiviral construct.

Although thought initially to be less immunogenic than other viral vectors (e.g. adenoviruses), there is a strong body of evidence suggesting that immune responses are mounted to recombinant lentiviral vectors *in vivo* [38,39]. Our data demonstrate that varicella zoster virus (VZV)-G pseudotyped lentiviral vectors strongly induce IFN-γ secretion in CIA and suppress antigen-specific IL-10 secretion. The role of IFN-γ in the pathogenesis of CIA is complex; in sum, it is believed that early exposure to IFN-γ in the CIA model leads to exacerbation of disease pathogenesis [40], while later exposure to IFN-γ leads to amelioration of disease, possibly via induction of indoleamine-2,3-dioxygenase [41]. Although IFN-γ is associated with inflammatory disease, the IFN-γ receptor knock-out mouse perversely develops more severe arthritis than the wild-type, similar to the IL-10 knock-out mouse [42] [43]. Both Lenti-GFP and Lenti-mBiP vectors induce IFN-γ: cells from Lenti-GFP-treated animals have significantly higher baseline and induced IFN-γ secretion in response to mitogenic stimulus, associated with a trend towards early worsening of disease activity between days 4 and 8 post-arthritis onset, but marginally improved arthritis scores compared to CIA controls at later time-points. Lenti-mBiP-treated animals show non-significant trends towards increased IFN-γ secretion, suggesting that the mBiP transgene is exerting an immunomodulatory effect over vector-induced IFN-γ secretion. The results from co-culture experiments with CII responder cells initially appear counterintuitive, but could be explained by fundamental changes in the expression of immunologically relevant cell surface molecules [e.g. CD80/CD86/major histocompatibility complex (MHC)] induced directly or indirectly by the lentiviral vector subsequently modulating cell–cell interactions and consequent cytokine profiles. The consequences of lentiviral vector-induced IFN-γ are complex and probably alter the immunological and clinical development of the CIA model, and we urge

caution when attributing immunological mechanisms to transgenes contained within future lentiviral-based gene therapies.

In summary, this study extends previous observations of the immunoregulatory properties of extracellular BiP. We demonstrate that lentivirus-derived BiP reduces disease activity in CIA, with associated up-regulation of sCTLA-4 and suppression of IL-17. Moreover, we provide evidence that CTLA-4 plays an important mechanistic role in BiP immunoregulation using the clinically relevant rhuBiP molecule. Currently, translation of BiP into a gene therapy for rheumatic disease is premature, and further development would necessitate a more complete understanding of the delicate balance between intracellular and extracellular BiP and the development of a suitable, safe gene therapy vector for human use.

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Disclosure

G. S. P. and V. M. C. are shareholders in a non-profit-making company which has the patent for the use of BiP in the treatment of rheumatoid arthritis. No other authors have any conflicts of interest.

Author contributions

A. M. S. performed the experiments (UK and USA), helped to design the study and wrote the paper, L. S. K. helped to design the study, provided reagents and reviewed the paper; M. A. helped to design the study; P. H. W. helped to design the study (USA); G. S. P. helped to design the study; H. L. C. helped to perform the animal experiments (UK) and design the study; S. J. T. helped to perform the animal experiments (UK) and design the study; V. M. C. performed some experiments, designed the study and wrote the paper.

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