CD19+CD24hiCD38hi B Cells Exhibit Regulatory Capacity in Healthy Individuals but Are Functionally Impaired in Systemic Lupus Erythematosus Patients

Paul A. Blair,1 Lina Yassin Noreña,1 Fabian Flores-Borja,1 David J. Rawlings,2 David A. Isenberg,1 Michael R. Ehrenstein,1 and Claudia Mauri.1,*

1Centre for Rheumatology Research, Department of Medicine, University College London, 46 Cleveland Street, London W1T 4JF, UK
2Departments of Pediatrics and Immunology, Seattle Children’s Research Institute and University of Washington, Seattle, WA 98101, USA
*Correspondence: c.mauri@ucl.ac.uk
DOI 10.1016/j.immuni.2009.11.009

SUMMARY

The immunosuppressive function of regulatory B cells has been shown in several murine models of chronic inflammation, including collagen-induced arthritis, inflammatory bowel disease, and experimental autoimmune encephalomyelitis. Despite interest in these cells, their relevance to the maintenance of peripheral tolerance in humans remains elusive. Here, we demonstrate that human CD19+ CD24hiCD38hi B cells possessed regulatory capacity. After CD40 stimulation, CD19+CD24hiCD38hi B cells suppressed the differentiation of T helper 1 cells, partially via the provision of interleukin-10 (IL-10), but not transforming growth factor-β (TGF-β), and their suppressive capacity was reversed by the addition of CD80 and CD86 mAbs. In addition, CD19+CD24hiCD38hi SLE B cells isolated from the peripheral blood of systemic lupus erythematosus (SLE) patients were refractory to further CD40 stimulation, produced less IL-10, and lacked the suppressive capacity of their healthy counterparts. Altered cellular function within this compartment may impact effector immune responses in SLE and other autoimmune disorders.

INTRODUCTION

Regulatory cells are important for maintenance of immunological homeostasis and self-tolerance (Hayday and Tigelaar, 2003; Mauri and Ehrenstein, 2008; Van Parijs and Abbas, 1998). In the majority of autoimmune diseases, B cells are generally considered to be pathogenic because of their capacity to secrete autoantibodies (Shlomchik et al., 1994; Yanaba et al., 2008b). However, it has emerged that specific subsets of B cells downregulate immune responses in mice, and their absence or loss results in exacerbated autoimmune responses (Fillatreau et al., 2002; Mauri et al., 2003; Mizoguchi et al., 1997). Different B cell subsets have been ascribed with regulatory function, such as CD5+B-1a cells (Carroll and Prodeus, 1998; Silverman et al., 2008), CD19+CD1d+CD5+ B cells (Matsushita et al., 2008; Yanaba et al., 2008a), or marginal zone (MZ) B cells (Gray et al., 2007; Lenert et al., 2005). We recently demonstrated that interleukin-10 (IL-10)+ B cells in a collagen-induced arthritis (CIA) model are found within the immature transitional two-marginal zone precursor B cell subset (T2-MZP) and display a CD19+CD21hiCD23hiCD24hiCD1dhi phenotype. Adoptive transfer of T2-MZP B cells from naive or convalescent mice prevented recipient syngeneic DBA/1 mice from developing arthritis (Evans et al., 2007). We have also identified regulatory B (Breg) cells with a similar phenotype (T2-like) in MRL/lpr mice, which spontaneously develop lupus-like disease, and demonstrated that they can be enriched upon short-term in vitro culture with agonistic CD40 monoclonal antibody (mAb) (Blair et al., 2009). Functional analysis of murine Breg cells showed that this population preferentially produce IL-10, but can also release transforming growth factor-β (TGF-β), interferon-γ (IFN-γ), and interleukin-12 (IL-12) (Blair et al., 2009; Evans et al., 2007; Fillatreau et al., 2002; Mizoguchi et al., 2002; Sugimoto et al., 2007; Tian et al., 2001). Detailed characterization in vitro and in vivo revealed that their suppressive capacity is mediated by the provision of IL-10 and by direct contact with CD4+ T cells (Mauri et al., 2003) (Mizoguchi et al., 2002). CD40 signaling is pivotal for the generation and function of Breg cells in experimental autoimmune encephalomyelitis (EAE), CIA, and lupus and inflammatory bowel disease models (Fillatreau et al., 2002; Mauri et al., 2003; Mizoguchi et al., 2000). In CIA, stimulation of B cells with an agonistic CD40 mAb gives rise to a population of B cells with potent suppressive capacity both in vivo and in vitro (Mauri et al., 2003). Chimeric mice constituted with CD40-deficient B cells, but having otherwise normal immune systems, are unable to recover from EAE because of the inability of the B cells to produce IL-10 (Fillatreau et al., 2002). Similarly, CD40-deficient B cells are unable to protect colitis prone TCRα knockout (KO) mice from developing inflammatory colitis, unlike wild-type B cells (Mizoguchi et al., 1997). Engagement of CD40 on human B cells leads preferentially, as in mice, to the production of IL-10 (Duddy et al., 2004). However, in contrast to the murine studies, there is a paucity of data regarding any potential role for an equivalent regulatory B lymphocyte population either in healthy individuals or in patients with autoimmune disease (Duddy et al., 2007) (Correale et al., 2008). In this study, we examined the functional regulatory properties of peripheral blood (PB)-derived human B cell subsets from...
Figure 1. CD19+CD24hi CD38hi B Cells from Healthy Individuals Suppress T Helper Cell Differentiation

(A) PBMCs were stained with CD19, CD24, and CD38 and gated as previously shown (Carsetti et al., 2004). Representative histograms showing the surface expression of IgM, IgD, CD5, CD10, CD27, and CD1d of CD19+CD24hiCD38hi, CD19+CD24+CD38dim, and CD19+CD24intCD38int B cell subsets. Results are representative of 14 different healthy controls.

(B) CD1d and CD5 expression on CD19+ B cells was assessed by immunofluorescence as previously shown in mice (Matsushita et al., 2008; Yanaba et al., 2008a).

(C) Representative dot plot showing CD19+CD1dhiCD5+ B cells (black dots) overlaid on a plot depicting CD24 and CD38 expression by total CD19+ B cells (gray dots).

(D) Flow cytometry-sorted CD19+CD24hiCD38hi, CD19+CD24+CD38dim, and CD19+CD24intCD38int DAPI− B cell subsets from healthy individuals were cultured 1:1 with autologous magnetic-bead-purified CD4+CD25− T cells for 72 hr with 0.5 μg/ml plate-bound CD3 mAb. PMA+iono was added in the last 6 hr of culture. Representative histograms for 1 out of 14 individuals, showing the frequencies of CD4+IFN-γ+ and CD4+TNF-α+ T cells are shown.

(E) Bar graphs show percentage inhibition of the frequency of CD4+IFN-γ+ and CD4+TNF-α+ T cells after coculture with specified B cells’ subset. Cumulative results expressed as mean ± SE (standard error of the mean). The results were compared with two-tailed t test analysis (CD4+CD24hiCD38hi versus other groups), *p < 0.01. One out three independent experiments using a minimum of six samples for each group is shown.
Characterization of Human Regulatory B Cells

CD19°CD24hiCD38hi B Cells Inhibit Proinflammatory Cytokine Production by CD4° T Cells

Analysis of 14 healthy donors revealed, as has been previously shown, three distinct B cell populations in the PB: CD19°CD38°CD24hi B cells (a population that includes immature B cells), CD19°CD38°CD24int B cells (primarily mature B cells), and CD19°CD24hiCD38° B cells (primarily memory B cells) (Carsetti et al., 2004; Sims et al., 2005). Further phenotypical identification confirmed that the majority of CD19°CD38hiCD24hi B cells were also IgM°IgD°CD5°CD10°CD20°CD27°CD1d°; whereas CD19°CD38intCD24int B cells were IgM°IgD°CD5°CD10°CD20°CD27°CD1d+. CD19°CD24hiCD38° B cells were CD5°CD10°CD27°CD1d+ and can have either an IgM°IgD° or IgM°IgD° phenotype (Figure 1A). Interestingly, the majority of the CD19°CD5°CD1d°B cells (71%), previously reported to be regulatory in experimental models of inflammation (Matsushita et al., 2008; Yanaba et al., 2008a), are contained within the CD24hiCD38hi B cell subset (Figures 1B and 1C).

To assess the functional regulatory properties of PB B cells, we sorted CD19°CD24hiCD38hi, CD19°CD24intCD38hi, or CD19°CD24hiCD38intDAPI B cells by flow cytometry from healthy donors and cultured them 1:1 with autologous magnetic-bead purified CD4°CD25° T cells. The gating criteria and postsort purity of B cell subsets are shown respectively in Figure 1A and Figure S1A available online. Cultures were stimulated with CD3 mAb, and the frequencies of CD4°IFN-γ° and CD4° tumor necrosis factor-α (TNF-α)° T cells were assessed by flow cytometry. CD4° T cells cultured with either CD19°CD24hiCD38hi or CD19°CD24intCD38hi B cells produced equivalent amounts of proinflammatory cytokines to cultures containing CD4° T cells alone. In contrast, culture of CD4° T cells with CD19°CD24intCD38hi B cells significantly suppressed the frequencies of CD4°IFN-γ° and CD4°TNF-α° T cells (Figures 1D and 1E). This inhibitory effect was cell dose dependent (Figure 1F). CD19°CD24hiCD38hi B cells were at least under these experimental conditions, unable to suppress Th17 cell differentiation (Figure S1B).

RESULTS

CD19°CD24hiCD38hi B Cells Are Enriched within the CD19°CD24hiCD38hi B Cell Subset

We next determined whether IL-10-producing B cells were enriched within any of the previously described B cell subset(s). CD19°CD24hiCD38hi, CD19°CD24intCD38hi, and CD19°CD24intCD38int B cells were sorted by flow cytometry from healthy donors, cultured with Chinese hamster ovary (CHO) cells that had been transfected with CD154 (CHO-CD154) or with control CHO cells, and stimulated briefly with phorbol myristate acetate (PMA) and ionomycin (PMA+iono). Intracellular staining for IL-10 demonstrated that a significantly higher percentage of CD19°CD24hiCD38hi B cells were IL-10° compared with CD19°CD24intCD38hi and CD19°CD24intCD38int B cells (Figures 2A and 2B). To complement the results obtained by intracellular staining, we measured IL-10 production by ELISA in the supernatants derived from the aforementioned experiments prior to stimulation with PMA+iono. The results in Figure 2C demonstrate that, upon CD40 engagement, there were higher amounts of IL-10 in the supernatants of cultured CD19°CD24hiCD38hi B cells compared to CD19°CD24intCD38hi or CD19°CD24intCD38int B cells. Moreover, after culture of healthy PBMCs with CHO-CD154 cells and stimulation with PMA+iono, the majority of IL-10-expressing B cells were enriched within the CD19°CD24hiCD38hi gating. In addition, the CD24hiCD38hi B cells defined the brightest IL-10-stained cells after CD40 engagement followed by PMA+iono stimulation (Figures 2D and 2E and Figure S2). Although back-calculation of the absolute numbers of IL-10° B cells suggest that there are similar numbers of IL-10°CD24hiCD38hi and IL-10°CD19°CD24hiCD38hi B cells present in the PB (Figure S3A), CD19°CD24intCD38int B cells still fail to suppress CD4° T cell activation even at a ratio of five B cells to one T cell (Figure S3B). Irradiated CHO cells cultured alone made no detectable IL-10 measured by ELISA. In addition there was negligible IL-10 production by any B cell subset in response to IgM (Fab)2, or IgM (Fab)2 in combination with CHO-CD154 cells, measured by ELISA or by intracellular staining [stimulation with IgM (Fab)2, or IgM (Fab)2 in combination with CHO-CD154 cells was followed by PMA+iono] (data not shown).

In vivo, activated T cells may be the cells that provide the necessary CD40 stimulation to CD19°CD24hiCD38hi B cells for the production of IL-10. We hypothesized that the upregulation of CD154 in response to CD3 mAb stimulation might be pivotal for the efficient generation of IL-10° CD19°CD24hiCD38hi B cells with regulatory capacity (Figure 2F). To assess this possibility, we cocultured flow cytometry-sorted CD19°CD24hiCD38hi B cells with magnetic-bead-purified CD4°CD25° T cells and stimulated them with CD3 mAb alone or with CD3 mAb in the presence of neutralizing CD154 mAb. Consistent with this idea, B cells cultured with unstimulated T cells did not produce any IL-10, whereas coculture with T cells activated with CD3 mAb, which upregulated CD154 expression, led to the generation of IL-10-expressing B cells (Figure 2G). The production of IL-10...
by CD19⁺CD24⁺CD38⁺ B cells in the T cell:B cell coculture was inhibited by the addition of blocking CD154 mAb.

**CD19⁺CD24⁺CD38⁺ B Cell Suppression Is Dependent on IL-10, CD80, and CD86, but Not TGF-β**

We next evaluated the potential effector mechanism(s) by which CD19⁺CD24⁺CD38⁺ B cells suppress cytokine production by CD4⁺ T cells. CD4⁺CD25⁻ T cells were cultured either alone or 1:1 with CD19⁺CD24⁺CD38⁺ B cells in the presence of IL-10 and IL-10 receptor (IL-10R) mAbs, TGF-β, CD80, or CD86 mAbs. Inhibition of TGF-β during coculture had no impact on the ability of CD19⁺CD24⁺CD38⁺ B cells to suppress CD4⁺ T cell proinflammatory cytokine production. In contrast, blockade of IL-10 restored TNF-α⁺ CD4⁺ T cells to the frequencies observed in cultures containing CD4⁺ T cells alone and also partially restored IFN-γ production by CD4⁺ T cells (Figures 3A and 3B; cumulative results of this and additional results are shown as bar charts on the right), suggesting that, similarly to the murine experimental models of autoimmunity, additional interactions are necessary for Breg cells to achieve their full suppressive potential (Mann et al., 2007; Mizoguchi et al., 2000). Addition of blocking antibodies against CD80, and to a lesser extent against CD86, inhibited CD19⁺CD24⁺CD38⁺ B cells from suppressing the frequency of TNF-α⁺ and IFN-γ⁺ CD4⁺ T cells (Figures 3A and 3B). Although blockade of IL-10 and IL-10R, CD80, or CD86 individually appeared to completely reverse the ability of CD19⁺CD24⁺CD38⁺ B cells to suppress the expression of TNF-α by CD4⁺ T cells, the blockade of these molecules individually was not sufficient to reverse the suppression of IFN-γ. However, a combination of mAbs against all of three molecules completely reversed the inhibition of IFN-γ expression by CD4⁺ T cells. Thus, analogous to murine experimental models, the suppressive effect of human Breg cells involves both the release of IL-10 and CD80 and CD86 costimulatory signaling.

**CD19⁺CD24⁺CD38⁺ B Cells Isolated from SLE Patients Do Not Suppress CD4⁺ T Cell Cytokine Production**

We next assessed whether the activity of Breg cells might be altered in human autoimmune disorders. As a first B cell candidate disorder, we evaluated patients with active SLE. Because of the difficulties of obtaining sufficient CD19⁺CD24⁺CD38⁺ B cell numbers from PB samples from SLE patients, we evaluated whether depletion of CD19⁺CD24⁺CD38⁺ B cells from healthy donors or patients with SLE might impact T cell cytokine production. CD19⁺CD24⁺CD38⁺ B cells were depleted from the PBMCs from healthy donors and patients with SLE by flow cytometry sorting. Undepleted PBMCs and B cell-depleted PBMCs from healthy donors were cultured for 72 hr with plate-bound CD3 mAb. We observed a significant increase in the frequency of CD4⁺IFN-γ⁺ and CD4⁺TNF-α⁺ T cells in CD19⁺CD24⁺CD38⁺ B cell-depleted compared with nondepleted PBMCs from healthy donors. The frequencies of CD4⁺ T cells were culture with flow cytometry-sorted CD19⁺CD24⁺CD38⁺ B cells for 72 hr with 0.5 μg/ml plate-bound CD3 mAb and isotype control or with CD3 mAb and blocking CD154 mAb. Intracellular cytokine staining showing the effect that blocking CD154 mAb has on the frequency of IL-10⁺CD19⁺ B cells. Data show the responses of three individuals. The experiment was performed three times.
CD4+IFN-γ+ and CD4+TNF-α+ T cells in healthy PBMCs depleted of either CD19+CD24dimCD38hi or CD19+CD24intCD38int B cells were not increased compared to undepleted PBMCs (Figure S4). In contrast to healthy donor PBMCs, depletion of CD19+CD38hiCD24hi B cells from the PBMCs of SLE patients did not lead to any significant increase in the percentages of CD4+IFN-γ+ or CD4+TNF-α+ T cells (Figures 4A and 4B). Our finding using healthy donors provides further support for a direct role for PB-derived CD19+CD24hiCD38hi cells in modulating T cell inflammatory responses. Our data also indicate that the equivalent cell population in patients with SLE lacks a similar degree of suppressive capacity. The inability of CD19+CD24hiCD38hi B cells to suppress CD4+ T cell cytokine production observed in the PBMCs of patients with SLE was not observed with samples from patients with other arthritic diseases (OAD) such as Sjögren’s syndrome or osteoarthritis. Coculture of CD19+CD24hiCD38hi B cells isolated from the PB of Sjögren’s or osteoarthritis patients with autologous CD4+ T cells led to a significant decrease in the frequency of CD4+IFN-γ+ and CD4+TNF-α+ T cells compared to CD4+ T cells cultured alone (Figures S5A).
CD19+CD24hiCD38hi B cells were purified by flow cytometry from the PB of patients with SLE or healthy controls. PBMCs isolated from patients with SLE or healthy controls were stained with CD4 and CD19 mAbs, permeabilized, and stained with TNF-α for the last 6 hr of culture. Depleted and nondepleted PBMCs were surface cultured for 72 hr with 0.5 μg/ml plate-bound CD3 mAb. PMA+iono was added for the last 6 hr of culture. Depleted and nondepleted PBMCs were surface stained with CD4 and CD19 mAbs, permeabilized, and stained with TNF-α or IFN-γ mAbs.

(A) Representative flow cytometry plots for CD4+IFN-γ+ and CD4+TNF-α+ T cell.

(B) Graphs showing the differences in the frequency of IFN-γ+CD4+ and TNF-α+CD4+ T cells between depleted and nondepleted PBMCs from the same individual. Healthy individuals are represented in black and SLE is represented in white. Results from three healthy individuals and three SLE patients are shown. The data are representative of four separate experiments. p values for differences in cytokine production in healthy comparing pre- and post-B cell depletion were calculated by two-tailed t test; **p < 0.01.

(C) Healthy CD19+CD24+CD38hi B cells suppress SLE CD4+ T cells. CD19+CD24+CD38hi B cells were purified by flow cytometry from the PB of healthy individuals and cultured 1:1 with either autologous magnetic-bead purified CD4+CD25+ T cells and autologous irradiated accessory cells or with CD4+ T cells and irradiated accessory cells isolated from patients with SLE in the presence of 0.5 μg/ml plate-bound CD3 mAb (1:10 irradiated PBMCs:CD4+ T cells). PMA+iono was added for the last 6 hr of culture. Cells were stained for the expression of CD4 and intracellularly for IFN-γ and TNF-α.

Expression of IFN-γ and TNF-α was assessed relative to isotype matched control mAbs. The bar graph shows mean ± SE percentage inhibition of IFN-γ and TNF-α expression by CD4+ T cells after culture with CD19+CD24+CD38hi B cells compared to SLE CD4+ T cells cultured alone.

**SLE Patients Display Similar Numbers of CD19+CD38hiCD24hi B Cells Compared with Healthy Individuals**

We next evaluated whether there was a numerical deficit in CD19+CD38hiCD24hi B cells in the PB of patients with SLE. The relative percentages of CD19+CD38hiCD24hi, CD19+CD38hiCD24int, and CD19+CD38intCD24hi B cell subsets were measured in the PB of healthy individuals versus SLE patients or versus age-matched control patients with OAD. As reported elsewhere, we observed significantly higher percentages of CD19+CD38hiCD24hi B cells in the PBMCs of patients with SLE compared to healthy individuals or to patients with OAD (Figure 5A) (Sims et al., 2005). However, the absolute cell numbers of CD19+CD38hiCD24hi B cells/liter in the PB of controls versus patients with SLE were not statistically different (Figure 5B). In contrast, the numbers of both CD19+CD38hiCD24int and CD19+CD38intCD24hi B cells were both significantly reduced in SLE patients. These results suggest that the inability of SLE CD19+CD38hiCD24hi B cells to suppress the expression of proinflammatory cytokines by CD4+ T cells is unlikely to be due to a numerical deficiency.

**CD19+CD24hiCD38hi B Cells Are “Refractory” to CD40 Engagement**

Next we evaluated whether CD19+CD38hiCD24hi B cells derived from SLE patients might exhibit cell-intrinsic deficits in response to CD40 stimulation followed by short period of PMA+iono stimulation (stimuli that upregulate IL-10 expression by B cells and S5B). The suppressive capacity of CD19+CD24hiCD38hi B cells isolated from OAD patients was comparable to the suppressive capacity of CD19+CD24hiCD38hi B cells isolated from healthy donors (Figures S5A and S5B). In addition, CD19+CD24hiCD38hi B cells isolated from OAD patients produce similar amounts of IL-10 compared to healthy CD19+CD24hiCD38hi B cells, reinforcing the association between B cell, IL-10 production, and suppressive capacity (Figures S5C and S5D). Thus, the lack of suppressive effect of SLE CD19+CD24hiCD38hi B cells does not simply reflect a feature of all arthritic diseases but may be specific to SLE patients. The lack of effect on T cell inflammatory cytokine production observed after depletion of CD19+CD38hiCD24hi B cells from the PBMCs of SLE patients could reflect either: (1) a B cell-extrinsic deficit(s) specific to SLE derived T cells, (2) numerical deficiency of the regulatory subset, and/or (3) a B cell-intrinsic functional impairment within this regulatory subset. To address these possibilities, we first investigated whether SLE effector T cells are resistant to the suppressive effect exerted by regulatory CD19+CD24hiCD38hi B cells derived from healthy donors. Flow cytometry-purified, CD19+CD24hiCD38hi B cells from healthy donors were cultured with either autologous CD4+ T cells or with CD4+ T cells isolated from SLE patients. Healthy CD19+CD24hiCD38hi B cells were able to suppress IFN-γ and TNF-α expression by SLE CD4+ T cell almost as efficiently as they were able to suppress cytokine expression by autologous healthy CD4+ T cells (Figure 4C).

Therefore, it is unlikely that the lack of suppression by SLE CD19+CD24hiCD38hi B cells observed in Figure 4A reflects intrinsic differences in effector T cell activity in SLE versus control donors.
isolated from healthy donors. Consistent with this idea, we observed a profound impairment in IL-10 production after coculture with CHO-CD154 cells followed by PMA+iono stimulation (Figures 5C and 5D). In particular, whereas healthy CD19$^{+}$CD24$^{hi}$CD38$^{hi}$ B cells responded to CD40 engagement, followed by PMA+iono stimulation, by upregulating IL-10 production, SLE CD19$^{+}$CD24$^{hi}$CD38$^{hi}$ B cells were refractory to the same combination of stimuli (Figures 5C and 5D). In contrast to the IL-10 production by SLE B cells after CD40 engagement, the levels of IL-10 production upon CpG+PMA+iono stimulation were restored to levels nearly equivalent to those observed in healthy controls. Stimulation of rodent splenocytes or human PBMCs with CPG was previously shown to upregulate the production of IL-10 by B cells (Barr et al., 2007; Brummel and Lenert, 2005; Ganttner et al., 2003). These findings show that, unlike healthy CD19$^{+}$CD24$^{hi}$CD38$^{hi}$ B cells, CD19$^{+}$CD24$^{hi}$CD38$^{hi}$ B cells isolated from SLE patients are unable to express IL-10 after activation via CD40, followed by PMA+iono stimulation (Figure 5D). We also tested whether this deficit reflected a defect in CD40 expression. CD40 was similarly expressed on B cells derived from healthy and SLE patients, indicating that the lack of IL-10 upregulation was not due simply to a decrease in CD40 expression (data not shown).

**Alteration of STAT3 Signaling after CD40 Engagement of CD19$^{+}$CD24$^{hi}$CD38$^{hi}$ SLE B Cells**

CD40 ligation results in the activation of multiple signaling cascades. Among CD40-activated signals, the JAK3 (Janus family of kinases) and STAT3 (signal transducer and activators of transcription family) cascade (Hanissian and Geha, 1997) provides a pathway that can be measured with flow cytometry. This provides a direct cell-based assessment of CD40-driven biochemical events in the limited numbers of CD24$^{hi}$CD38$^{hi}$ B cells obtainable within peripheral blood samples. Therefore, we investigated whether CD24$^{hi}$CD38$^{hi}$ B cells, by virtue of their ability to upregulate IL-10 upon CD40 engagement, might display a different pattern of STAT3 phosphorylation (p-STAT3) compared to other B cell subsets. In vitro stimulation of PB B cells derived from healthy donors with an agonistic CD40 mAb led to significantly higher (p < 0.05) p-STAT3 expression in CD24$^{hi}$CD38$^{hi}$ B cells compared with either CD24$^{hi}$CD38$^{hi}$ or CD24$^{hi}$CD38$^{hi}$ B cells (Figures 5E and 5F). No significant increases in the phosphorylation of STAT3 in any SLE-derived B cell subset were detected. Furthermore, we observed consistently higher basal STAT3 phosphorylation (p < 0.05) in healthy donor-derived versus SLE-derived CD24$^{hi}$CD38$^{hi}$ B cells.

These findings clearly demonstrate that STAT3 is phosphorylated in CD24$^{hi}$CD38$^{hi}$ B cells in response to CD40 ligation and suggest that this and/or other CD40-dependent proximal signals are impaired in CD24$^{hi}$CD38$^{hi}$ B cells isolated from SLE patients. Other downstream events of CD40 signaling, such as the upregulation of the activation markers CD80 and CD86, did not appear to be altered in CD19$^{+}$CD24$^{hi}$CD38$^{hi}$ B cells from SLE patients (Figure S6).

**DISCUSSION**

The delicate balance between pathogen-induced effector functions and endogenous tolerance-mediating mechanisms is of vital importance to the integrity of a host in the course of an immune response (Kyewski and Klein, 2006). So far maintenance of this balance has been mainly attributed to regulatory T (Treg) cells (Belkaid and Tarbell, 2009). Our results however, demonstrate that human PB also contains a population of B cells with regulatory capacity. CD19$^{+}$CD24$^{hi}$CD38$^{hi}$ B cells significantly inhibit the differentiation of proinflammatory cytokine expressing CD4$^{+}$ T cells in a dose- and contact-dependent manner.

Human PB CD19$^{+}$CD24$^{hi}$CD38$^{hi}$ B cells have previously been identified as immature transitional B cells (Palanichamy et al., 2009; Sims et al., 2005; Plebani et al., 2007). Here, we have ascribed these cells with a regulatory capacity. We propose that within the subset of PB B cells with a CD19$^{+}$CD24$^{hi}$CD38$^{hi}$ phenotype, there are both functionally immature transitional B cells and B cells that are functionally regulatory. Whether these functions are shared by ontologically different B cell subsets is yet to be established. It is therefore possible that the observed T cell suppression is the consequence of a developmentally regulated response of CD19$^{+}$CD24$^{hi}$CD38$^{hi}$ B cells to CD40 engagement (Masood et al., 1995; Rousset et al., 1992). However, we cannot exclude the possibility that PB CD19$^{+}$CD24$^{hi}$CD38$^{hi}$ B cells are comprised of two functionally and even developmentally distinct subpopulations: “conventional” CD19$^{+}$CD24$^{hi}$CD38$^{hi}$ immature B cells programmed to become mature B cells and CD19$^{+}$CD24$^{hi}$CD38$^{hi}$-like regulatory B cells.

As with regulatory T cells, there may be a number of distinct regulatory B cell subsets. We have found that although defining regulatory B cells with a CD19$^{+}$CD24$^{hi}$CD38$^{hi}$ phenotype enriches for a population of IL-10$^{+}$ regulatory B cells, other B cell subsets, such as CD19$^{+}$CD24$^{hi}$CD38$^{hi}$ B cells, in the peripheral blood also express IL-10. Our results suggest that, in addition to IL-10 production, other soluble factors or costimulatory molecules that might be present specifically on the CD24$^{hi}$CD38$^{hi}$ B cell subset could be required for suppression. It remains to be formally proven whether the IL-10-producing B cells contained within these other B cell subsets are functionally suppressive. At present we have no means of purifying exclusively those B cells that express IL-10 in sufficient quantities to perform functional studies. It is therefore important to continue to address the existence of potential additional regulatory B cell subsets, given that they might play a role in the maintenance of the balance between tolerance and autoimmunity.

Despite phenotypic and functional similarities, our studies revealed several key differences between murine and human regulatory B cells. The suppressive activity of murine Breg cells is primarily, if not exclusively, IL-10 dependent (Bouaziz et al., 2008; Mauri and Ehrenstein, 2008; Mizoguchi and Bhan, 2006). In contrast, whereas neutralization of IL-10 in human B cell and T cell cocultures completely restored TNFα production by target CD4$^{+}$ T cells, IL-10 inhibition alone did not fully restore T cell IFNγ production. In addition, whereas in mice TGFβ has been shown to mediate Breg cell-suppressive activity in experimental diabetes (Tian et al., 2001), human CD19$^{+}$CD24$^{hi}$CD38$^{hi}$ B cell-suppressive capacity is not dependent on TGFβ production.

An important feature of the suppressive capacity of human CD19$^{+}$CD24$^{hi}$CD38$^{hi}$ B cell is the involvement of CD80 and CD86. In a TCRα KO mouse model of intestinal inflammation, CD86 in particular has been reported to mediate the suppressive
Figure 5. CD19+CD24hiCD38hi B Cells from SLE Patients Express Lower Amounts of IL-10 and Lower Amounts of Phosphorylated STAT-3 in Response to CD40 Followed by PMA+iono Stimulation, Compared to Healthy Controls

PBMCs isolated from patients with SLE and healthy controls were stained directly ex vivo for the expression of CD19, CD24, and CD38. 

(A) Representative flow cytometry plot of B cell subset gating for a SLE sample showing CD19+CD24hiCD38hi, CD19+CD24intCD38int, and CD19+CD24hiCD38hi B cells. Scatter plots show mean percentages of B cells within each subset in the peripheral blood of 14 healthy individuals and 25 patients with active SLE (for information about clinical status of SLE patients, refer to Table 1). p values were calculated by two-tailed t test; **p < 0.01.

(B) Bar charts showing the mean absolute numbers ± SE (cells/liter of blood) of B cells within each subset for the same individuals and patients; *p < 0.05. IL-10 expression by healthy versus SLE B cell subsets is shown. PBMCs from healthy individuals and patients with SLE were stimulated for 72 hr with CHO-CD154 cells, control CHO cells, or CpG (0.1 μM). PMA+iono were added for the last 6 hr of culture. Cells were surface stained with CD19, CD24, and CD38 mAbs and intracellularly stained for IL-10. Expression of IL-10 was assessed relative to isotype matched control mAbs. B cell subsets were gated as in (A).
Characterization of Human Regulatory B Cells

Immunity

effects of B cells (Mann et al., 2007; Mizoguchi et al., 2000). Moreover, it has been shown that blockade of CD80 impairs Treg cell-suppressive capacity (Hajoui et al., 2004). Our results suggest that CD80 and CD86 interactions between B cells and CD4+ T cells work synergistically with B cell IL-10 production to suppress CD4+ T cell cytokine production. CD28 and CTLA-4, both expressed by a variety of T cell populations, including regulatory T cells, are both ligands for CD80 and CD86 (Salomon and Bluestone, 2001; Tang and Bluestone, 2008). A previous study revealed that CD80 expressed on DCs acts preferentially as a ligand for CTLA-4 and mediates Treg cell suppression (Zheng et al., 2004). However, whether this exact mechanism is utilized by Breg cells will be subject for future investigations.

Defects in regulatory T cells have been identified in several autoimmune diseases including rheumatoid arthritis (RA), multiple sclerosis (MS), and SLE (Ehrenstein et al., 2004; Viglietta et al., 2004). Here, we propose that regulatory B cells may also be defective in SLE patients. Because of the profound lymphopenia that characterizes the majority of patients with SLE, and the small volumes of SLE PB available from active patients, we have not been able to directly test the suppressive capacity of purified CD19+CD24hiCD38hi B cells derived from SLE patients (using coculture assays with CD4+ T cells). Nevertheless, our data clearly show that depletion of CD19+CD24hiCD38hi B cells from the PBMCs of SLE patients fails to promote an increase in CD4+ T cell inflammatory cytokine production as it does in healthy controls or patients with OAD. Our results with SLE T cells contrast with previous reports indicating that effector T cells in SLE patients are refractory to Treg cell suppression (Vargas-Rojas et al., 2008). This may be due to the different stimuli used to activate effector cells (CD3 mAb and irradiated PBMCs in our study versus CD3 mAb and CD28 mAb in previous studies), as well as differences in treatment regimes. Our data showing that healthy CD19+CD24hiCD38hi B cells were capable of efficiently suppressing cytokine production by SLE effector CD4+ T cells indicates that an alteration in T cell resistance in SLE is unlikely to explain the failed response to CD19+CD24hiCD38hi B cell depletion in our assays.

B cells in patients with SLE display a variety of abnormalities including abnormal expression or function of key signaling molecules, costimulatory pathways, cytokines, and perturbations in developmental subsets (Grammer and Lipsky, 2003). Here, we report an additional defect in SLE patients associated with CD40 signaling cascades in CD19+CD24hiCD38hi B cells. Unlike healthy B cells, CD19+CD24hiCD38hi B cells from SLE patients display impaired IL-10 production when activated via CD40. This deficit correlates with lower levels of STAT-3 phosphorylation. However, upregulation of CD80 and CD86 in response to CD40 stimulation did not appear to be altered in CD19+CD24hiCD38hi B cells from SLE patients. This suggests that the defective response to CD40 signaling in these cells may be limited to certain cellular functions, in particular Stat-3 phosphorylation and IL-10 production. Previous studies have reported an increase in CD154 expression on SLE T cells (Koshy et al., 1996). It is conceivable that this change might result in the overstimulation of SLE Breg cells, perhaps rendering them less capable of an appropriate IL-10 response upon CD40 engagement, followed by PMA+iono stimulation, in a context where they would otherwise have a regulatory function. Our results also shed further light on previous data showing that the strength of CD40 stimulation may dictate the direction (activation versus inhibition) of B cell responses (Mathur et al., 2004; Miyashita et al., 1997).

Our combined studies provide important new data defining the phenotype and suppressive activity for a potential regulatory B cell population in healthy humans and for a defect in the activity of such cells in an autoimmune disease setting. At present, our data are insufficient to determine whether the defective response to CD40 stimulation in CD19+CD24hiCD38hi SLE B cells and the failed suppressive capacity of this population reflects an initiating, as opposed to a secondary, effect of SLE disease processes. Ongoing studies designed to test whether regulatory B cell function can be restored after B cell depletion therapy in SLE may help to address this question. In association with previous reports showing defects in Treg cell activity in SLE, our data suggests that a broad range of deficiencies in immune regulatory cell function may be present in this and, possibly, other autoimmune diseases.

**EXPERIMENTAL PROCEDURES**

**Patients and Controls**

SLE patients fulfilling the American College of Rheumatology revised classification criteria for lupus (Tan et al., 1982) were assessed for disease activity with the British Isles Lupus Assessment Group index (Hay et al., 1993). Only patients with active SLE (British Isles Lupus Assessment global score higher than 4) were included (mean age, 39 years; age range, 19–76 years). Table 1 summarizes the characteristics of patients with SLE. Healthy individuals (mean age, 38 years; age range, 24–64 years) were studied in parallel as controls. Age-matched patients with Sjögren’s syndrome disease and patients with osteoarthritis (mean age 63) were included in this study as disease control (OAD). The ethics committee of the University College London Hospitals National Health Service Trust approved this study; patients and healthy volunteers were recruited after obtaining informed consent.

**Human Cell Isolation**

PBMCs were isolated by Ficoll-Paque Plus (Amersham Biosciences, Uppsala, Sweden) gradient centrifugation. Isolated PBMCs were resuspended in FCS (C) Representative dot plot showing the frequency of CD19+CD24hiCD38hi, CD19+CD24intCD38int, and CD19+CD24+CD38 B cells positive for IL-10 staining in one SLE patient.

(D) Cumulative results from three independent experiments with five samples per group. Bar charts represent mean ± SE percentages of IL-10+ B cells within CD19+CD24hiCD38hi, CD19+CD24intCD38int, and CD19+CD24+CD38 B cells at 0 and 5 min poststimulation by flow cytometry after stimulating PBMC from healthy controls (n = 6) and SLE patients (n = 6) in vitro with agonistic CD40 mAb (0.5 μg/106 cells). Cells were surface stained with CD19, CD24, and CD38 mAbs.

(E) STAT-3 phosphorylation at 5 min poststimulation with CD40 mAb is depicted. Phosphorylation is plotted as mean percentage increase ± SE in pSTAT-3 compared to the unstimulated control at time zero. Difference between pairs was evaluated by two-way ANOVA; * p <0.05.

(F) Representative histograms of pSTAT-3 expression by CD19+CD24hiCD38hi, CD19+CD24intCD38int, and CD19+CD24+CD38 B cells at 0 and 5 min poststimulation with CD40 mAb for one SLE patient and one healthy control.
were calculated for healthy controls by multiplying 10
Gladbach, Germany). Absolute numbers of cells within each B cell subset/liter
isolated by magnetic-bead purification with MACS kits (Miltenyi, Bergisch
Cell Culture
fraction of PBMCs contained within each subset.
numbers were determined by multiplying clinical lymphocyte counts by the
each subset as determined by flow cytometry. SLE patient B cell subset
lymphocytes isolated/100 ml blood by the fraction of PBMCs contained within
UK) were grown in DMEM containing 4500
cells (kindly provided by Prof R. Mageed, Queen Mary’s, University of London,
CD154 expressing, and untransfected control Chinese hamster ovary (CHO)
Ringmer, UK) in 96-well U-bottom plates (Nunc, Langenselbold, Germany).

Table 1. Summary of SLE Patient Clinical Data
Subject  Gender  Symptoms  Therapies  BILAG  C3, g/l  dsDNA, IU/ml  Anti-ENA
1  F  f, a, obs  nt  13  0.89  295  negative
2  F  a, ser, sv  hcq, mtx,  15  0.88  244  negative
3  F  a, ser, lym  pred, hcq, mtx, aza,  14  0.95  570  Ro, RNP
4  F  a, ser, n, sv  n.t.  12  0.15  352  Sm, RNP
5  F  a, ser, n, sv  pred, hcq, aza, mmf  26  0.5  2866  RNP
6  F  a, ser, n, sr  pred, hcq, aza,  13  0.64  2560  RNP
7  F  a, ser, n, sv  pred, hcq, mtx, aza,  24  0.83  3879  Ro, La, RNP
8  F  at, h, an, n  pred, hcq, aza, mmf,  16  0.4  510  negative
9  M  a, ser, n  pred, aza,  11  0.52  218  Ro, La, RNP
10  F  a, ser, sr  n.t.  14  0.53  39  Sm, RNP
11  F  f, sr, s, n  pred, aza, cyc, cya, mmf  12  0.44  13  Ro
12  F  a, n  pred, hcq,  12  0.88  118  Ro, Sm, RNP
13  F  f, a, n  n.t.  20  0.65  10  negative
14  F  at  pred, hcq, aza  3  1.34  21  negative
15  F  f, a, vas, n  pred, aza, mmf  19  0.21  1364  negative
16  M  h, a, n  pred, aza  12  0.81  15  negative
17  F  n  pred, aza, mmf  6  0.89  203  negative
18  F  a, n  pred, aza, mmf  17  0.35  522  Sm, RNP
19  F  f, a, n, sr  pred, hcq, aza, mmf  8  0.68  267  Ro
20  F  a, s, n  hcq, aza, mmf  6  1.15  124  Ro, La, Sm, RNP
21  F  a  hcq, mtx, aza,  12  1.39  10  Ro
22  F  a, n  hcq, aza  12  0.61  1058  Sm, RNP
23  F  f, a, n, obs  nt  45  0.42  4386  Ro, Sm, RNP
24  F  f, a, s, sv  hcq, mtx, aza,  13  1.5  31  RNP

The following abbreviations are used: arthritis (a), serositis (ser), skin vasculitis (sv), lymphadenopathy (lym), skin rash (sr), hemolytic (h), anemia (an), nephritis (n), fever (f), organic brain syndrome (obs), autoimmune thrombocytopenia (at), predinsolone (pred), hydroxychloroquine (hcq), methotrexate (mtx), azathioprine (aza), mycophenolate (mmf), and not treated (nt).

Cell Culture
Human cells were cultured in RPMI 1640 containing L-glutamine and NaHCO3 (Sigma-Aldrich, St. Louis, USA) supplemented with, 100 U/ml penicillin/streptomycin (Life Technologies, Carlsbad, USA), and 10% FCS (Biosera, Ringmer, UK) in 96-well U-bottom plates (Nunc, Langenelsbeld, Germany). CD154 expressing, and untransfected control Chinese hamster ovary (CHO) cells (kindly provided by Prof R. Mageed, Queen Mary’s, University of London, UK) were grown in DMEM containing 4500 μg/L glucose, 110 μg/L sodium pyruvate and L-glutamine (Sigma-Aldrich) with 100 U/ml penicillin and streptomycin and 5% FCS. CD154 expression was routinely checked by flow cytometry analysis after staining with CD154-PE mAb. For coculture experiments, CHO cells were first X-ray irradiated (240kV, 10 mA, 10.19 mGy/s) in 25 cm² cell culture flasks for 99.9 min then cultured with human PBMCs at a ratio of 1:10 CHO:PBMC. For experiments with purified SLE CD4⁺ T cells, autologous irradiated PBMCs (40 Gy dose), depleted of CD4⁺ T cells by magnetic-bead purification, were added at a ratio of 1:10 irradiated PBMCs:CD4⁺ T cells.

Cytokine Detection
For analysis of human intracellular cytokine production, PBMCs were stimulated with either 0.5 μg/ml purified plate-bound CD3 mAb (Hit-3a) (BD Biosciences) for 3 days for T cell cocultures, or with CD154 expressing CHO cells (1:10 CHO:B cells or PBMCs) or 0.1 μg/ml CpG ODN2006 (Invivogen, San Diego, USA) for 72 hr for B cells. GolgiPlug (BD Biosciences) was added for the last 6 hr along with PMA+Iono. For intracellular staining, cells were stained with combinations of CD4-Alexa Fluor488, CD19-PE-Cy7, CD38-FITC, CD38-Cy5, CD24-PE, CD24-biotin, and CD24-FITC mAbs (BD Biosciences). Cells were washed, fixed, permeabilized, and stained for detection of intracellular cytokines with IFN-γ-APC, TNFα-APC, and IL-10-APC mAbs (BD Biosciences). Appropriate APC-conjugated isotype controls were used for gate setting for cytokine expression. Alternatively, IL-10 was measured in the culture supernatants by ELISA (BD Biosciences) with aliquots of supernatant taken from cell cultures before the addition of GolgiPlug and PMA+Iono to the cultures.

Cell Signaling
PBMCs were rested for 1 hr in medium. Cells were then stimulated by incubation with 5 μg/ml purified stimulatory mouse-anti-human CD40 mAb (clone-SC3, BD Biosciences) (0.5 μg CD40 mAb/10⁶ cells) for 30 min on ice in the dark. Cells were washed with ice-cold PBS, 5 μg/ml goat anti-mouse IgG F(ab')₂ was added, and finally the cells were incubated at 4°C for 20 min. Cells were stained with appropriate antibodies for 30 min in ice-cold PBS at 4C. Cells were washed with ice-cold PBS, resuspended in warm PBS, and

(Biosera, Ringmer, UK) with 5% DMSO at a concentration of 1 × 10⁸/ml for freezing. B cell subsets, CD4⁺CD25⁻ T cells, and PBMCs depleted of B cell subsets were isolated by FACSAria (Becton Dickinson, Franklin Lakes, USA) on the basis of their expression of CD4 or CD19, CD24, and CD38 and DAPI (BD Biosciences, San José, USA). Untouched CD4⁺CD25⁻ T cells were also isolated by magnetic-bead purification with MACS kits (Miltenyi, Bergisch Gladbach, Germany). Absolute numbers of cells within each B cell subset/liter were calculated for healthy controls by multiplying 10x the total number of lymphocytes isolated/100 ml blood by the fraction of PBMCs contained within each subset as determined by flow cytometry. SLE patient B cell subset numbers were determined by multiplying clinical lymphocyte counts by the fraction of PBMCs contained within each subset.
incubated for up to 30 min at 37°C and fixed. Phosphorylated Stat3-PE mAb Phosflow antibody (BD Biosciences) was used at a 1:50 dilution of stock in Permwash (BD Biosciences) with 50 μl/well.

B Cell Functional Assays
Cocultures of 2.5 x 10^5 B cells and 2.5 x 10^5 CD4^+ T cells (1:1) were stimulated with plate-bound CD3 mAb (0.5 μg/ml) for 72 hr. GolgiPlug (BD Biosciences) was added for the last 6 hr along with PMA+iono. Cells were surfaced stained, permeabilized, stained intracellularly for IFN-γ, TNF-α, and analyzed by flow cytometry. For experiments with purified SLE CD4^+ T cells, autologous irradiated PBMCs (40 Gy dose), depleted of CD4^+ T cells by magnetic-bead purification, were also added at a ratio of 1:10 irradiated PBMCs:CD4^+ T cells.

Statistics
All values are expressed as mean ± SEM. We performed analysis of significance in Prism (GraphPad, La Jolla, USA) by the two-tailed t test analysis and by two-way ANOVA.

SUPPLEMENTAL INFORMATION
Supplemental Information includes six figures and can be found with this article online at doi:10.1016/j.immuni.2009.11.009.

ACKNOWLEDGMENTS
We thank J. Evans for providing technical assistance with flow cytometry sorting and M. Maini for helpful discussions and for critically reviewing this manuscript. This project and F.F.B. are funded by the Arthritis Research Campaign (ARC) Programme grant MP/17707. P.A.B is funded by the Oliver manuscript. This project and F.F.B. are funded by the Arthritis Research Campaign (ARC) Programme grant MP/17707. P.A.B is funded by the Oliver manuscript. This project and F.F.B. are funded by the Arthritis Research Campaign (ARC) Programme grant MP/17707. P.A.B is funded by the Oliver manuscript. This project and F.F.B. are funded by the Arthritis Research Campaign (ARC) Programme grant MP/17707. P.A.B is funded by the Oliver manuscript. This project and F.F.B. are funded by the Arthritis Research Campaign (ARC) Programme grant MP/17707. P.A.B is funded by the Oliver manuscript. This project and F.F.B. are funded by the Arthritis Research Campaign (ARC) Programme grant MP/17707. P.A.B is funded by the Oliver manuscript. This project and F.F.B. are funded by the Arthritis Research Campaign (ARC) Programme grant MP/17707. P.A.B is funded by the Oliver manuscript. This project and F.F.B. are funded by the Arthritis Research Campaign (ARC) Programme grant MP/17707. P.A.B is funded by the Oliver manuscript. This project and F.F.B. are funded by the Arthritis Research Campaign (ARC) Programme grant MP/17707. P.A.B is funded by the Oliver manuscript. This project and F.F.B. are funded by the Arthritis Research Campaign (ARC) Programme grant MP/17707. P.A.B is funded by the Oliver manuscript. This project and F.F.B. are funded by the Arthritis Research Campaign (ARC) Programme grant MP/17707. P.A.B is funded by the Oliver manuscript. This project and F.F.B. are funded by the Arthritis Research Campaign (ARC) Programme grant MP/17707. P.A.B is funded by the Oliver manuscript. This project and F.F.B. are funded by the Arthritis Research Campaign (ARC) Programme grant MP/17707. P.A.B is funded by the Oliver manuscript. This project and F.F.B. are funded by the Arthritis Research Campaign (ARC) Programme grant MP/17707. P.A.B is funded by the Oliver manuscript. This project and F.F.B. are funded by the Arthritis Research Campaign (ARC) Programme grant MP/17707. P.A.B is funded by the Oliver manuscript. This project and F.F.B. are funded by the Arthritis Research Campaign (ARC) Programme grant MP/17707. P.A.B is funded by the Oliver manuscript. This project and F.F.B. are funded by the Arthritis Research Campaign (ARC) Programme grant MP/17707. P.A.B is funded by the Oliver manuscript. This project and F.F.B. are funded by the Arthritis Research Campaign (ARC) Programme grant MP/17707. P.A.B is funded by the Oliver manuscript. This project and F.F.B. are funded by the Arthritis Research Campaign (ARC) Programme grant MP/17707. P.A.B is funded by the Oliver manuscript. This project and F.F.B. are funded by the Arthritis Research Campaign (ARC) Programme grant MP/17707. P.A.B is funded by the Oliver manuscript. This project and F.F.B. are funded by the Arthritis Research Campaign (ARC) Programme grant MP/17707. P.A.B is funded by the Oliver manuscript. This project and F.F.B. are funded by the Arthritis Research Campaign (ARC) Programme grant MP/17707. P.A.B is funded by the Oliver manuscript. This project and F.F.B. are funded by the Arthritis Research Campaign (ARC) Programme grant MP/17707. P.A.B is funded by the Oliver manuscript. This project and F.F.B. are funded by the Arthritis Research Campaign (ARC) Programme grant MP/17707. P.A.B is funded by the Oliver manuscript. This project and F.F.B. are funded by the Arthritis Research Campaign (ARC) Programme grant MP/17707. P.A.B is funded by the Oliver manuscript. This project and F.F.B. are funded by the Arthritis Research Campaign (ARC) Programme grant MP/17707. P.A.B is funded by the Oliver manuscript. This project and F.F.B. are funded by the Arthritis Research Campaign (ARC) Programme grant MP/17707. P.A.B is funded by the Oliver manuscript. This project and F.F.B. are funded by the Arthritis Research Campaign (ARC) Programme grant MP/17707. P.A.B is funded by the Oliver manuscript. This project and F.F.B. are funded by the Arthritis Research Campaign (ARC) Programme grant MP/17707. P.A.B is funded by the Oliver manuscript. This project and F.F.B. are funded by the Arthritis Research Campaign (ARC) Programme grant MP/17707. P.A.B is funded by the Oliver manuscript. This project and F.F.B. are funded by the Arthritis Research Campaign (ARC) Programme grant MP/17707. P.A.B is funded by the Oliver manuscript. This project and F.F.B. are funded by the Arthritis Research Campaign (ARC) Programme grant MP/17707. P.A.B is funded by the Oliver manuscript. This project and F.F.B. are funded by the Arthritis Research Campaign (ARC) Programme grant MP/17707. P.A.B is funded by the Oliver manuscript. This project and F.F.B. are funded by the Arthritis Research Campaign (ARC) Programme grant MP/17707. P.A.B is funded by the Oliver.


